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Original article

Genetic stability of wild *Rubus sachalinensis* Léveillé cultivated using tissue culture and rapid propagation techniques examined with the ISSR-PCR system

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Summary

Background – **Rapid plant growth and high yield can be achieved via the adoption of tissue culture and rapid propagation systems. Hence, the influence of external factors can be eliminated, and the problems caused by the scarcity of wild resources can be addressed. The comparative ISSR molecular marker analysis of test-tube and wild plantlets can reveal the genetic stability of test-tube plantlets. Thus, it is of great significance for studying germplasm resources, such as those of *Rubus sachalinensis* Léveillé. This study was conducted to acquire a large quantity of *Rubus sachalinensis* Léveillé plantlets and provide a basis for its large-scale production.** *Result* – **A regeneration system for *Rubus sachalinensis* Léveillé was established in this study using axillary buds as explants in vitro. Tissue culture seedlings were amplified using PCR with simple repeat intervals. Axillary buds of *Rubus sachalinensis* Léveillé sterilized in 75% alcohol for 40 s and then in 0.1% $HgCl_2$ for 8 min were the most effective. Their contamination and browning rates were rather low, and they reached a survival rate of 78.34%. The optimum medium compositions for proliferation, strong plant culture, and root induction were MS + 6-BA 2.0 mg L^{-1} + KT 0.5 mg L^{-1} + NAA 0.1 mg L^{-1} + IBA 0.05 mg L^{-1} (proliferation coefficient: 7.71), MS + 6-BA 2.0 mg L^{-1} + KT 0.5 mg L^{-1} + NAA 0.1 mg L^{-1} + IBA 0.1 mg L^{-1} + GA_3 0.5 mg L^{-1} (proliferation coefficient: 6.60), and ½ MS + IBA 1.5 mg L^{-1} (rooting rate: 97.04%), respectively. The most suitable transplanting medium for test-tube plantlets was a mixed medium of humus, vermiculite, and perlite (2:1:1 v/v), and the survival rate following transplantation was 100%.** *Conclusion* – **Rubi Ramulus is the dried stem of *Rubus sachalinensis* Léveillé. It is used to treat cold, immature fever, stroke fever, cough, "heyi" fever and other diseases. Since the twentieth century, with the rapid development of Mongolian medicine industry and the increasing amount of Mongolian medicinal materials, the wild resources are decreasing day by day. It severely restricts the healthy development of Mongolian medicine industry. In order to protect the wild resources, this study established the tissue rapid propagation system for the first time. Using adventitious buds as explants, it can cultivate high quality seedlings in a short period of time. It overcomes the scarcity of wild *Rubus sachalinensis* Léveillé and protects the ecological environment. At the same time, the tissue culture seedlings with good reproducibility, low mutation rate and high genetic stability were obtained by PCR amplification with simple repeat interval. It not only opened up a new way for the rapid propagation of raspberry tissue, but also laid a foundation for the preservation of fine characters and the protection of resources.**

Significance of this study

What is already known on this subject?

- Plants in the genus *Rubus* have a long history of medicinal and fruit use, with a history of cultivation in Europe and America. The fruit of Kuye Hook is used in clinical practice, with a long history of application and no toxicity reports.

What are the new findings?

- This study established an in vitro regeneration system using adventitious buds as explants. The system was cultured under human control, resulting in rapid growth and high yield, improving the productivity of medicinal materials to meet market demand.
  An ISSR-PCR reaction system was established, and orthogonal experimental design was used to optimize the template DNA, primer content, Mix enzyme content, deionized formamide content, and bovine serum albumin content in the reaction system.
  Finally, genetic stability testing was conducted on the obtained tissue culture seedlings.

What is the expected impact on horticulture?

- The combination of multiple molecular marker technologies can be used to improve species identification ability. Researchers can make choices based on different purposes, species, and requirements.

Keywords
Rubus sachalinensis Léveillé, axillary buds, tissue culture, rapid propagation, ISSR-PCR

Introduction

Rubi Ramulus is the dried stem of *Rubus sachalinensis* Léveillé, a plant widely distributed in China, Japan, Korea,

the Soviet Union, and Europe (Chinese Flora, 1988). Alpine hookwood is often used in Chinese herbal medicine (Caidan et al., 2016). It has anti-inflammatory, antibacterial, antioxidant, and other biological activities (Ren et al., 2015). It is primarily used to treat colds and coughs (Inner Mongolia Botanical Medicine, 1989) and has a high medicinal value, different parts have different effects. For fruit, the main effect is solid fine, urine shrinkage; stem and leaf, the main effect is to eliminate rheumatism, detoxification; root, the main effect is to stop bleeding; dry stems (called *Rubus mongolicus*), the main effect is "Heyi" hot, immature heat, cold, cough (Ren et al., 2015), more research in Mongolian clinical medicine. Rubi Ramulus was included in the "Drug Standards of the Ministry of Health of the People's Republic of China, Mongolian Medicine Volume" in 1998. In the classic Mongolian medicine plague heat syndrome basic prescription 'Quattuor sapidity elecampane inula root powder' as the first batch of national medicine contained in the 'Chinese Pharmacopoeia 2010 Edition' in an upside-down variety, under the Mongolian prescription 'Four-flavor Tu mu xiang Powder'; there are more than 20 commonly used clinical prescriptions and prescriptions containing this product. Among them, wulan thirteen herbs' powder, Kegan Eridun Tablets, Kegan Eridun Capsules, Incense tranquilization Scatter, Elecampane inula root five flavours juscul jusculum powder, Clear odynolysis eleven sapidity pill are all included in the 1998 edition of the "Drug Standards of the Ministry of Health of the People's Republic of China, Mongolian Medicine Volume" (Zhang et al., 2013), and there are Mongolian pharmaceutical companies producing warehouse pages with high medicinal value.

In recent years, the Mongolian medicine industry has been growing, the market is in short supply, and the problem of decreasing wild resources caused by unreasonable use of resources and environmental changes has become increasingly prominent. Some Mongolian medicine clinical institutions and Mongolian medicine production enterprises can only use substitutes, which limits the healthy development of the Mongolian medicine industry. Our research group has been engaged in the antiviral clinical research of 'Quattuor sapidity elecampane inula root powder' for a long time, and has carried out in-depth research on its medicinal material Rubi Ramulus. Through literature review, there has been no report on sexual reproduction or asexual reproduction of *Rubus sachalinensis* Léveillé. Plant tissue culture technology is at the core of the field of plant science innovation (Kang et al., 2011). It has become a reliable technology for the large-scale production of plant materials and rapid and efficient plant reproduction (Dias et al., 2015). Using this experimental technique, a large number of cells can be obtained from explants, and the process is unaffected by season, temperature, and moisture levels (Pande and Gupta, 2013; Shahzad et al., 2017). Plant hormones such as auxin and cytokinin also play a crucial role in the developmental pathways of plant cells and tissues in culture media (Sasidharan et al., 2017).

