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Genetic Variation of *Anoectochilus formosanus* Revealed by ISSR and AFLP Analysis

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ABSTRACT

Anoectochilus formosanus is an expensive medicinal folk herb in Taiwan. The plant has been used to treat hypertension, diabetes, and hepatitis by native people for a long time. In this study, 4 typical lines of *A. formosanus*, red-stem, green-stem, narrow-leaf, and round-leaf, were collected and screened to identify the line-specific SSR (single sequence repeat) markers. In addition, we found the narrow-leaf line exhibited the highest polysaccharide content (3.39 ± 0.74%, polysaccharide wt/plant dried wt) which is more than twofold of the lowest content of the red-stem line (1.27 ± 0.41%). In the culture program construction, the extent of the somaclonal variation among 20 regenerated shoots from the same parental individual of a narrow-leaf line was revealed using the amplified fragment length polymorphism (AFLP) technique. The aim of this study is to preliminarily investigate the genetic variation of *A. formosanus* through polymorphic marker screening, which will benefit to the cultivation of *A. formosanus* with high polysaccharide content in future work. The results showed that 6 out of 81 primers screened, generating a total of 26 markers, were able to differentiate the 4 lines in the inter single sequence repeat (ISSR) analysis. On the other hand, 17 sets of AFLP primers were chosen to detect the somatic variation among the excised shoots derived from the same individual. The genetic variation among the regenerated shoots ranged from 0.00% to 5.43%. The primer pair B6 exerts the most powerful selection ability in the AFLP analysis among 20 somaclonal samples used, whereas the E4 primer pair the lowest. The AFLP primer pairs become useful tools for selecting the lowest somaclonal variation from the regenerated shoots of *A. formosanus* under the tissue culture program.

Key words: *Anoectochilus formosanus*, Orchidaceae, ISSR, AFLP, somaclonal variation

INTRODUCTION

The herb genus *Anoectochilus* (Orchidaceae) in Taiwan contains two species, *A. formosanus* and *A. koshunensis*. Crude extracts of *A. formosanus* were orally administered to rats for pharmaceutical evaluation in several studies recently. They exhibited antioxidation and hepatoprotection[(1)], anti-flammatory[(2)], and antihyperliposis[(3)], and antihyperglycemia activities[(4)]. In our previous study, water extract of *A. formosanus* was orally administered to BALB/c mice to investigate its immunomodulating activity[(5)]. The results indicated that mice so treated might undergo murine immune responses, such as stimulation of the proliferation of lymphoid tissues and activation of the phagocytosis of peritoneal macrophages against *Staphylococcus aureus*. We further investigated the effects of its crude polysaccharide extract and a significantly higher antitumor and immunoregulation activities were observed in the treated mouse inoculated with human colon tumor cell line CT-26 and pathogen *S. aureus*, respectively. Kyeong *et al*. reported that the acidic polysaccharide extracted from Panax ginseng possessed immunomodulating activity, which has been demonstrated to involve nitric oxide[(6)]. The polysaccharide content of farm-raised *A. formosanus* was thus investigated. Four lines of *A. formosanus* in the field, red-stem, green-stem, narrow-leaf (3-5 cm in width, 7-10 cm in length), and round-leaf (4-6 cm in width, 5-7 cm in length) could be easily distinguished based on morphological characteristics. The major line is the narrow-leaf line. A more than two-fold deviation of the polysaccharide content among the four lines was found when they grew in the same environment[(7)]. The narrow-leaf line exhibited the highest average polysaccharide content, but not all individuals within it exhibited high content. We, therefore, tried to screen for intraspecial molecular markers within the species to differentiate the four lines, in the hope that the selection of germplasm with high polysaccharide content from the four lines can be facilitated.

Due to the high degree of polymorphism in the microsatellite sequences and simple sequence repeats

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of the eukaryotic genome[8,9], ISSR fingerprinting is designed to increase the primer specificity by adding one to four nucleotides on the 3' or 5' end of the SSR primer. This will result in a more specific and clearer pattern for the amplified polymerase chain reaction (PCR) products. Twenty six polymorphic markers produced by two ISSR primers along with markers produced by three AFLP primer pairs were employed to classify the taxonomic status of some *Miscanthus* species collected[10]. The AFLP technique[11], an approach combining the advantages of the RFLP and PCR techniques, was used as a novel tool to detect the genome variation. AFLP markers are thought more efficient for polymorphism detection between genomes than random amplified polymorphic DNA (RAPD) or SSR markers are[8]. In the present study, ISSR and AFLP techniques were employed to preliminarily investigate intraspecial genetic diversity in *A. formosanus*. In another part of this study, a somaclonal variation among the excised shoots deriving from an original individual was also analyzed by AFLP.

