

# Application of SRAP markers in the identification of *Stylosanthes guianensis* hybrids

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**Abstract** Sequence-related amplified polymorphism (SRAP) is a new molecular marker technology developed based on polymerase chain reaction. The authenticity of 84 progenies of 8 hybrid combinations of *Stylosanthes guianensis* was identified by SRAP markers to select the true hybrids used in the present study. A total of 35 SRAP primer combinations were selected from the parents of 8 hybrid combinations. The selected polymorphism primer combinations were applied to identify the authenticity of all progenies. The male parents of the primer combinations had specific markers, whereas the female parents did not. 68 progenies exhibited male parent-specific bands, which were identified as true hybrids. The rest of the progenies were considered self-hybrids because of the absence of male parent-specific bands. The results of hybrid identification provided solid evidence for further studies of hybrids and demonstrated SRAP molecular markers as a useful technology for assessing the purity of *S. guianensis* hybrids.

**Keywords** *Stylosanthes guianensis* · Hybrid identification · SRAP markers · Male parent-specific markers

## Introduction

The genus *Stylosanthes* belongs to the family Fabaceae and consists of 48 species that are naturally distributed in the tropical, subtropical, and temperate regions of the Americas, Africa, and Southeast Asia [1]. Each species has rich variations in morphology, physiology, and genetics. These abundant genetic resources establish an important foundation for improved hybrid breeding and morphological characteristics. *S. guianensis* (Aubl.) Sw. ( $2n = 20$ ) is the most widespread *Stylosanthes* species and exhibits remarkable phenotypic variations [2, 3]. This species is one of the most important tropical forage legumes currently known and is native to South and Central America and Africa, where it is widely distributed, although not in the equatorial zone [2]. *S. guianensis* has been used successfully as a pasture legume in many parts of the tropics and subtropics [4].

A range of *Stylosanthes* species was introduced from Australia into tropical southern China in the early 1980s [5, 6]. *Stylosanthes* is well-adapted to the environment of Guangdong and Hainan province. A series of *Stylosanthes* cultivars has been grown by selective breeding, including *S. guianensis* cv. Reyan No. 2 in 1991, *S. guianensis* cv. Reyan No. 5 in 1999, and *S. guianensis* cv. Reyan No. 20 in 2010. Cross breeding is commonly used to improve the characteristics of *Stylosanthes* and overcome the time and labor limitations of selective breeding. Thus, the hybridity of new seedlings must be verified at an early stage to assure the uniformity and stability of the field performance and yield of the crop and optimize planting time and costs.

DNA molecular marker technology, which is based on sequence variations of specific genomic regions, provides a powerful tool for hybrid identification and seed verification. This technology has the advantages of time savings,

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reduced labor consumption, and higher efficiency compared with other methods [7–11].

The main DNA marker techniques currently in application are restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), and sequence-related amplified polymorphism (SRAP). SRAP preferentially amplifies open reading frames, which are coding sequences in the genome. SRAP can disclose numerous co-dominant markers with a large amount of polymorphic loci and allows easy isolation of bands for sequencing. It is based on two-primer amplification. The primers are 17 or 18 nucleotides long and consist of the following elements. Core sequences, which are 13–14 bases long, where the first 10 or 11 bases starting at the 5' end, are sequences of no specific constitution ("filler" sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by 3 selective nucleotides at the 3' end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long [12]. This technique can generate more polymorphic fragments that reveal genetic diversity than SSR, ISSR, or RAPD markers [13]. Ferriol et al. [14] showed that the information provided by SRAP markers is more consistent with the morphological variability and evolutionary history of the morphotypes than that obtained from AFLP markers. Therefore, SRAP markers are ideal molecular markers for genotype identification, map construction, gene tagging, and genomic and cDNA fingerprinting, such as in the diversity analysis of buffalo grass [13, 15, 16]. Thus far,

few studies that use SRAP markers for true hybrid identification have been reported.