In order to effectively accelerate the protection of wild

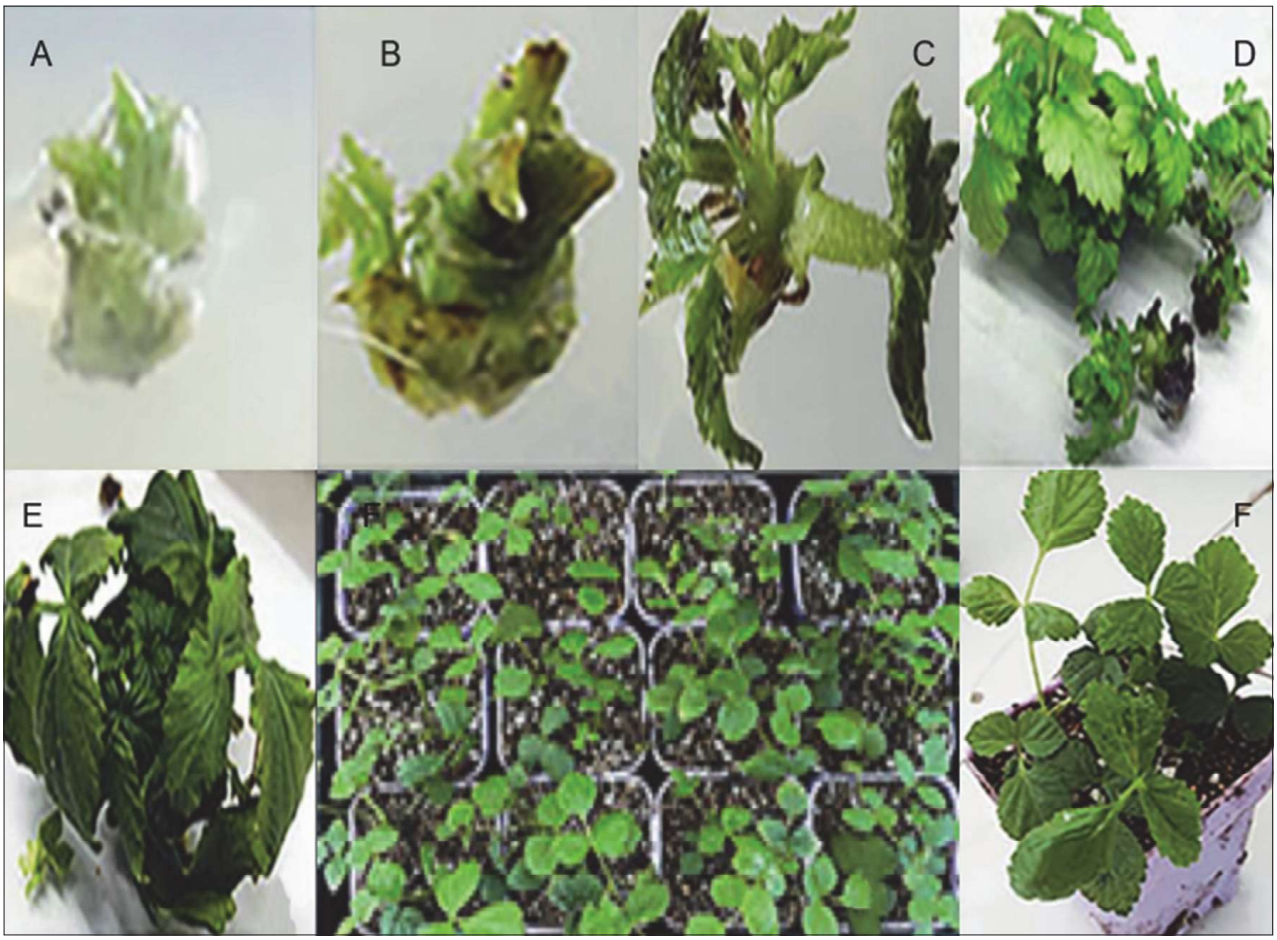

Figure 1. A: Axillary buds of *Rubus sachalinensis* Léveillé used as explants; B: Growth of axillary buds occurring after 5 days of culture; C: Expansion and elongation of explant occurring after 15 days of culture; D: Multiple buds seen after culture for 30 days; E: Growth of multiple buds into multiple shoots after 30 days; F: Whole-genome DNA extraction from different parts of wild *Rubus sachalinensis* Léveillé.

resources, on the one hand, this study established an in vitro regeneration system, using adventitious buds as explants, through a series of aseptic operation processes such as disinfection and germination, proliferation, strong seedling and rooting of explants, and cultured under human regulation. Rapid growth, high yield, greatly improving the productivity of medicinal materials to meet market demand. On the other hand, the ISSR-PCR reaction system was established. The orthogonal test design was used to optimize the template DNA, primer content, Mix enzyme content, deionized formamide content and bovine serum albumin content in the reaction system, and the accurate and sensitive bands were obtained. Finally, the genetic stability of the obtained tissue culture seedlings was tested to ensure the authenticity and consistency of the medicinal materials and maintain the original excellent characteristics of the mother plant. It not only effectively overcomes the problem of resource scarcity caused by the unreasonable utilization of wild resources, but also lays a foundation for the large-scale and efficient cultivation of *Rubus sachalinensis* Léveillé.

Materials and methods

Experimental materials

Rapid propagation: In the early summer period, plants with strong growth and no pests or diseases were selected. Adventitious buds and their outer spore scales were completely removed from the branches, wrapped in a wet paper towel, and placed in a culture bottle for transport to the laboratory. Tissues were stored at 4°C under sufficient moisture.

ISSR-PCR: Fresh wild *Rubus sachalinensis* Léveillé leaves and stems were obtained, frozen in liquid nitrogen, and stored in an ultra-low temperature refrigerator.

Rapid propagation technique

1. Preparation and conditions of culture medium. All experiments were carried out using 3% sucrose (w/v) and 0.7% agar (w/v). The pH was adjusted to 5.8 before autoclaving. Media were sterilized by autoclaving at 121°C under 15 psi pressure for 20 min. All cultures were maintained at 25±1°C in a culture room under white fluorescent lamps at an intensity of 90 µmol m^{-2} s^{-1} with a 16-h photoperiod.

2. Disinfection of explants. The outer scales of the obtained adventitious buds were peeled off until the layer closest to the bud was reached, and the explants were washed with 1% detergent (w/v) for 3 h. Peel off the last layer of scales from the explants. The explants were then sterilized with 75% alcohol for 20 s to 40 s and with 0.1% (w/v) $HgCl_2$ plus a drop of Tween 80 for 5 min to 9 min. Finally, the explants were washed five times with sterile distilled water. The sterile explants were freshly cut under laminar airflow and inoculated on the primary culture medium (Figure 1A). The composition of the primary culture medium was as follows: MS + 6-BA 0.5 mg L^{-1} + KT 0.1 mg L^{-1} + NAA 0.1 mg L^{-1}. The germination and contamination rates of the explants were recorded after 15 days.

3. Shoot multiplication. The primary cultures were transferred to the shoot multiplication medium. The shoot multiplication medium was MS medium supplemented with a combination and different 6-BA (0.5, 1.0, and 2.0 mg L^{-1}), KT (0, 0.1, and 0.5 mg L^{-1}), NAA (0.05, 0.1, and 0.2 mg L^{-1}), and IBA (0.05, 0.1, and 0.2 mg L^{-1}) concentrations for shoot multiplication. Three repeats were set for each treatment, with 30 bottles grafted per repeat. Bud growth was observed, and the proliferation coefficient was calculated after 30 days.

4. Shoot growth culture. Small and weak axillary buds from the multiplication medium were transferred to the growth culture medium. The growth culture medium was multiplication medium supplemented with GA_3. Three replicates were set up for each treatment and 30 vials were grafted for each replicate. Bud growth was observed, and the proliferation coefficient was calculated after 30 days.

5. Root induction. Strong seedlings with a stem length of about 1.5 cm were selected and transferred to the rooting medium. The root induction medium was ½ MS medium supplemented with various combinations of auxins like IBA and NAA. Thirty bottles were grafted per repeat. The rooting parameters (number of roots produced by each explant and root length) were recorded after 40 days of root initiation.

6. Hardening and acclimatization. When the root length was over 3 cm, the lid was opened and the plantlets were moved into a greenhouse (temperature 25 ± 2°C, humidity about 90%) for acclimatization. The in vitro rooted plantlets were carefully removed from the culture bottles and washed thoroughly with distilled water to remove any traces of the medium. The plantlets were transplanted into different mixed medium soils for hardening the seedlings. The survival rate was recorded after 30 days.

Statistical analysis

The experimental design was completely random. Data were analyzed using analysis of variance (ANOVA) to calculate statistical significance. Significant differences in treatment data (mean ± standard error) were assessed at the $p < 0.05$ level using Bonferroni's multi-range test. Germination rate = number of germinal explants/number of inoculated explants × 100%. Contamination rate = number of contaminated explants/number of inoculated explants × 100%. Proliferation coefficient = (total number of buds – number of inoculated buds)/number of inoculated buds × 100%. Rooting rate = number of roots/number of inoculated roots × 100%. Survival rate = number of living explants/number of inoculated explants × 100.