MATERIALS AND METHODS

I. *Materials*

A. formosanus plants were collected from the Puli area of Taiwan and transplanted to the greenhouse of Taipei Medical University. These plants were identified according to the characteristics of their flowers[12]. Plants typical of each line were chosen based on the morphological criteria, such as red stem, green stem, narrow leaf, and round leaf. Three individuals of each line were used for ISSR and polysaccharide content assay. On the other hand, the seeds of *A. formosanus* collected from the mountain area in Puli were germinated on half-strength (1/2) MS basal medium supplemented with 0.2% activated charcoal and 8% banana homogenate for four months. Germinated seedlings were cultured in 1/2 liquid MS medium containing 2 mg/L N^6-benzyladenine (BA) in 125-mL Erlenmeyer flasks for two months. Before harvest, seedlings with well-developed rhizomes and shoots were cultured on 1/2 MS medium with 0.2% activated charcoal, 8% banana homogenate, 2 mg/L BA and 0.5 mg/L a-naphthaleneacetic acid for another four months. Twenty regenerated shoots from a single seedling were collected for AFLP analysis. The fresh leaves of the samples were ground into powder with mortar and pestle in liquid nitrogen and stored at -70°C. All voucher specimens (Accession No. SP 021108) were prepared and deposited in the herbarium of Taipei Medical University.

II. *DNA Extraction*

Genomic DNA was extracted from the sample by CTAB method[13]. One milliliter of cetyl-trimethyl ammonium bromide (CTAB) extraction buffer (0.1 M Tris-HCl, pH 8.0; 20 mM ethylenediaminetetraacetic acid (EDTA); 2% CTAB; 1.4 M NaCl; 0.2% β-mercaptoethanol) was added to 0.1 g of the powdered sample. The suspension was incubated with RNase A (1 mg/mL) in water bath at 60°C for 60 min with occasional shaking. The mixture was then cooled to room temperature and extracted with 1 vol. of phenol/chloroform/isoamyl alcohol (25:24:1) twice. After centrifugation at 14,000 ×g for 10 min, the two phases were separated by centrifugation, and 1 vol. of isopropanol was added to the aqueous phase. The mixture was stored overnight at -20°C. The pellet was collected after centrifugation at 14,000 ×g for 40 min, washed with the wash buffer (0.2 M sodium acetate; 75% ethanol) twice, and dissolved in 100 µL of sterilized 1/10X TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The resulting solution was used for the PCR reaction.

III. *ISSR-PCR Reaction*

In this analysis, 81 ISSR primers were screened. The primers were purchased from the University of British Columbia, Vancouver, Canada. PCR was performed in a total volume of 12.5 µL, which consisted of 0.48 µM each of the primer, 0.5 mM of dNTP, 40 ng genomic DNA template, and 1.0 U T*aq* polymerase (Life Technologies, USA) in 1.4× PCR buffer. DNA amplification was performed using a Perkin Elmer Cetus 9700 DNA thermocycler programmed for 40 cycles of 0.5 min at 94°C, 0.5 min at 52°C, and 0.5 min at 72°C, and then terminated with 7 min at 72°C. The ISSR products were resolved on a 1.5% agarose gel by electrophoresis in 0.5× TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA, pH8.3).

IV. *AFLP Procedure*

Following restriction digestion of genomic DNA (250 ng) by E*co* RI and M*se* I enzymes, adaptors specific to E*co* RI and M*se* I overhangs were ligated. Pre-amplification of the adapter-ligated DNA was performed using the primer pair combination, E*co* RI+A and Mse I+C, for 20 cycles of 30 sec at 94°C, 60 sec at 56°C, and 60 sec 72°C. All the chemicals used were purchased from Life Technologies. Selective-amplification was performed with the primer pair having three selective nucleotides (Table 1) for 12 cycles of 30 sec at 94°C, 30 sec at 65°C with 0.7°C lowering for each cycle, and 1 min at 72°C, followed by 27 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C. The amplified products were resolved by electrophoresis on a 5% denaturing polyacrylamide gel and visualized by silver-staining (Promega, USA).