Many methods for hybrids identification most focused on SSR, RAPD and AFLP molecular markers, few focused on SRAP molecular makers. Considering these advantages, the present study detects true hybrids of *S. guianensis* using SRAP markers. The specific goals of the study are: (1) to assess the feasibility of the SRAP technique in the study of *S. guianensis* and (2) to identify true *S. guianensis* hybrids using SRAP markers.

## Materials and methods

### Plant materials

Table 1 shows the 84 hybrids generated by 8 hybrid combinations of 12 parents [1,979(D) × 109(P), 1979(D) × 106(P), 3079(D) × 45(P), 3183(D) × 96(P), 3206(D) × 16(P), 3265(D) × 16(P), 3268(D) × 16(P), and 3321(D) × 109(P)] that were used in this study. The female parents were male-sterile. Each hybrids and the parents (n = 3) were grown in 20 cm diameter pots under uniform conditions in the greenhouse of the Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences (Hainan Island).

### DNA extraction

Total genomic DNA was isolated according to the modified hexadecyltrimethylammonium bromide (CTAB) DNA extraction procedure described by Huang et al. [17]. The

**Table 1** Hybrid combinations and progenies of *S. guianensis*

Name of hybrid combinations	Hybrid combinations	No. of hybrid progenies	Total numbers of hybrid progenies
2	1,979(D) × 109(P)	2-01, 2-07	2
4	1,979(D) × 106(P)	4-01, 4-02, 4-03, 4-08, 4-09, 4-11, 4-12, 4-13, 4-15, 4-16	10
7	3,079(D) × 45(P)	7-01, 7-03, 7-08, 7-17, 7-18, 7-19	6
16	3,183(D) × 96(P)	16-04, 16-06, 16-07, 16-08	4
19	3,206(D) × 16(P)	19-06, 19-07, 19-08, 19-09, 19-10, 19-11, 19-12, 19-13, 19-14, 19-15, 19-16	11
21	3,265(D) × 16(P)	21-01, 21-02, 21-03, 21-04, 21-05, 21-14, 21-16, 21-19, 21-20, 21-22, 21-23, 21-28, 21-29, 21-31, 21-32, 21-37, 21-40, 21-41, 21-44, 21-46, 21-51, 21-52, 21-53, 21-54, 21-60, 21-69, 21-70, 21-71	28
22	3,268(D) × 16(P)	22-01, 22-03, 22-04, 22-06, 22-07, 22-09, 22-10, 22-11, 22-12, 22-13, 22-14, 22-15, 22-16, 22-17, 22-18, 22-19, 22-20, 22-21	18
28	3,321(D) × 109(P)	28-01, 28-03, 28-04, 28-05, 29-07	5
Total	8 combinations		84

**Table 2** List of the forward and reverse SRAP primers used in the present study

Name	Forward primer (5′–3′)	Name	Reverse primer (5′–3′)
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGA ATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me7	TGAGTCCAAACCGGTAG	Em6	GACTGCGTACGAATTGCA
Me8	TGAGTCCAAACCGGTAA	Em7	GACTGCGTACGAATTCGA
Me9	TGAGTCCAAACCGGTCC	Em8	GACTGCGTACGAATTCAA
Me10	TGAGTCCAAACCGGTGC	Em9	GACTGCGTACGAATTCTG
Me11	TGAGTCCAAACCGGT	Em10	GACTGCGTACGAATTAGC

**Table 3** The 100 SRAP primer combinations used in the present study

	Me1	Me2	Me3	Me4	Me5	Me7	Me8	Me9	Me10	Me11
Em1	01	02	03	04	05	06	07	08	09	10
Em2	11	12	13	14	15	16	17	18	19	20
Em3	21	22	23	24	25	26	27	28	29	30
Em4	31	32	33	34	35	36	37	38	39	40
Em5	41	42	43	44	45	46	47	48	49	50
Em6	51	52	53	54	55	56	57	58	59	60
Em7	61	62	63	64	65	66	67	68	69	70
Em8	71	72	73	74	75	76	77	78	79	80
Em9	81	82	83	84	85	86	87	88	89	90
Em10	91	92	93	94	95	96	97	98	99	100

quality and quantity of genomic DNA were estimated by measuring A260/A280 using a UV spectrophotometer. The intactness of the genomic DNA was verified by gel electrophoresis [18]. The DNA concentration was adjusted to 50 ng/μL to facilitate polymerase chain reaction (PCR) amplification. DNA samples were stored at −20 °C until use.