Establishment of ISSR-PCR system

Genomic DNA extraction

The stems and leaves of 200 mg fresh *Rubus sachalinensis* Léveillé were weighed, fully washed with double distilled water, and dried in the shade. Then, 3% PVP was added, and the stems and leaves were ground into a fine powder in liquid nitrogen. The fine powder was transferred to a 5.0 mL centrifuge tube. Subsequently, 2,000 µL buffer LP 1 and 12 µL RNase A (10 mg mL^{-1}) were added, vortexed for 1 min, and placed at room temperature for 60 min. Following this, 130 µL buffer LP 2 was added. The contents were mixed well, vortexed for 1 min, and centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a sterilized centrifuge tube, and this procedure was repeated 5 times. A 1.5-fold volume of buffer LP 3 (anhydrous ethanol was added before use) was added, and the contents were immediately mixed well for 15 s. At this time, flocculent precipitation occurred in some cases. The supernatant was transferred to an adsorption column CB 3 (CB 3 was placed in the collection tube), and centrifuged at 12,000 rpm for 30 s. The waste liquid was discarded, and the adsorption column CB 3 was placed in a collection tube. Then, 600 µL rinsing solution PW (add anhydrous ethanol before use) was added to adsorption column CB 3 and centrifuged at 12,000 rpm for 30 s. The waste liquid was discarded, and the adsorption column CB 3 was added

to a collection tube. The procedure was repeated four times. Then, CB 3 was put back into the collection tube and centrifuged at 12,000 rpm for 2 min, and the waste liquid was discarded. The adsorption column CB 3 was placed at room temperature for 15 min. CB 3 was transferred to a clean centrifuge tube, with the adsorption film suspended in the middle. Then, 50 µL TE was added and the mixture was placed at room temperature for 5 min. Subsequently, the mixture was centrifuged at 12,000 rpm for 2 min.

Detection of DNA purity and concentration

Weighing 200 mg of fresh *Rubus sachalinensis* Léveillé stem segments and leaf blades. Then, 2.5 µL of extracted DNA was added to a tube and its purity was detected using 0.8% agarose gel electrophoresis. The extracted genomic DNA was stored at -20°C.

Establishment of ISSR-PCR system

Referring to the method of Tang et al. (2014), template DNA with high purity was selected for ISSR-PCR. Using primers, template DNA, enzyme mix, bovine serum protein, and deionized formamide as factors, the ISSR-PCR system was developed based on an orthogonal design L16 (4) 5. The concentrations of each factor are shown in Table 1, and the treatment combinations are shown in Table 2. Each tube was supplemented with ddH_2O to make up a volume of 20 µL, with three parallel experiments per treatment conditions. Based on the three experiments, the treatment conditions providing the highest amplification and the clearest bands were selected as the optimal conditions for PCR amplification.

ISSR-PCR amplification

Referring to the methods of Mu et al. (2006), the primers UBS 844 (sequence CTC TCT CTC TCT CTC TRC) were used preliminarily, and DNA from the leaves of wild *Rubus sachalinensis* Léveillé was used as the template DNA. The amplification procedure was as follows: pre-denaturation at 94°C for 2 min, followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. A total of 30 cycles were performed, with extension at 72°C for 30 s and preservation at 4°C.

Screening of ISSR-PCR primers and determination of the optimal annealing temperature

Based on the establishment of the optimal ISSR-PCR system for *Rubus sachalinensis* Léveillé, DNA from the leaves of wild *R. leptospiroides* was used as template DNA. Primers for reference molecular markers of *Rubus sachalinensis* Léveillé were screened (Liu et al., 2018; He et al., 2012). The primer sequences were provided by the University of Columbia (UBC), Canada, and the primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Table 3).

Data analysis

ISSR-PCR amplification data were statistically analyzed. Bands were tagged as "1" if they were present on the electro-

Table 1. Constituents of all tested ISSR-PCR systems.

Sr. No.	Experimental factors					ddH_2O
	A Primer concentration (µmol L^{-1})	B DNA template (ng)	C Enzyme mix (µL)	D Bovine serum albumin (µg $µL^{-1}$)	E Deionized formamide (%)	
1	0.2	10	9	1.5	0.5	Complement 20 µL
2	0.3	20	10	2.0	0.75	
3	0.4	25	11	2.5	1.0	
4	0.5	30	12	3.0	1.5	

Table 2. Orthogonal test table L_{16} (4^5).

Number	Experimental factors					ddH_2O
	A	B	C	D	E	
1	1	1	1	1	1	Complement 20 µL
2	1	2	2	2	2	
3	1	3	3	3	3	
4	1	4	4	3	4	
5	2	1	2	3	4	
6	2	2	1	4	3	
7	2	3	4	1	2	
8	2	4	3	2	1	
9	3	1	3	4	2	
10	3	2	4	3	1	
11	3	3	1	2	4	
12	3	4	2	1	3	
13	4	1	4	2	3	
14	4	2	3	1	4	
15	4	3	2	4	1	
16	4	4	1	3	2	

phoresis gel, and as "0" if no bands were seen (Zhang et al., 2014). Inconsistent bands across samples were considered variation bands (including deletion and increase).

Table 3. ISSR-PCR primers and their sequences.

Primer	Sequence (5'–3')
UBC-807	AGAGAGAGAGAGAGAGT
UBC-811	GAGAGAGAGAGAGAGAC
UBC-814	CTCTCTCTCTCTCTCTA
UBC-826	ACACACACACACACACC
UBC-834	AGAGAGAGAGAGAGAGYT
UBC-835	AGAGAGAGAGAGAGAGYC
UBC-844	CTCTCTCTCTCTCTCTRC
UBC-846	CACACACACACACACART
UBC-848	CACACACACACACACARG
UBC-856	ACACACACACACACACYA
UBC-873	GACAGACAGACAGACA
UBC-880	GGAGAGGAGAGGAGA
UBC-890	VHVGTGTGTGTGTGTGT
UBC-891	HVHTGTGTGTGTGTGTG

Product detection

Leaf DNA from wild and tissue culture seedlings of *Rubus sachalinensis* Léveillé was amplified using PCR with 9 sets of primers according to the PCR program**.** The amplified products were electrophoresed on a 0.8% agarose gel. Staining was performed after electrophoresis, and the stained gel was observed and photographed using a gel imaging system.

Results

Rapid propagation technique

1. Establishment of disinfection protocol. After the explant was inoculated with the primary culture medium for 5 days, axillary buds began to germinate and grow (Figure 1B). After 15 days, the explants expanded and elongated (Figure 1C). Explant sterilization experiments (Table 4) demonstrated that the duration of treatment with 75% alcohol has significant effects on the germination rate and contamination rate. The germination rate and contamination rate were the poorest after 20 s of treatment ($P < 0.05$). When the treatment duration of 0.1% $HgCl_2$ remained constant, the germination rate increased with an increase in the 75% alcohol treatment duration, and the contamination rate decreased. The sterilization effect was optimal under experi-

Table 4. Effects of different sterilization protocols on explants sterilization.

Sr. No.	75% alcohol treatment time (s)	0.1% $HgCl_2$ treatment time (min)	Germination rate	Contamination rate
1	20	5	17.66±0.71^{e}	71.97±2.15^{a}
2	20	7	20.25±0.19^{e}	61.15±1.77^{b}
3	20	9	34.44±1.57^{d}	50.48±0.52^{c}
4	30	5	44.30±2.89^{cd}	32.44±2.53^{d}
5	30	7	51.71±1.14^{bc}	27.75±1.03^{d}
6	30	9	41.23±4.35^{d}	29.67±4.11^{d}
7	40	5	60.11±2.59^{b}	23.25±2.47^{d}
8	40	7	78.34±1.67^{a}	5.527±3.45^{e}
9	40	9	34.67±1.44^{d}	21.21±0.92^{d}

Data are shown as the mean ± SE.
Means with the same letters along the column are not significantly different at $p \leq 0.05$.

Table 5. Effect of different concentrations of PGRs on the shoot multiplication of *Rubus sachalinensis* Léveillé.

Sr. No.	PGRs (mg L^{-1})				Proliferation coefficient	Growth status
	6-BA	KT	NAA	IBA		
1	0.5	0	0.05	0.05	3.67±0.68^{bc}	Strong growth, high buds, and fresh leaves
2	0.5	0.1	0.1	0.1	5.12±0.92^{b}	Strong growth, high buds, and fresh leaves
3	0.5	0.5	0.2	0.2	4.53±0.29^{b}	Weak growth, short buds, and yellow leaf tips
4	1.0	0	0.1	0.2	2.06±0.52^{c}	Weak growth, short buds, and yellow leaves
5	1.0	0.1	0.2	0.05	2.31±0.40^{c}	Weak growth, short buds, and yellow leaf tips
6	1.0	0.5	0.05	0.1	3.49±0.36^{bc}	Strong growth, short buds, and fresh leaves
7	2.0	0	0.2	0.1	3.92±0.11^{bc}	Ordinary growth and short buds
8	2.0	0.1	0.05	0.2	3.68±0.66^{bc}	Ordinary growth and fresh leaves
9	2.0	0.5	0.1	0.05	7.71±0.65^{a}	Significantly high bud proliferation, short buds, and fresh leaves
K1	4.44	3.22	3.61	4.56		
K2	2.62	3.70	4.96	4.18		
K3	5.10	5.24	3.59	3.42		
R	2.48	2.03	1.38	1.14		

Data are represented as the mean ± SE.
Means with the same letters along the column are not significantly different at $p \leq 0.05$.