V. *Data Analysis*

AFLP makers ranging in size from 100 to 2000 base pairs were scored 0 for absence or 1 for presence across the somaclonal individuals for the 17 primer pair combi-

Table 1. The 17 primer pairs of AFLP analysis used in the study

Code	Primer pair combination	Code	Primer pair combination
A2	E-AAG / M-CAA	D4	E-ACC / M-CAT
A3	E-ACA / M-CAA	D5	E-ACG / M-CAT
B2	E-AAG / M-CAC	D6	E-ACT / M-CAT
B3	E-ACA / M-CAC	E4	E-ACC / M-CTA
B6	E-ACT / M-CAC	E7	E-AGC / M-CTA
C1	E-AAC / M-CAG	G3	E-ACA / M-CTG
C3	E-ACA / M-CAG	G6	E-ACT / M-CTG
C6	E-ACT / M-CAG	G7	E-AGC / M-CTG
C8	E-AGG / M-CAG		

nations. The frequencies of bands present in the patterns of the AFLP assays were statistically analyzed by means of Euclidean distance[14] using UPGMA (unweighted pair group method with arithmetic averages). The genetic variation between the individuals was evaluated based on pair-wise comparisons according to the equation: n/(m+n) where m is the number of matched bands, and n is the number of mismatched bands.

RESULTS

In ISSR analysis, 6 out of 81 screened primers were clear and in reproducible polymorphic patterns. The 6 ISSR primer numbers are 808, 827, 835, 836, 841, and 842. Their sequence information was listed in Table 2. A total of 26 markers were generated from the 6 primers, as outlined in Table 3. The primer 808 generates two polymorphic markers, 690 and 1250 bp in length. The 690-bp marker is specific to the red-stem line, and absent in the remaining lines. However, the 1250-bp marker is present in all lines in the study but the green-stem. Three polymorphic markers, 820, 1700, and 1750 bp in length, are present in the fingerprint of primer 827. The 820-bp marker is present in the lines of narrow- and round-leaf, but absent in the lines of red- and green-stem. The 1700-bp marker is in all but the green-stem lines in the study. The marker of 1750 bp is specific to the green-stem line and not found in the remaining lines. Six polymorphic markers, 520, 680, 780, 850, 1400, and 1750 bp in length, are generated in the reaction of primer 835. The 520-bp marker is specific to the red-stem line, and the 780-bp marker is specific to narrow-leaf line. The markers of 850- and 1400-bp are specific to the round-leaf line and are not found in the remaining lines. The 680-bp marker is present in all lines but the green-stem in the study. Four polymorphic markers, 550, 630, 950, and 1900 bp in length, were found in the fingerprint of primer 836. Like the 820-bp marker of primer 827, the 1750-bp marker of primer 835, and the 950-bp marker of primer 836, the marker of 950-bp is specific to the narrow- and round-leaf lines. However, the 550-bp marker is red line-specific marker, the 630-bp marker round-leaf line-specific, and 1900-bp is narrow-leaf line-specific. Out of five polymorphic markers of primer 841, 520, 1100, 1350 markers are specific to the lines of round-leaf and red-stem. The 1450-bp marker is present only in the green-stem line. The 1500-bp marker is in all

Table 2. The attributes of the 6 ISSR primers generating polymorphisms among the lines used in this study

Primer code	Sequence[a] (5′ to 3′)	Repeat
808	AGA GAG AGA GAG AGA GC	$(AG)_8C$
827	ACA CAC ACA CAC ACA CG	$(AC)_8G$
835	AGA GAG AGA GAG AGA GYC	$(AG)_8YC$
836	AGA GAG AGA GAG AGA GYG	$(AG)_8YG$
841	GAG AGA GAG AGA GAG AYC	$(GA)_8YC$
842	GAG AGA GAG AGA GAG AYG	$(GA)_8YG$

[a]In the primer sequences, Y = C or T.