#### SRAP PCR amplification

A total of 100 primer combinations, including 10 forward and 10 reverse primers, from Sheng Gong Inc. (Shanghai, China) were tested by PCR (Tables 2, 3) [12].

Each 25 μL PCR reaction mixture contained 80 ng of genomic DNA, 0.4 μM primer, 250 μM dNTPs, 1.5 mM MgCl<sub>2</sub>, 1.5 units of Taq polymerase, and 2.5 μL of 1 × PCR buffer. The mixtures were overlaid with 20–30 μL of mineral oil. DNA amplification was performed using a Thermal Cycler (Bio-Rad S1000™, USA) under the following conditions: initial denaturation at 94 °C for 4 min, followed by 5 cycles of 1 min denaturation at 94 °C, 1 min annealing at 35 °C, and 30 s elongation at 72 °C. Denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and elongation at 72 °C for 1 min were conducted in the following 30 cycles. Each cycle ended

with an elongation step for 10 min at 72 °C. The amplified products were stored at 4 °C until analysis. The amplification products were separated by electrophoresis on 1.5 % (w/v) agarose gel in 1.0 × TBE buffer (0.09 mol/L Tris-H<sub>3</sub>BO<sub>3</sub>, 0.002 mol/L EDTA, pH 8.0) at a constant voltage of 100 V for approximately 1.5 h. GoldView (0.5 μg/mL) was added to facilitate UV light visualization. Molecular weights were estimated using a 100 bp DNA ladder.

## Results

#### SRAP analysis

The primers were selected for their ability to yield clear, polymorphic, and reproducible patterns of amplification. Polymorphisms observed between male and female parents were used as markers to assess the purity of the hybrids. Only 35 of the 100 SRAP primer combinations tested generated multiple fragments between the 8 hybrid combinations of 12 parents (Table 4), 75 were rejected for failing to produce amplification or displaying monomorphic patterns. These 35 primer combinations were chosen for further analysis on the basis of their capability to facilitate good amplification. SRAP analysis results in

**Table 4** The 35 SRAP primer combinations used in the present study

Code	Primer combinations	Code	Primer combinations	Code	Primer combinations	Code	Primer combinations
01	Me1-Em1	95	Me5-Em10	57	Me8-Em6	12	Me2-Em2
41	Me1-Em5	07	Me8-Em1	87	Me8-Em9	92	Me2-Em10
71	Me1-Em8	06	Me7-Em1	97	Me8-Em10	09	Me10-Em1
03	Me3-Em1	16	Me7-Em2	08	Me9-Em1	29	Me10-Em3
13	Me3-Em2	66	Me7-Em7	28	Me9-Em3	39	Me10-Em4
23	Me3-Em3	46	Me7-Em5	48	Me9-Em5	69	Me10-Em7
83	Me3-Em9	56	Me7-Em6	58	Me9-Em6	89	Me10-Em9
14	Me4-Em2	17	Me8-Em2	68	Me9-Em7		
34	Me4-Em4	47	Me8-Em5	02	Me2-Em1		

**Table 5** Filtering results of the primer combinations

Name of hybrid combinations	Total number of primer combinations with male parent-specific markers	Primer combinations with male parent-specific markers	Total number of male parent-specific markers	Percentage of primer combinations with male parent-specific markers (%)
2	4	71, 13, 23, 12	4	11.43
4	24	01, 83, 14, 06, 16, 66, 46, 56, 07, 17, 47, 57, 97, 08, 28, 48, 58, 68, 02, 09, 29, 39, 69, 89	40	68.57
7	4	23, 95, 47, 92	5	11.43
16	3	71, 95, 17	3	8.57
19	4	95, 17, 92, 69	5	11.43
21	5	01, 41, 14, 08, 92	9	14.29
22	6	01, 03, 14, 95, 07, 92	10	17.14
28	3	71, 83, 87	5	8.57