Table 6. Effect of different concentrations of PGRs on the shoot growth of *Rubus sachalinensis* Léveillé.

Sr. No.	PGRs (mg L^{-1})					Proliferation coefficient	Growth status
	6-BA	KT	NAA	IBA	GA_3		
1	2.0	0	0.05	0	0.5	3.33±0.34^{bc}	Ordinary growth and fresh leaves
2	0.5	0	0	0.1	0.5	4.94±0.50^{ab}	Strong growth, high buds, and fresh leaves
3	1.0	0	0.1	0	0.5	2.41±0.23^{c}	Strong growth, high buds, and fresh leaves
4	2.0	0.5	0.1	0.1	0.5	6.60±0.69^{a}	Significantly high bud proliferation, high buds, and fresh leaves
5	1.0	0.25	0.05	0.05	0.5	4.81±0.35^{ab}	Strong growth, high buds, and fresh leaves
6	0.5	0.25	0.05	0.05	0.5	4.23±0.76^{bc}	Strong growth, high buds, and fresh leaves

Data are represented as the mean ± SE.
Means with the same letters along the column are not significantly different at $p \leq 0.05$.

mental condition no. 8. Hence, the best sterilization conditions for axillary buds of *Rubus sachalinensis* Léveillé included treatment with 75% alcohol for 40 s, followed by treatment with 0.1% $HgCl_2$ for 7 min. Under these conditions, the germination rate of explants was 78.34%, and the contamination rate was 5.527%.

2. Orthogonal test for cluster bud propagation in shoot multiplication medium. The primary cultures were transferred to the shoot multiplication medium. New axillary buds arose after 6 days, and after 30 days, the proliferation coefficients were 2.06–7.71. The bud proliferation results (Table 5) showed that 6-BA had the best effect on the proliferation of hook buds, followed by KT, NAA, and IBA. The range provided by 6-BA was 2.48 and the range provided by KT was 1.14. Experimental conditions no. 1 and no. 2 yielded strong growth, high buds, and fresh leaves. Meanwhile, no. 3, no. 4, and no. 5 yielded weak growth, short buds, and yellow leaf tips. Under condition no. 9, the proliferation coefficient was 7.71, and bud proliferation was significantly higher than that under other conditions ($P < 0.05$) (Figure 1D). Hence, the optimal concentration of the proliferation medium was chosen as MS + 6-BA 2.0 mg L^{-1} + KT 0.5 mg L^{-1} + NAA 0.1 mg L^{-1} + IBA 0.05 mg L^{-1}.

3. Effect of gibberellin (GA_3) on multiple bud rejuvenation. The seedlings obtained after propagation in the multiplication medium showed slow growth and were weak. Thus, they were unfit for root induction. The growth culture medium was obtained by supplementing the multiplication medium with GA_3. Bud proliferation results (Table 6) demonstrated that experimental condition no. 4 provided significantly higher bud proliferation than the other experimental conditions ($P < 0.05$) (Fig. 1E), whereas condition no. 3 yielded the lowest bud proliferation ($P < 0.05$). The optimal growth medium was thus MS + 6-BA 2.0 mg L^{-1} + KT 0.5 mg L^{-1} + NAA 0.1 mg L^{-1} + IBA 0.1 mg L^{-1} + GA_3 0.1 mg L^{-1}.

4. Effects of different IBA and NAA concentrations on rooting. Root induction results (Table 7) showed that experimental condition no. 3 provided significantly higher root induction than the other conditions ($P < 0.05$). The rooting rate after 40 days was 97.04%, average root number 8.05, and average root length 3.05 cm. The root germination time was the longest and the rooting rate was the lowest in condition no. 1. In the absence of the hormone NAA, the rooting rate increased with an increase in the mass concentration of the hormone IBA. This indicated that IBA can effectively promote root induction in *Rubus sachalinensis* Léveillé.

5. Effects of different soil substrates and acclimation durations on the survival rate of seedlings. Robust tissue culture seedlings were selected and transplanted into different soil matrixes after hardening (3, 5, 7, and 10 d). As shown in Table 8, the survival rate of the three soil matrixes was high when the cap was opened for 7 days (Figure 1F). In

Table 7. Effect of different concentrations of IBA and NAA on root induction.

Sr. No.	PGRs (mg L^{-1})		Rooting rate (%)	No. of roots/ microcutting	Root length (cm)	Growth status
	IBA	NAA				
1	0.5	0	29.63±2.09^{d}	3.42±0.17^{b}	0.68±0.09^{d}	Few roots, short and thin, fibril growth seen
2	1.0	0	46.67±1.82^{c}	4.46±0.22^{b}	1.07±0.17^{cd}	Few roots, short and thin, roots
3	1.5	0	97.04±2.77^{a}	8.05±0.60^{a}	3.05±0.19^{a}	Roots grew quickly and were long and strong
4	0	0.2	36.67±1.11^{d}	3.19±0.26^{b}	1.78±0.33^{bc}	Roots grew slowly and were short and thin
5	0.1	0.5	72.59±3.78^{b}	5.14±0.99^{b}	2.16±0.24^{b}	Many roots, long and strong

Data are represented as the mean ± SE.
Means with the same letters along the column are not significantly different at $p \leq 0.05$.

Table 8. Effect of various soil matrixes and seeding times on survival rate.

Sr. No.	Soil matrixes	Survival rate of 3-day seedlings (%)	Survival rate of 5-day seedlings (%)	Survival rate of 7-day seedlings (%)	Survival rate of 10-day seedlings (%)
1	Humus:vermiculite:perlite (2:1:1, V/V)	75.00%	100%	100%	91.67%
2	Sand:vermiculite:perlite (2:1:1, V/V)	33.33%	75.00%	95.83%	70.83%
3	Humus:sand (1:1, V/V)	54.17%	100%	100%	79.17%

Data are represented as the mean ± SE.
Means with the same letters along the column are not significantly different at $p \leq 0.05$.



Figure 2. Whole-genome DNA extraction from different parts of wild *Rubus sachalinensis* Léveillé.
M: 100 bp DNA ladder; 1: Stem; 2: Leaf.

contrast, the survival was the lowest when the cap was opened for 3 days. This may be because the seeding had little ability to adapt to the external environment because of the short duration of hardening. When the cap was opened for 10 days, a lot of bacteria may have been introduced into the medium, which is not conducive to plant growth. Plants grown in sand:vermiculite:perlite (2:1:1, v/v) had a lower survival rate than those grown under the other two combinations. This may have been due to the high hydrophobicity and weak water retention ability of the medium, which reduced the survival rate of hardened seedlings.

Establishment of ISSR-PCR system

1. DNA extraction from different parts of wild hook seedlings. Agarose gel electrophoresis was performed to detect the purity of genomic DNA extracted from different parts of wild *Rubus sachalinensis* Léveillé seedlings (Figure 2). None of the DNA electrophoresis bands showed any tailing phenomenon, and the sample wells in each lane were relatively clean (Yang et al., 2008). A comparison of bands between stem and leaf DNA samples showed that leaf DNA bands were brighter and clearer.

2. Purity and concentration of DNA obtained from different parts of the plant. The concentration and purity of whole-genome DNA extracted from leaf and stem samples were detected using UV spectrophotometry (Cao et al., 2008). As shown in Table 9 data, whole-genome DNA extracted from different parts of the plant showed different A260/A280 ratios on UV spectrophotometry. The average value of the A260/A280 ratio in leaf and stem DNA samples was 1.8184 and 1.6291, respectively, indicating that the DNA purity was higher for leaf samples. Leaf DNA concentration was also higher than stem DNA concentration. Therefore, the leaves were the best source of DNA for further PCR experiments.

3. Establishment of the ISSR-PCR system. The experimental results were analyzed according to the method described by He et al. (1998). Based on the number of amplified bands and degree of separation, brightness, and clarity of PCR amplification bands, an optimal ISSR-PCR amplification system was developed.