Table 3. A summary of the ISSR markers absent and present among the samples of the 4 lines of *A. formosanus*

Primer	Marker	Line			
	(bp)	Round leaf	Narrow leaf	Red stem	Green stem
808	690	–	–	+	–
	1250	+	+	+	–
827	820	+	+	–	–
	1700	+	+	+	–
	1750	–	+	–	–
835	520	–	–	+	–
	680	+	+	+	–
	780	–	+	–	–
	850	+	–	–	–
	1400	+	–	–	–
	1750	+	+	–	–
836	550	–	–	+	–
	630	+	–	–	–
	950	+	+	–	–
	1900	–	+	–	–
841	520	+	–	+	–
	1100	+	–	+	–
	1350	+	–	+	–
	1450	–	–	–	+
	1500	+	–	+	+
842	525	+	–	+	–
	700	+	+	+	–
	920	–	+	–	
	1100	+	–	+	+
	1450	+	–	+	+
	1750	–	–	–	+

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but the narrow-leaf lines in the study. In the fingerprint of the sixth primer 842, six markers, 525, 700, 920, 1100, 1450, and 1750 bp in length, were found to be polymorphic. The markers of 525, 1100, and 1450 bp, are specific to the lines of round-leaf and red-stem. The 1750-bp marker is a green-stem line-specific marker, the marker of 700 bp is present in all but the green-stem lines. In conclusion, the ISSR primers numbered 827, 835, and 836 could differentiate the four lines of *A. formosanus* used in the study. Primer 808 could not distinguish the round-leaf from the narrow-leaf line whereas primers 841 and 842 could not distinguish the round-leaf from the red-stem line.

In AFLP analysis, 17 out of 24 primer pairs were chosen for evaluating somaclonal variation among the 20 regenerated shoots from one original individual. The AFLP pattern of E-ACA and M-CAG primer pair (C3) was shown in Figure 1. 515.5 polymorphic markers in total per individual generated from the 17 AFLP primer pairs were scored in the analysis. The size of the AFLP markers scored ranged from 100 to 2000 bp. The frequencies of the somaclonal variation between the regenerated shoots and the original plant are shown in Table 4. It was revealed that the frequencies ranged from 0.00% to 5.43% among the 20 regenerated shoots. The overall average was 2.65%. Out of the primer pairs used, the B6 (E-ACT / M-CAC) exhibited the most powerful detection ability in polymorphism among the samples. A total of 572 makers was generated by using B6 primer pair in the AFLP analysis. However, 37 polymorphic markers scored among the 20 somaclonal individuals. The polymorphism frequency of 6.47% (37/572) was obtained, which is the highest among the primer sets used in this study. The lowest frequency is only 0.20% (1/505) generated by E4 (E-ACC / M-CTA). Among the 20 somaclonal sheets (P1 to P20), P2 was found with a total of 28 polymorphic markers by all the primer pairs we used in the analysis. The frequency of the sample in overall polymorphism is calculated 5.43% (28/515.5). In other words, the individuals P2 and P15 exhibit the largest genetic variation among the 20 somaclonal individuals. However, we do not find any unique marker in P1 sample.

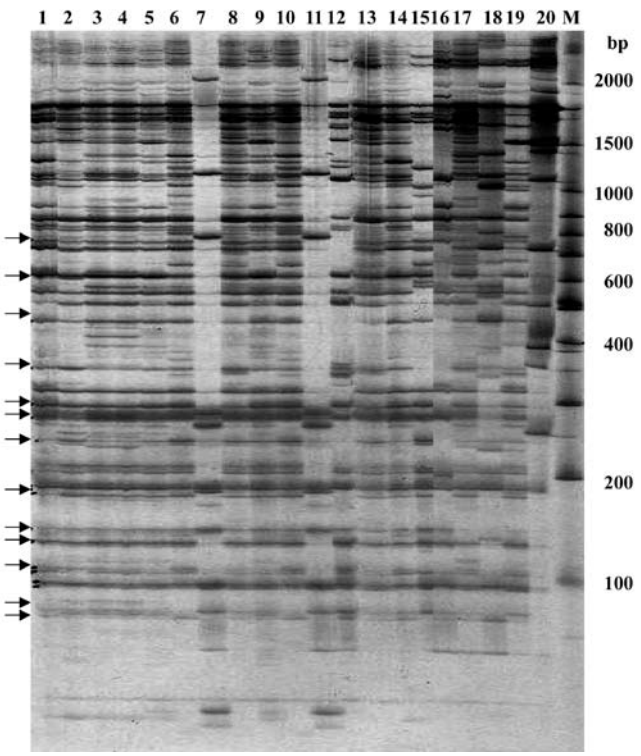

Figure 1. The AFLP pattern of E-ACA and M-CAG primer pair (C3). 1-20: somaclonal sheets P1- P20. M: DNA marker. The 13 markers are labeled by arrows.

