

Study on Genetic Stability of Wild *Rubus sachalinensis* Leveille Based on Tissue Rapid Propagation Techniques and ISSR-PCR Reaction System

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Study on Genetic Stability of Wild *Rubus sachalinensis* Leveille Based on Tissue Rapid Propagation Techniques and ISSR-PCR Reaction System

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Abstract

Background : By establishing the tissue rapid propagation system, the rapid propagation and large yield were not affected by external factors, and the scarcity of wild resources was effectively solved. Analysis of test-tube plantlets and wild plantlets by ISSR molecular marker technology to detect the stability of genetic characteristics of test-tube plantlets is of great significance to the study of germplasm resources and medicinal value of *Leptospiraea japonica*.

Result: The regeneration system of *Rubus sachalinensis* Leveille was established in this paper by using axillary buds as explants in vitro culture. The results showed that

axillary buds of *Rubus sachalinensis* Leveille were sterilized in 75% alcohol for 40 s and then in 0.1% HgCl₂ for 8 min had the best effect, the pollution and the browning rate of the explants reached a rather low level, with the survival rate of 78.34%. The optimum compositions for proliferation culture, strong plant culture and root inducing were MS + 6-BA 2.0 mgL⁻¹ + KT 0.5 mgL⁻¹ + NAA 0.1 mgL⁻¹ + IBA 0.05 mgL⁻¹, MS + 6-BA 2.0 mgL⁻¹ + KT 0.5 mgL⁻¹ + NAA 0.1 mgL⁻¹ + IBA 0.1 mgL⁻¹ + GA₃ 0.5 mgL⁻¹ and 1/2 MS + IBA 1.5 mgL⁻¹. The proliferation coefficient and rooting rate on these media were up to 7.71, 6.60, and 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 of transplanting was 100 %. This study was conducted to acquire a large quantity of wide *Rubus sachalinensis* Leveille plantlets and provide a basis for large-scale production. The tissue culture seedlings were amplified by PCR with simple repeat interval.

Conclusion: The results showed that the tissue culture seedlings had high genetic stability and low mutation rate compared with wild seedlings. This study not only opens up a new way for the tissue rapid propagation technology of *L. japonica*, but also lays a foundation for the maintenance of excellent traits and resource protection of *L. japonica*.

Krywords: *Rubus sachalinensis* Leveille, axillary buds, tissue culture, rapid propagation, ISSR-PCR

Introduction

Rubi Ramulus is the dried stem of *Rubus sachalinensis* Leveille, distributed in China, Japan, Korea, the Soviet Union and Europe (Chinese flora 1988). Alpine hookworm is often used in herbal medicine in China (Caidan et al., 2016). It has anti-inflammatory, antibacterial, antioxidant and other biological activities (Ren et al., 2015). It is mainly used to treat colds and coughs (Inner Mongolia Botanical Medicine 1989), and has high medicinal value. Since the 20th century, the Mongolian medicine industry has developed rapidly. Researchers and manufacturers have excavated a large number of wild medicinal materials, resulting in the depletion of wild resources. Some enterprises have adopted substitutes, which has seriously restricted the healthy development of the Mongolian medicine industry. In order to make the wild resources no longer be destroyed, this topic for the first time to establish a tissue rapid propagation system for the original plant *R.papyrifera*, using adventitious buds as explants for induction, can quickly and stably breed a large number of excellent tissue culture seedlings, field cultivation and planting experiments have also achieved good results, effectively overcoming the scarcity of wild resources and protecting the natural ecological environment. At the same time, the tissue culture seedlings were amplified by PCR using simple repetitive series intervals. The obtained tissue culture seedlings had high genetic stability and low variation rate compared with wild seedlings. This study not only opened up a new way for the rapid propagation technology of *Rubus sachalinensis* Leveille , but also laid a foundation for the maintenance of excellent traits and the protection of

resources.

Plant tissue culture technology is the core of the application of plant science innovation area (Kang et al,2011). It has become a reliable technology for large-scale production of plant materials for rapid and efficient reproduction of plants (Dias et al., 2015). This is an experimental technique for producing a large number of cells from explants, which is not affected by season, temperature and moisture (Pande and Gupta et al,2013 ; shahzad et al., 2017). Plant hormones such as auxin and cytokinin play a crucial role in the developmental pathways of plant cells and tissues in culture media (Sasidharan et al., 2107). In this study, a series of experiments were carried out by tissue culture method for the first time, aiming to obtain a large number of high-quality plants in a short time, and provide a basis for resource protection, development and utilization.