The electrophoresis bands obtained using the 16 orthogonal design treatments were clear and showed no trailing phenomenon (Figure 3). However, there were significant differences in PCR amplification results among the 16 treat-

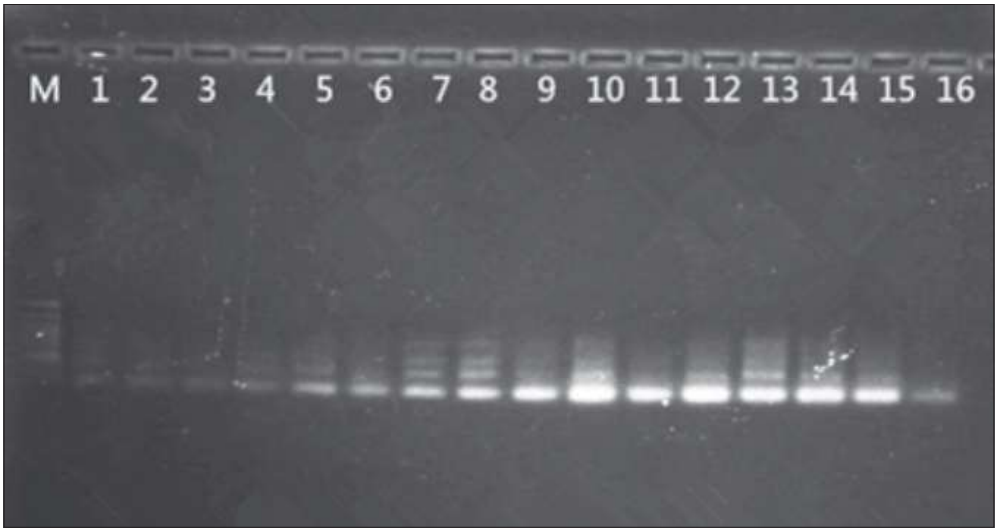

Figure 3. Orthogonal test of the ISSR-PCR system.

Table 9. Determination of the DNA concentration and purity of different parts.

Absorbance of extracted fractions	Blade				Stem			
	X_1	X_2	X_3	X	X_1	X_2	X_3	X
A_{230}	0.0877	0.0942	0.0923	0.0914	0.1252	0.1803	0.1791	0.1615
A_{260}	0.2409	0.2593	0.2406	0.2469	0.1383	0.1427	0.1504	0.1438
A_{280}	0.1329	0.1412	0.1332	0.1358	0.0854	0.0871	0.0923	0.0883
A_{260}/A_{230}	2.7469	2.7527	2.6067	2.7021	1.1046	0.7915	0.8398	0.9120
A_{260}/A_{280}	1.8126	1.8364	1.8063	1.8184	1.6194	1.6383	1.6295	1.6291
DNA concentrations (ng μL^{-1})	120.45	129.65	120.30	123.45	69.15	71.35	75.2	71.9

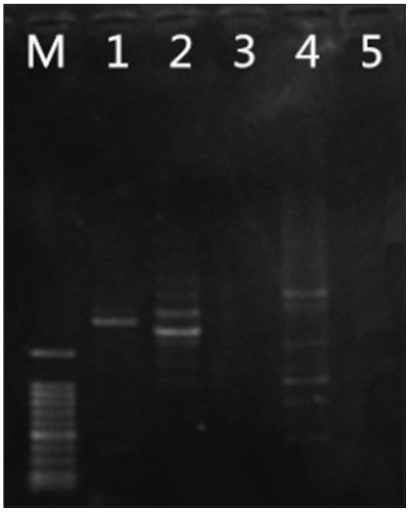

Figure 4. Amplification using primer UBC-807 to primer UBC-834.
M: 100 bp DNA ladder; 1: Primer UBC-834; 2: Primer UBC-826; 3: Primer UBC-814; 4: Primer UBC-811; 5: Primer UBC-807.

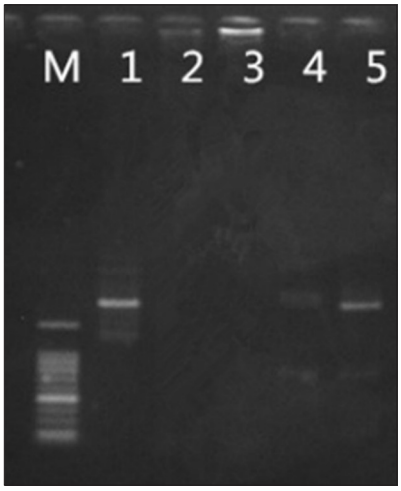

Figure 5. Amplification using primer UBC-835 to primer UBC-856.
M: 100 bp DNA ladder; 1: Primer UBC-856; 2: Primer UBC-848; 3: Primer UBC-846; 4: Primer UBC-844; 5: Primer UBC-835.

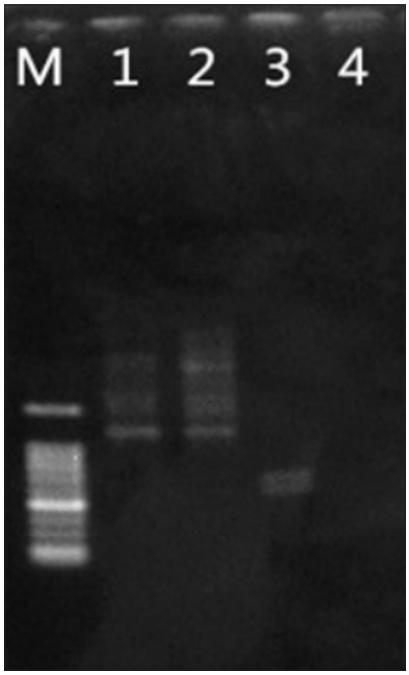

Figure 6. Amplification using primer UBC-873 to primer UBC-891.
M: 100 bp DNA ladder; 1: Primer UBC-891; 2: Primer UBC-890; 3: Primer UBC-880; 4: Primer UBC-873.

ment conditions due to different concentrations and combinations of 5 factors. The bands amplified by combinations 2, 3, 6, and 16 were not obvious and their brightness was weak. The bands amplified by combinations 1, 4, and 5 were obvious, although the brightness was still somewhat low. While the bands amplified by combinations 9, 10, 11, 12, 13, 14, and 15 were pronounced, the background was fuzzy, and the bands were difficult to distinguish. The bands amplified by combinations 7 and 8 were clear and stark. Repeat tests showed that combination no. 8 yielded bands with higher stability and reproducibility than combination no. 7. Therefore, through a comprehensive analysis of various factors in the ISSR-PCR system, condition no. 8 was finally chosen as the optimal reaction system combination. The parameters were as follows: total reaction volume of 20 µL with primer content 0.3 µmol L^{-1}, template DNA content 30 ng, enzyme mix content 11 µL, deionized formamide content 0.75%, and bovine serum albumin content 3 µg mL^{-1}; and PCR conditions involving pre-denaturation at 94°C for 2 min, followed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, with a total of 30 cycles, extension at 72°C for 30 s, and storage at 4°C.

5. ISSR-PCR primer screening. Primers were selected based on the optimal ISSR-PCR system. As can be seen from Figures 4–6, 9 out of 14 primers yielded bands that were high in brightness, low in variation, and suitable for amplification. Thus, UBC-811, UBC-826, UBC-834, UBC-835, UBC-844, UBC-856, UBC-880, UBC-890, and UBC-891 were used as primers for the reaction. Among them, UBC-826, UBC-856, UBC-890, and UBC-891 had the largest number of bands at 7, 8, 9, and 8, respectively.

6. Effect of annealing temperature on ISSR-PCR. In the

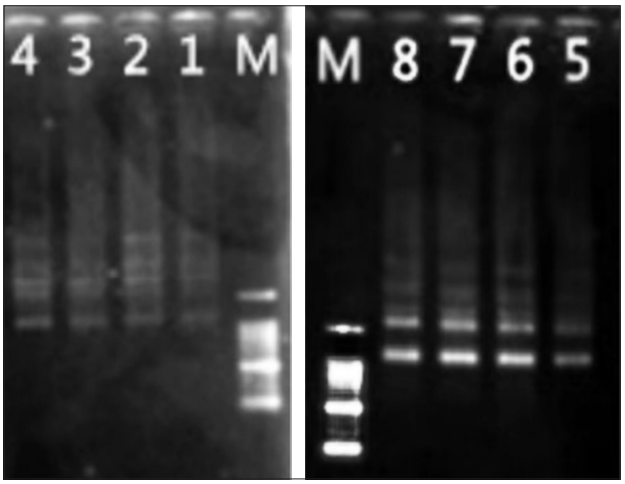

Figure 7. Effect of annealing temperature on primer UBC-811-based amplification.
1–8: Annealing temperature 43.1°C, 43.4°C, 44.1°C, 45.3°C, 48.8°C, 50.0°C, 50.7°C, and 51.0°C, respectively.