DISCUSSION

In this study, ISSR and AFLP markers were employed to evaluate the genetic variation within species of *A. formosanus*. ISSR is commonly found in the genomes of animal and plants. The polymorphism of the repeat sequence was frequently used to distinguish the genome deviation among individuals within the same species. It is reported that three species, *Nothofagus nervosa*, *N. obliquan*, and *N. dombeyi*, were differentiated using ten RAPD primers and ten ISSR primers, respectively. They found that 22.8 bands in average were amplified by each ISSR primer, but only 12.5 bands by RAPD primer. For detection ability comparison, 83% is polymorphic in ISSR markers versus 63% in RAPD markers. With the advantages of simplicity, high reproducibility and lack of need for sequence information, the ISSR technique is thought as a more powerful tool of molecular fingerprinting. In this study, 18 RAPD primers were employed to differentiate the four lines of *A. formosanus*, however with no success (data not shown). On the other hand, 26 out of 174 (15%) markers generated from 81 ISSR primers showed polymorphic results among the four lines in the study. Since the ISSR primer is longer than RAPD, the specificity and reproducibility of ISSR is thought higher[15]. In our ISSR analysis, more polymorphic markers were found to be amplified when the sequence of the primer used were with $(GA)_n$ or $(TA)_n$. However, fewer polymorphic markers were generated, and they were appeared smeared when the primer with $(AT)_n$ was used. In our study, five ISSR markers were produced in the reaction with primer 841, which has sequence with $(GA)_n$. Some primers, such as 837, 838, or 839, with high AT repeats generated few products. The PCR reaction is thought to be hindered by the structures of dimmer or hair-pin formed in the primer.

The polysaccharide of *A. formosanus* exhibited immunomodulating activity in vivo after oral administration to mice in our laboratory. The polysaccharide exists in a major part of the water extract of *A. formosanus*. We discovered the polysaccharide contents vary among individuals of *A. formosanus* in the field. The

Table 4. Summary of AFLP analysis for the investigation of genetic variation among the 20 somaclonal shoots of *A. formosanus*

Primer	Markers	Sample																				Frequency of
	/individual	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	polymorphism (%)
A2	18.3	0	0	0	0	0	1	0	0	0	0	0	3	5	0	0	2	0	0	1	1	3.56 (13/365)[a]
A3	18.7	0	3	0	0	0	0	3	0	0	2	1	0	0	0	0	0	0	0	4	0	3.32 (13/374)
B2	37.4	0	0	0	0	6	1	1	6	0	0	0	0	0	0	0	3	1	0	6	3	3.61 (27/748)
B3	33.0	0	4	0	0	0	4	0	0	0	0	1	0	0	2	6	1	0	1	0	0	2.88 (19/660)
B6	28.6	0	0	0	0	0	3	0	0	0	2	0	0	1	10	4	1	6	2	6	2	6.47 (37/572)
C1	30.8	0	0	4	0	0	0	4	0	0	0	5	0	0	0	1	2	3	4	1	5	4.71 (29/616)
C3	35.2	0	0	0	0	0	0	1	0	0	0	1	2	0	0	0	1	0	0	0	8	1.85 (13/704)
C6	35.2	0	1	0	0	0	0	2	0	0	0	0	0	0	1	0	1	0	0	0	0	0.71 (5/704)
C8	34.1	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0.59 (4/681)
D4	30.8	0	2	0	1	0	0	0	0	0	0	0	0	6	0	1	0	0	1	0	0	1.79 (11/616)
D5	29.7	0	3	0	0	0	3	2	0	0	0	0	1	1	0	0	0	0	0	0	0	1.68 (10/594)
D6	37.4	0	2	0	0	0	6	0	0	0	2	0	0	0	9	0	0	0	0	0	0	2.54 (19/748)
E4	25.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0.20 (1/505)
E7	27.5	0	2	4	0	0	3	6	0	0	0	0	0	0	0	0	0	1	0	0	0	2.91 (16/550)
G3	29.7	0	4	0	0	0	2	0	1	3	1	0	2	1	0	8	0	0	0	0	0	3.70 (22/594)
G6	31.9	0	1	0	0	6	1	0	0	1	0	0	0	1	0	4	1	0	0	1	0	2.51 (16/638)
G7	31.9	0	4	0	0	0	3	0	0	0	0	1	0	6	0	4	0	0	0	0	0	2.82 (18/638)
Total bands	515.5																					
Frequency of		0.00		1.74		2.33		3.68		0.78		1.74		4.07		5.43		2.13		3.68		
polymorphism (%)			5.43[b]		0.19		5.23		1.36		1.36		1.55		4.26		2.71		1.55		3.68	