Materials and Methods

experimental material

rapid propagation: In the early summer period, select the strong growth and no pests and diseases of the plant, the adventitious buds and their outer spore scales completely removed from the branches, wrapped in a wet paper towel completely, into the culture bottle back to the laboratory, 4 °C moisturizing storage.

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

Rapid propagation technique

Preparation and Conditions of Culture Medium

All the experiments were carried out using 3% sucrose (w/v) and 0.7% agar (w/v). Adjust the pH to 5.8 before autoclaving. Media were sterilised by autoclaving at 121 °C temperature with 15 psi pressure for 20 min. All cultures were maintained at 25±1°C in a culture room under white fluorescent lamps at intensity of 90 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with 16 hrs photoperiod.

Disinfection of explants

The obtained adventitious buds were peeled off the outer scales until the layer closest to the bud was retained, and the explants were washed with 1 % detergent (w / v) for 3 hours. Use tweezers to remove scales from the last layer of explants. The explants were then sterilized with 75 % alcohol for 20-40 seconds and with 0.1 % (w / v) HgCl_2 plus a drop of Tween 80 for 5-9 minutes. Finally, the explants were washed 5 times with sterile distilled water. The sterile explants were freshly cut under laminar airflow and inoculated on the primary culture medium (Fig.1). The primary culture medium was MS + 6-BA 0.5 mg L⁻¹ + KT 0.1 mg L⁻¹ + NAA 0.1 mg L⁻¹. The germination rate and contamination rate of explants were recorded after 15 days.

Fig 1 The axillary buds of *Rubus sachalinensis* Leveille used as explants



Shoot Multiplication

The primary cultures were transferred on shoot multiplication medium. The shoot multiplication medium were inoculated on MS medium supplemented with combination and different concentrations of 6-BA (0.5, 1.0, 2.0 mg·L⁻¹), KT (0, 0.1, 0.5 mg·L⁻¹), NAA (0.05, 0.1, 0.2 mg·L⁻¹) and IBA (0.05, 0.1, 0.2 mg·L⁻¹) for shoot multiplication. Set three repeats for each treatment. Graft 30 bottles with each repeat. Observe the growth of the buds and calculate proliferation coefficient after 30 days.

Shoot Growth Culture

The small and weak axillary buds from multiplication medium were transferred on growth culture medium. The growth culture medium supplemented GA₃ on the basis of multiplication medium. Set three repeats for each treatment. Graft 30 bottles with each repeat. Observe the growth of the buds and calculate proliferation coefficient after 30 days.

Root Induction

The strong seedlings with stem length of about 1.5 cm were selected and transferred to rooting medium. The root induction medium were inoculated on 1/2 MS medium supplemented with various combination of auxins like IBA and NAA. Graft 30 bottles with each repeat. The rooting parameters (number of roots produced by each explants and root length) were recorded after 40 days of initiation of roots.

Hardening and acclimatization

When root length is over 3 cm, open lid and move it into greenhouse (temperature is $25\pm 2^{\circ}\text{C}$, humidity is about 90%) and train seedlings. The in vitro rooted plantlets were carefully removed from the culture bottles and washed thoroughly with distilled water to remove traces of the medium. The plantlets were transplanted into different mixed medium soils for hardening-seedlings. The survival rate was recorded after 30 days of plants.