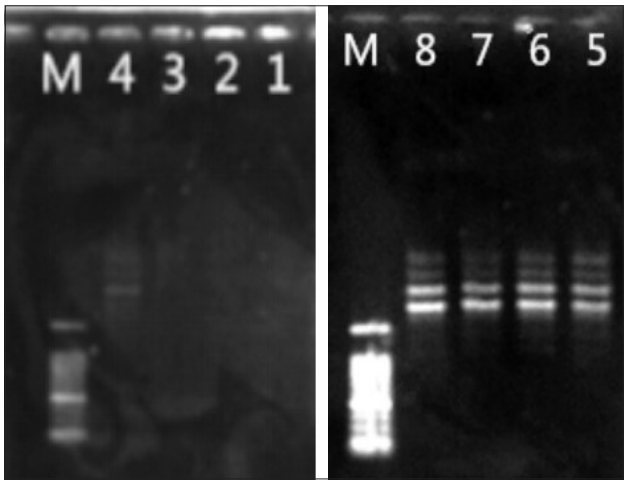

Figure 8. Effect of annealing temperature on primer UBC-826-based amplification.
1–8: Annealing temperature 49.1°C, 49.4°C, 50.1°C, 51.3°C, 52.8°C, 54.0°C, 54.7°C, and 55.1°C, respectively.

reaction system, the specific binding of primers and templates is affected by the annealing temperature. Thus, the annealing temperatures of 9 suitable primers were screened based on an optimal combination of reaction conditions. According to Liu et al. (2009), the annealing temperature usually fluctuates by 3°C based on the Tm value. The 8 gradients automatically generated by the PCR instrument were denoted as 1–8 for our temperature test. Finally, the optimal annealing temperature was determined based on the number and brightness of bands. The number of

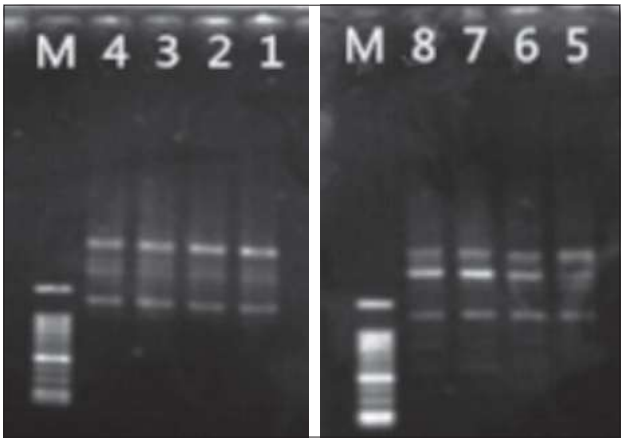

Figure 9. Effect of annealing temperature on primer UBC-834-based amplification.
1–8: Annealing temperature 45.1°C, 45.4°C, 56.1°C, 47.3°C, 48.8°C, 50.0°C, 50.7°C, and 51.0°C, respectively.

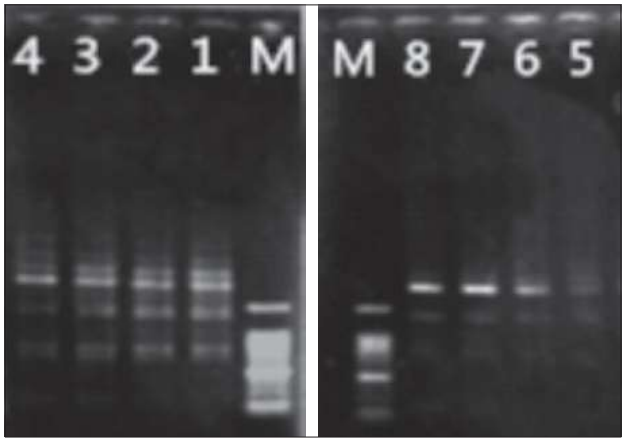

Figure 10. Effect of annealing temperature on primer UBC-835-based amplification.
1–8: Annealing temperature 47.1°C, 47.4°C, 48.4°C, 49.3°C, 50.8°C, 52.0°C, 52.7°C, and 53.1°C, respectively.

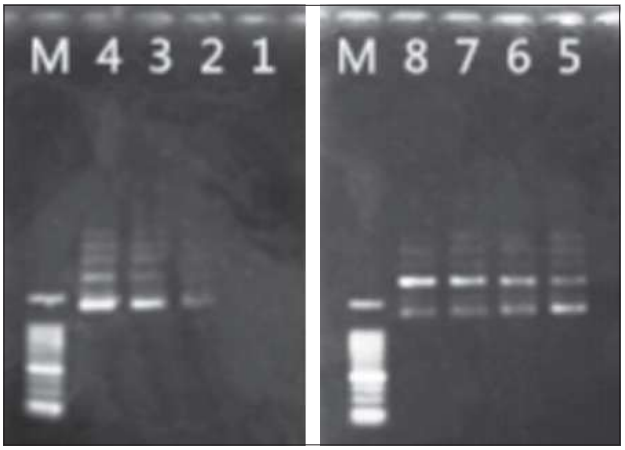

Figure 11. Effect of annealing temperature on primer UBC-844-based amplification.
1–8: Annealing temperature 45.1°C, 45.4°C, 46.1°C, 47.3°C, 48.8°C, 50.0°C, 50.7°C, and 51.0°C, respectively.

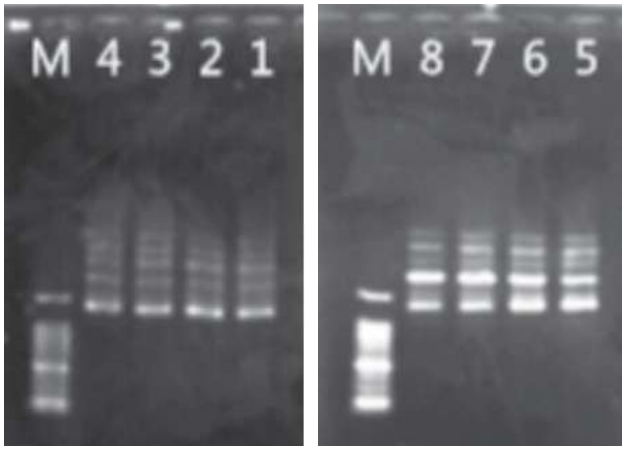

Figure 12. Effect of annealing temperature on primer UBC-856-based amplification.
1–8: Annealing temperature 49.1°C, 49.4°C, 50.1°C, 51.3°C, 52.8°C, 54.0°C, 54.7°C, and 55.1°C, respectively.

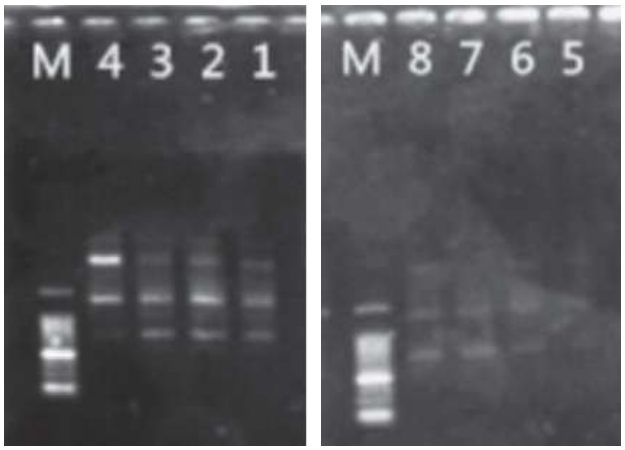

Figure 13. Effect of annealing temperature on primer UBC-880-based amplification.
1–8: Annealing temperature 44.1°C, 44.4°C, 45.1°C, 46.3°C, 47.8°C, 49.0°C, 49.7°C, and 50.0°C, respectively.