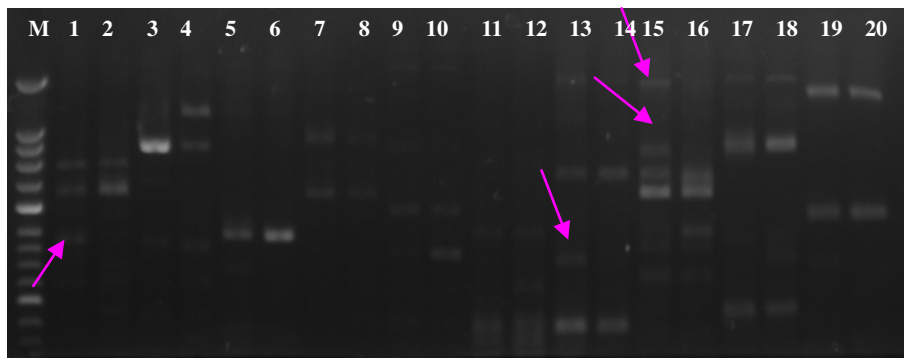
Table 5 show that: (1) only 4 (11.43 %) of the 35 primer combinations generated male parent-special markers in hybrid combination 2 (1,979 × 109), with a total of 4 male parent-special markers; (2) 24 primer combinations (68.57 %) produced male parent-special markers in hybrid combination 4 (1,979 × 106), with a total of 40 male parent-special markers; (3) 4 primer combinations (11.43 %) produced male parent-special markers in hybrid combination 7 (3,079 × 45), with a total of 5 male parent-special markers; (4) 3 primer combinations (8.57 %) produced male parent-special markers in hybrid combination 16 (3,183 × 96), with a total of 3 male parent-special markers; (5) 4 primer combinations (11.43 %) produced male parent-special markers in hybrid combination 19 (3,206 × 16), with a total of 5 male parent-special markers; (6) 5 primer combinations (14.29 %) produced male parent-special markers in hybrid combination 21 (3,265 × 16), with a total of 9 male parent-special markers; (7) 6 primer combinations (17.14 %) produced male parent-special markers in hybrid combination 22 (3,268 × 16), with a total of 10 male parent-special markers; and (8) 3 primer combinations (8.57 %) produced male parent-special markers in hybrid

combination 28 (3,321 × 109), with a total of 5 male parent-special markers.

Figure 1 shows that three primer combinations, namely, Me1-Em1, Me3-Em9, and Me4-Em2, could be used to determine hybrid purity because they exhibit polymorphic bands between the male and female parents in hybrid combination 4 (1,979 × 106).

#### Hybrid identification

Only the primer combinations that amplified male parent-specific bands of each hybrid were considered to verify hybrid purity. Table 6 shows the following: (1) Primer combination Me1Em8 verifies that 2-01 is a true hybrid whereas 2-07 is not a true hybrid in hybrid combination 2 (1,979 × 109). (2) Primer combination Me4-Em2 can verify hybrid purity in hybrid combination four (1,979 × 106) because it exhibits two bands (950 and 1,450 bp) in the progenies, which are specific to the respective male parents in all cases. The 9 progenies of hybrid combination 4 (1,979 × 106) have 1 or 2 male-special bands except for 4-08. Thus, these progenies may be verified as true hybrids



**Fig. 1** Polymorphic bands of SRAP primer combinations between parents in hybrid combination 4 ( $1,979 \times 106$ ) Note: M: 50 bp ladder marker; the arrowhead indicates the polymorphic bands in the male parent; the odd numbers represent male parents, whereas the even