[a]A total of 365 markers among the 20 somaclonal individuals were calculated in the AFLP analysis with the A2 primer pair. Thirteen of the 365 markers were polymorphic.
[b]A total of 515.5 markers per individual generated from the 17 AFLP primer pairs were calculated in the analysis. Twenty eight markers of the 515.5 markers were present in the sample P2.

four lines of *A. formosanus* were collected from different areas and cultivated in the same greenhouse for three months. The polysaccharide content was quantified after hot water extraction. We found the narrow-leaf line exhibited the highest content (3.39 ± 0.74%, polysaccharide wt./plant dried wt.) which is more than twofold that of the red-stem line (1.27 ± 0.51%)[7]. We have identified several ISSR markers, enough to differentiate the four lines in the assay. Four of the polymorphic markers, the 1750-bp marker of primer 827, the 780-bp marker of primer 835, the 1900-bp marker of primer 836, and the 920-bp marker of primer 842, were specific to narrow-leaf line only. In the population of the narrow-leaf line, not all the individuals exhibited high polysaccharide content even though they had a higher average value. Therefore, screening more markers with locations close to the loci of the genes involved in polysaccharide biosynthesis and collecting more narrow-leaf line samples with high polysaccharide content to assess the linkage of the ISSR markers will be key goals for further study. The plant of the narrow-leaf line was thought to have a stronger growth potential in the field, so the markers we obtained which highly link to the high polysaccharide content will be helpful to the culture program.

The advantages of the AFLP technique, including the small amount of DNA needed, the ability to work without sequence information, high reproducibility, and high allelic locus that can be detected, make it frequently employed to evaluate the level of the somaclonal variation in the process of tissue culture[16]. The DNA fragments digested with two kinds of restriction enzymes underwent two selective amplifications in order to get a clearer and more specific result. It was reported that somatic variation was analyzed among the 51 regenerated shoots from the same parental individual of *Arabidopsis thaliana* using 12 AFLP primer pairs[17]. The result showed 778 bands were produced in the analysis. The average size of the bands was 175 bp in length. More than 30% of the bands were less than 100 bp in length. Compared to our study of 20 regenerated shoots of *A. formosanus*, 850 bands produced from 17 AFLP primer pairs showed more diversity in *A. formosanus* than in *Arabidopsis thaliana*. The overall somaclonal variation of *A. formosanus* was at 2.89% while that of *Arabidopsis thaliana* was 0.59%. The difference of somaclonal variation may vary from the size of the plant genome and characteristics of the plant itself.

CONCLUSIONS

The 4 lines of *A. formosanus* were distinctly differentiated by the 26 ISSR markers we obtained. Some of the ISSR markers were specific to the narrow-leaf line only. We will continue to find more narrow-leaf line-specific markers and assess their high polysaccharide content linkage in future studies. We are also trying to find highly distinguishable ISSR and AFLP markers to detect the variations in the genomes among the individuals of somaclonal shoots or intra-species of *A. formosanus* in the study. In the further investigation, a new tool, the cDNA-AFLP technique[18], will be adopted to search for transcriptome data of medicinal plants involved in the biosynthesis of secondary metabolite. The work is becoming more important in the quality control of traditional Chinese medicines.

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