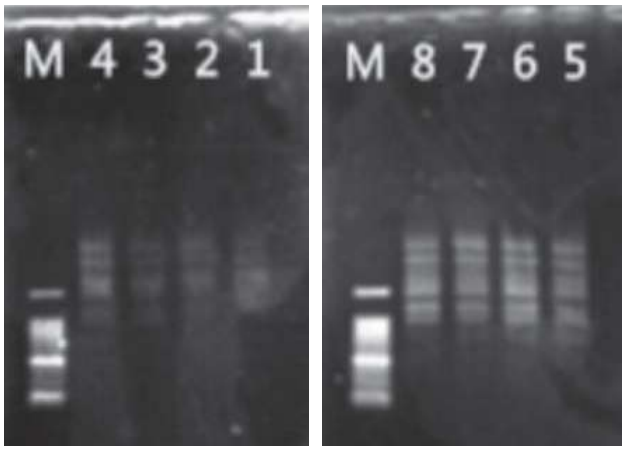

Figure 14. Effect of annealing temperature on primer UBC-890-based amplification.
1–8: Annealing temperature 48.1°C, 48.4°C, 49.1°C, 50.3°C, 51.8°C, 53.0°C, 53.7°C, and 54.1°C, respectively.

Table 10. The number of bands at 8 annealing temperatures corresponding to 9 primers.

	Primer	Tm (°C)	Annealing template concentration grades (1~8)							
			1	2	3	4	5	6	7	8
Band numbers	UBC-811	46.67	**8**	6	5	3	5	4	3	4
	UBC-826	52.63	0	0	0	4	8	**9**	8	6
	UBC-834	48.74	6	5	6	6	**6**	5	5	5
	UBC-835	50.19	10	10	**10**	9	7	7	5	4
	UBC-844	48.60	0	4	7	8	**8**	8	8	7
	UBC-856	52.35	8	7	8	7	**9**	8	8	8
	UBC-880	47.93	8	**8**	7	7	3	2	2	2
	UBC-890	51.99	0	3	4	8	9	10	10	**11**
	UBC-891	53.39	9	8	**10**	10	4	5	5	6

Table 11. Statistical table of 9 primers on DNA amplification bands of 5 samples and 1 parent.

Primer	Number of amplified bands / strip						Total number of amplified bands	Variation bands	Mutation probability (%)
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	dam			
UBC-811	8	8	8	8	8	8	48	0	0
UBC-826	10	9	9	8	9	9	54	2	3.70
UBC-834	7	6	6	6	6	6	37	1	2.70
UBC-835	10	10	10	10	10	10	60	0	0
UBC-844	8	8	8	8	8	8	48	0	0
UBC-856	9	9	9	9	9	9	54	0	0
UBC-880	8	8	8	8	8	8	48	0	0
UBC-890	11	11	11	11	11	11	66	0	0
UBC-891	10	10	10	10	10	10	60	0	0
Sum							475	3	0.63

electrophoresis bands yielded by each primer pair at annealing temperatures 1–8 are shown in Figures 7–15 and in Table 10.

The annealing temperature is an important determinant of PCR specificity. Visual analysis of electrophoresis gels was used to understand the effect of 8 annealing temperatures automatically generated by the PCR instrument. There were differences in the number of bands and band brightness at different temperatures. The primer pair with the highest number of bands was UBC-890 (11 bands), and its optimal annealing temperature was 54.1°C. However, the background was fuzzy, and the bands were difficult to identify. Primer pairs UBC-835 (annealing temperature, 48.4°C) and UBC-891 (annealing temperature, 51.1°C) yielded 10 clear bands. Further, at least 6 bands with good clarity were generated with the primer pair UBC-834 and an annealing temperature of 48.8°C. When the number of bands at different annealing temperatures was the same, the optimal annealing temperature could be determined by the brightness, clarity, and degree of separation between bands. For example,

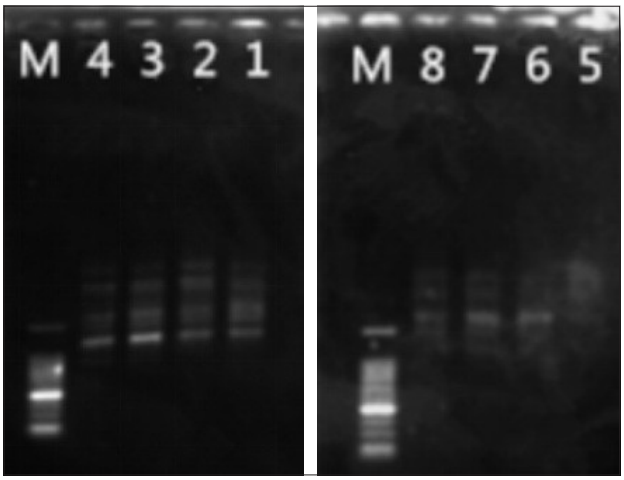

Figure 15. Effect of annealing temperature on primer UBC-891-based amplification.
1–8: Annealing temperature 50.1°C, 50.4°C, 51.1°C, 52.3°C, 53.8°C, 55.0°C, 55.7°C, and 56.1°C, respectively.

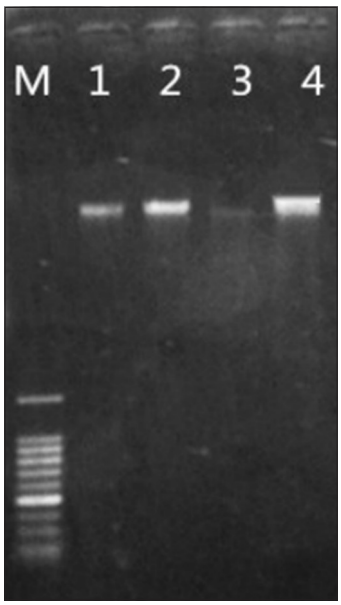

Figure 16. Whole-genome DNA extraction from different parts of wild bramble and tissue culture seedling plants.
M: 100 bp DNA ladder;
1: Stem segment of tissue culture seedlings;
2: Leaves of tissue culture seedlings;
3: Stem segment of wild seedlings;
4: Leaves of wild seedlings.

for the primer UBC-835, when the annealing temperatures were 47.1°C, 47.4°C, and 48.4°C, 10 bands were obtained. However, the bands at 48.4°C were more clearly separated than those at the other two temperatures.

Study of genetic stability

1. DNA extraction from different parts of wild *Rubus sachalinensis* Léveillé and tissue culture seedlings. Agarose gel electrophoresis was used to detect the purity of whole-genome DNA extracted from different parts of wild seedlings and tissue culture seedlings of *Rubus sachalinensis* Léveillé. The results are shown in Figure 16. There was no tailing in the electrophoretic bands, and the wells were all clean. By comparing the bands obtained from tissue culture seedlings and wild seedlings, we found that the DNA bands from the leaves of tissue culture seedlings and wild seedlings were brighter and clearer than those obtained from stems. Stem DNA from tissue culture seedlings was brighter and clearer than that obtained from wild seedlings, while leaf DNA showed similar brightness and clarity between the two samples.

2. Genetic stability analysis of tissue culture seedlings. The 9 selected primers were used for PCR amplification of the DNA extracted from the female parent and tissue culture samples. The amplification results are shown in Figure 17. Statistical analysis of the 9 gels (Table 11) revealed a total of 475 clearly identifiable bands following PCR amplification. Among the tested samples and female parents, samples 1 and 4 amplified by primer UBC-826 each showed a variant band, and sample 1 amplified by primer UBC-834 had a variant band. The other bands were consistent with those of the parent. Therefore, only 0.63% of the DNA bands of the test tube seedlings showed genetic alterations, indicating their high genetic stability.

Discussion

Establishment of rapid propagation system for *Rubus sachalinensis* Léveillé

In this study, the axillary buds of *Rubus sachalinensis* Léveillé were used as explants without any callus tissue, and seedlings with suitable genetic properties were obtained in a short period of time. During multiplication culture, the differentiated buds were weak and thin, which is extremely detrimental to further rooting culture. Therefore, it is necessary to improve the hormone concentration and increase the rate of adventitious bud induction (Wei Ying et al., 2015). The phytohormone GA_3 promotes stem elongation and cell division in plants (Zhou et al., 2016; Hedden and Sponsel, 2015). GA_3 can promote plant growth and increase crop yield, without a ceiling of optimum concentration. Even if the GA_3 concentration is high, an excellent promotion effect can be seen (Li et al., 2011). In this study, 0.5 mg L^{-1} GA_3 was added to the multiplication medium. Although the proliferation coefficient decreased slightly, there were more leaves of adventitious buds and the stems were stronger. Therefore, adding GA_3 can significantly increase the rooting and survival rate of seedlings in the tissue culture of *Rubus sachalinensis* Léveillé.