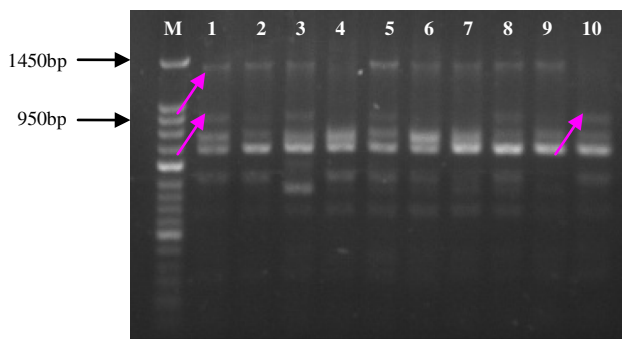
numbers signify female parents. The primer combinations were Me1-Em1, Me1-Em5, Me1-Em8, Me3-Em1, Me3-Em2, Me3-Em3, Me3-Em9, Me4-Em2, Me4-Em4, and Me5-Em10

**Table 6** Hybrid identification results

Name of hybrid combinations	Numbers of the hybrids	Primer combinations	True hybrids	Total numbers of the true hybrids
2	2	Me1-Em8	2-01	1
4	10	Me4-Em2	4-01, 4-02, 4-03, 4-09, 4-11, 4-12, 4-13, 4-15, 4-16	9
7	6	Me5-Em10	7-01,7-18	2
16	4	Me1-Em8	16-04, 16-06, 16-07, 16-08	4
19	11	Me5-Em10	19-07	1
21	28	Me9-Em1	21-01, 21-02, 21-03, 21-04, 21-05, 21-14, 21-16, 21-19, 21-20, 21-22, 21-23, 21-28, 21-29, 21-31, 21-32, 21-37, 21-40, 21-41,21-44, 21-46, 21-51, 21-52, 21-53, 21-54, 21-60, 21-69, 21-70, 21-71	28
22	18	Me5-Em10	22-01, 22-03, 22-04, 22-06, 22-07, 22-09, 22-10, 22-11, 22-12, 22-13, 22-14, 22-15, 22-16, 22-17, 22-18, 22-19, 22-20, 22-21	18
		Me10-Em7	22-01, 22-03, 22-04, 22-06, 22-07, 22-09, 22-10, 22-11, 22-12, 22-13, 22-14, 22-15, 22-16, 22-17, 22-18, 22-19, 22-20, 22-21	
		Me8-Em1	22-01, 22-03, 22-04, 22-06, 22-07, 22-09, 22-10, 22-11, 22-12, 22-19, 22-20, 22-21	
		Me4-Em2	22-01, 22-03, 22-04, 22-06, 22-07, 22-09, 22-10, 22-11, 22-12, 22-13, 22-14, 22-15, 22-16, 22-17, 22-18, 22-19, 22-20, 22-21	
28	5	Me8-Em9	28-01, 28-03, 28-04, 28-05, 29-07	5
		Me5-Em10	28-01, 28-03, 28-04, 28-05, 29-07	
Total	84			68

(4-01, 4-02, 4-03, 4-09, 4-11, 4-12, 4-13, 4-15, 4-16). Hybrid 4-08 is not a pure hybrid (Fig. 2). (3) Two progenies are pure hybrids (7-01, 7-18), whereas 4 progenies (7-03, 7-08, 7-17, 7-19) are not true hybrids in hybrid combination 7

( $3,079 \times 45$ ). Hybrid purity may be verified by Me5Em10. (4) Four progenies in hybrid combination 7 ( $3,079 \times 45$ ) are pure hybrids (16-04, 16-06, 16-07, 16-08), as verified by Me1Em8. (5) Only 19-07 is a true hybrid in hybrid



**Fig. 2** SRAP patterns obtained from hybrid combination 4 (1,979 × 106), as determined by the Me4-Em2 primer combination. Note: M: standard DNA (50 bp ladder marker). Lanes 1–10: fragments from hybrids; the *arrowhead* indicates male parent-specific markers. The hybrids obtained include 4-01, 4-02, 4-03, 4-08, 4-09, 4-11, 4-12, 4-13, 4-15, 4-16