Acclimatization and transplanting play an important role in determining whether aseptic seedlings can be used in production (Zhu and Li, 2022). Aseptic seedlings grow at high humidity, constant temperature, and weak light conditions (Zou et al., 2021). Hardening gradually enables the seedlings to adapt to their external environment. In this study, aseptic

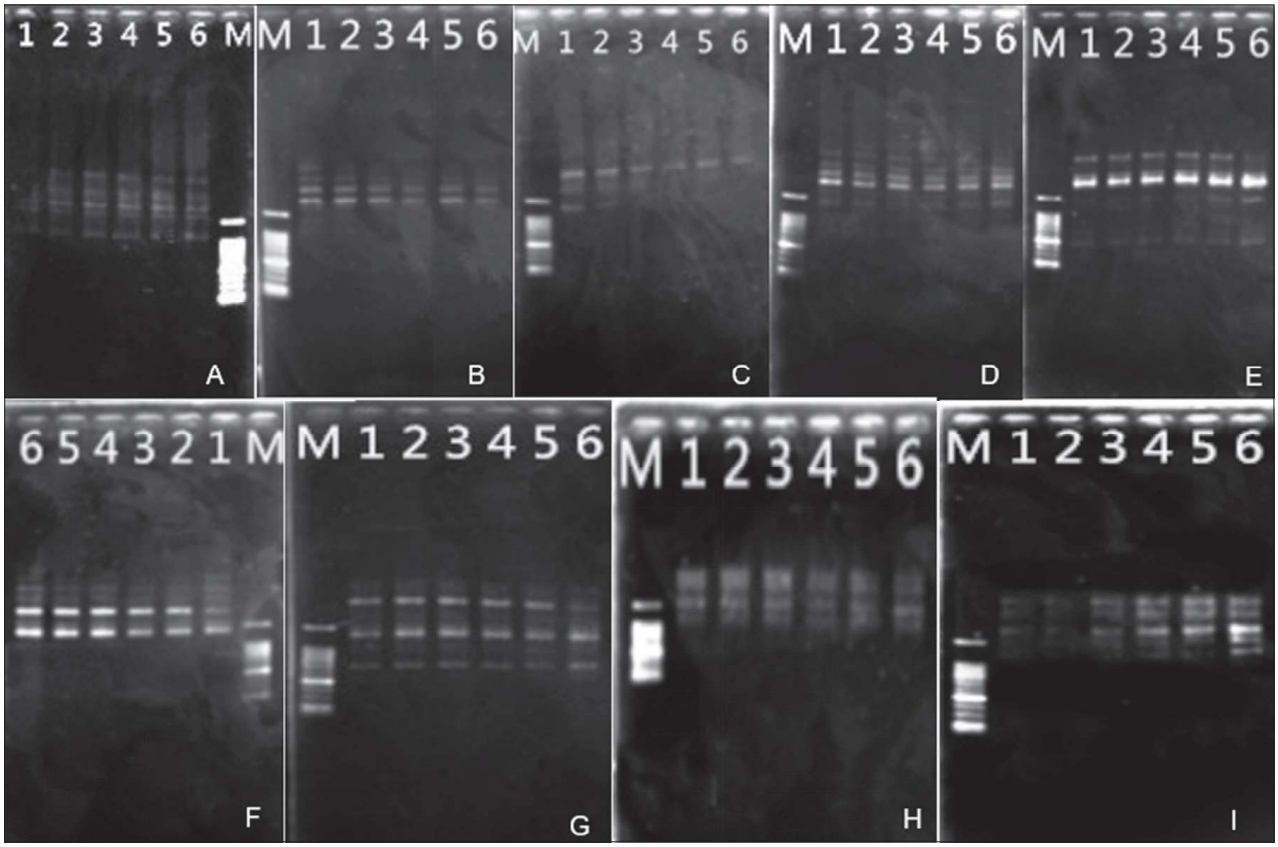

Figure 17. A: Primer UBC-811; B: Primer UBC-826; C: Primer UBC-834; D: Primer UBC-835; E: Primer UBC-844; F: Primer UBC-856; G: Primer UBC-880; H: Primer UBC-890; I: Primer UBC-891.
M: 100 bp DNA ladder; 1–5: Tissue culture seedlings; 6: Wild seedlings.

seedlings were cultured with water for some time. The most suitable conditions for hardening the seedlings were selected, which greatly improved their survival rate.

In the study, axillary buds of *Rubus sachalinensis* Léveillé were used as explants for tissue culture for the first time. This study effectively addresses the problem associated with the limited resources of *Rubus sachalinensis* Léveillé and could help in protecting the ecological environment while still obtaining plants for medicine. It not only opens a new technological avenue for the tissue culture of *Rubus sachalinensis* Léveillé, but also lays the foundation for its large-scale cultivation.

Establishment of ISSR-PCR system

In this study, whole-genome DNA was extracted from wild seedlings using the centrifugal column method. The results showed that the quality of leaf DNA was higher than that of stem DNA. This may have been because the stem was too hard to grind into a fine powder, and DNA could not be isolated sufficiently, resulting in poorer DNA quality. Ultraviolet spectrophotometry was used to detect the purity and concentration of DNA. If DNA samples were contaminated by protein, phenols, or small molecules, their absorbance was affected. The A260/A280 UV absorbance value of pure DNA should be 1.6–1.9, and the A260/A230 value should be greater than 2.0. An A260/A280 value greater than 1.9 indicates RNA contamination and a value less than 1.6 indicates protein or phenol contamination. Likewise, if the A260/A230 value is less than 2.0, it indicates that residual salt and small molecular impurities such as nucleotides, amino acids, and phenols are present in the DNA extract (Mu et al., 2006). Three parallel experiments demonstrated that the DNA purity and concentration were greater when DNA was extracted from leaves than from stems. Therefore, the leaves were the best part for extracting and detecting DNA from *Rubus sachalinensis* Léveillé.

Orthogonal experimental designs are widely used in practical work. Each test number is representative, which can reduce the number of tests and experimental effort (Tang et al., 2014). In this study, the orthogonal test design was used to optimize template DNA, primer, enzyme mix, deionized formamide, and bovine serum albumin content in the reaction system. Finally, an ISSR-PCR system suitable for *Rubus sachalinensis* Léveillé was established: for a total reaction volume of 20 μL, the primer content was 0.3 μmoL L^{-1}, template DNA content was 30 ng, enzyme mix content was 11 μL, deionized formamide content was 0.75%, and bovine serum albumin content was 3 μg mL^{-1}. For the ISSR-PCR system, 9 of 14 primers provided high amplification and good variation. Among them, UBC-826, UBC-856, UBC-890, and UBC-891 provided the largest number of amplified bands, i.e., 7, 8, 9, and 8, respectively. The annealing temperature of different primers is different, and the annealing temperature has a great influence on the primer reaction. Therefore, based on an optimal reaction system combination, the optimal annealing temperature for each primer was screened to obtain more reliable test results.

Study on the genetic stability of tissue culture seedlings of *Rubus sachalinensis* Léveillé

ISSR molecular marker technology is widely used in genetics, bioinformatics, and other aspects of research. The genetic stability and diversity of common plants such as *Gentiana straminea* after ultra-low temperature regeneration (Gao et al., 2015) and Guangxi sweet tea germplasm genetic diversity have been examined (Chen et al., 2017).

In this experiment, 9 primers providing clear amplification bands were screened from 14 primers using the established reaction system. A total of 475 clear and identifiable bands were obtained by analyzing the genomic DNA of the sample and the female parent, and 3 variant bands were detected. The mutation rate was low at 0.63%, consistent with the study by Gao et al. (2015). Hence, our cultured plants had high genetic stability. The findings thus guide strategies for the maintenance of excellent traits in *Rubus sachalinensis* Léveillé and the protection of its resources.

Authors' contributions

Zhang Ping, Yue Xin and Bao Baoquan designed the experiment; Zhu Wenjing, Wang Yanli, Zhao Hongmei and Zhang Ying conducted research. Zhu Wenjing, Wang Yanli, Xue Yan and Zhang Jinqiao analyzed the data. Zhu Wenjing and Wang Yanli wrote this manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The materials used in this study were collected from the Sumushan Forest Farm in Xinghe County, Wulanchabu City, Inner Mongolia Autonomous Region in May 2017. The original plant was identified by Yue Xin, teacher of Inner Mongolia Medical University. *Rubus corchorifolius* belongs to Chinese medicinal materials. This study conforms to the laws and regulations of the People's Republic of China.

Competing interests

The authors declare that they have no competing interests.

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