combination 19 (3,206 × 16). All other progenies are not true hybrids (19-06, 19-07, 19-08, 19-09, 19-10, 19-11, 19-12, 19-13, 19-14, 19-15, 19-16), as verified by Me5Em10. (6) Twenty-eight progenies of hybrid combination 21 (3,265 × 16) are true hybrids (21-01, 21-02, 21-03, 21-04, 21-05, 21-14, 21-16, 21-19, 21-20, 21-22, 21-23, 21-28, 21-29, 21-31, 21-32, 21-37, 21-40, 21-41, 21-44, 21-46, 21-51, 21-52, 21-53, 21-54, 21-60, 21-69, 21-70, 21-71); these findings may be verified by Me9Em1. (7) Eighteen progenies in hybrid combination 22 (3,268 × 16) are true hybrids (22-01, 22-03, 22-04, 22-06, 22-07, 22-09, 22-10, 22-11, 22-12, 22-13, 22-14, 22-15, 22-16, 22-17, 22-18, 22-19, 22-20, 22-21), as verified by Me5Em10, Me10Em7, Me8Em1, and Me4Em2. (8) Five progenies in hybrid combination 28 (3,321 × 109) are true hybrids (28-01, 28-03, 28-04, 28-05, 29-07), as verified by Me5Em10 and Me8Em9.

## Discussion

Identification of hybrid authenticity is important in cross-breeding. The rapid and accurate identification of hybrid authenticity is an important basis of cross-breeding. However, not all types of markers are suitable and/or feasible for identification and characterization of the hybrids. Traditionally, grow-out trails (GOTs) has been widely applied to hybrid identification in a variety of crops, which involve growing a representative sample of the seed followed by analysis of several morphological characteristics in different developmental stages. However, GOTs is time-consuming and costly, and requires extensive use of land. Furthermore, GOTs is easily affected by growing conditions which making the determination difficult [19]. Isozyme electrophoresis technologies also have been employed for hybrid identification, but the limitation of this method is their failure to detect polymorphisms in

closely related lines. Thus, more sensitive methods are necessary to test and determine hybrid purity. This has shifted the focus to DNA-based molecular markers.

With the advantages of time- and resource-saving, less labor-consumption and more precision, molecular markers are becoming vital tools for cultivar identification and seed quality control in many crops. Recently, several molecular markers, such as SSR [19–21], RAPD [10, 22, 23], ISSR [24, 25], and AFLP [26] have been extensively used for the identification of hybrids. However, studies that use SRAP for true hybrid identification have rarely been reported. The present study developed a method to identify *S. guianensis* hybrids. The authenticity of 84 progenies of 8 hybrid combinations of *S. guianensis* was identified by SRAP markers. Sixty-eight (80.95 %) progenies exhibited male parent-specific bands and were thus identified as true hybrids. These true hybrids could be used for map construction and cross-breeding in *S. Guianensis*.

This study is the first to apply DNA markers to identify the purity of *S. guianensis* hybrids. A new, simple, and low-cost method was presented in this study. The proposed method may be used in *S. guianensis* breeding programs worldwide. Our experimental results indicate that SRAP technology is a powerful and efficient approach for hybrid identification. Several studies that employ the SRAP technique to assess the genetic diversity of several crops, such as *Pennisetum purpureum* Schumach [27], *Lactuca* sp. [28], *Salvia miltiorrhiza* Bge [29], and *Cynodon Radiatus* [30], have been published. SRAP markers offer several advantages over other available molecule techniques, including production of highly specific polymorphic fragments and low requirements of DNA amount and quality.

The SRAP profiles of 84 hybrids and their respective parents show that the SRAP marker technique has good potential application in the identification of *S. guianensis* hybrids. Potential primers depicting distinct and good polymorphic markers may be used for the identification, registration, and protection of *S. guianensis*.

## Conclusions

This study demonstrates the feasibility of applying SRAP markers to identify the authenticity of hybrid progenies. A total of 84 progenies were determined. Sixty-eight (80.95 %) of these progenies that exhibited male parent-specific bands were identified as true hybrids. The remaining 16 progenies were self-hybrids because of the absence of male parent-specific bands. These true hybrids will be used for map construction and cross-breeding in *S. Guianensis*.

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