Statistical analysis

The experimental design was completely random. Data were analyzed by analysis of variance to calculate statistical significance. Significant differences in treatment data (mean $\pm$ standard error) were assessed at the $p < 0.05$ level by Bonferroni 's multi-range test. Germination rate = number of germinal explants / number of inoculated explants $\times$ 100 % contamination rate = number of contaminated explants /

$$\text{number of inoculated explants} \times 100 \% \text{ proliferation coefficient} = (\text{total number of buds} - \text{number of inoculated buds}) / \text{number of inoculated buds} \times 100 \%$$

$$\text{rooting rate} = \text{number of roots} / \text{number of inoculated roots} \times 100 \%$$

$$\text{survival rate} = \text{number of living explants} / \text{number of inoculated explants} \times 100.$$

Establishment of ISSR-PCR Reaction System

Genomic DNA extraction

The stems and leaves of 200 mg fresh *Rubus sachalinensis* Leveille were weighed. The leaves and stems were fully washed with double distilled water and dried in the shade. 3 % PVP was added and ground into fine powder in liquid nitrogen. The fine powder was transferred to a 5.0 mL centrifuge tube. 2000 μL buffer LP 1 and 12 μL RNase A (10 mg / mL) were added, vortexed for 1 min, and placed at room temperature for 60 min. Add 130 μL buffer LP 2, mix well, vortex shake for 1 min, centrifuge at 12000 rpm for 5 min, transfer the supernatant to the sterilized centrifuge tube, repeat 5 times. The 1.5-fold volume of buffer LP 3 (anhydrous ethanol was added before use) was added, and immediately shaken and mixed well for 15 s. At this time, flocculent precipitation may occur. The supernatant was transferred to an adsorption column CB 3 (CB 3 was placed in the collection tube), centrifuged at 12000 rpm for 30 s, brushed off the waste liquid, and the adsorption column CB 3 was placed in the collection tube ; add 600 μL rinsing solution PW (add anhydrous ethanol before use) to the adsorption column CB 3, centrifuge at 12000 rpm for 30 s, pour the waste liquid, and put the adsorption column CB 3 into the collection tube,

repeat 4 times ; cB 3 was put back into the collection tube, centrifuged at 12000 rmp for 2 min, and the waste liquid was discarded. The adsorption column CB 3 was placed at room temperature for 15 min. CB 3 transferred to a clean centrifuge tube, adsorption film suspended in the middle, 50 μ L TE at room temperature for 5 min, 12 000 rmp centrifuge 2 m.

Detection of DNA purity and concentration

The stems and leaves of 200 mg wild *Rubus sachalinensis* Leveille were weighed. 2.5 μ L of extracted DNA was sucked, the purity of DNA was detected by 0.8 % agarose gel electrophoresis, and the extracted genomic DNA was stored at -20° C.

Establishment of ISSR-PCR Reaction System

According to the method of Tang et al. , the template DNA with high purity was selected for ISSR-PCR. With primers, template DNA, Mixase, bovine serum protein, and deionformamide as factors, the ISSR-PCR reaction system was set by orthogonal design L16 (4) 5. The concentration levels of each factor are shown in Table 1, and the treatment combinations are shown in Table 2. Each treatment was supplemented with ddH₂O to 20 μ L, and each treatment was subjected to three parallel experiments. According to the results of three experiments, the most clear and most amplified bands were selected, and the optimal conditions for PCR amplification were obtained.

Table 1 Influence factors and level concentration of ISSR-PCR system

Number	experimental factors					ddH ₂ O (μ L)
	A primer concentration (μ mol/L)	B DNA template (ng)	C Mix enzyme (μ L)	D bovine serum albumin (μ g/ μ L)	E Deionized formamide (%)	
1	0.2	10	9	1.5	0.5	Complement 20
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 L₁₆ (4⁵)

Number	experimental factors					ddH ₂ O
	A	B	C	D	E	
1	1	1	1	1	1	Complement 20 μ L
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	

ISSR-PCR amplification

According to the methods of Mu et al. , the primers UBS 844 (sequence CTC TCT CTC TCT CTC TRC) were preliminarily used, and the leaves of wild *R. leptophylla* were used as template DNA. The amplification procedure was 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, 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 reaction system for *R. leptospiroides*, the leaves of wild *R. leptospiroides* were used as template DNA, and the primers were screened for reference molecular markers of *R. leptospiroides* (Liu et al.2018,He et al.2012). According to the primer sequences provided by the University of Columbia (UBC) of Canada, the primers and their sequences were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Table 3).

Table 3 ISSR-PCR primers and their sequences

primer	sequence (5' -3')
UBC-807	AGAGAGAGAGAGAGAGT
UBC-811	GAGAGAGAGAGAGAGAC
UBC-814	CTCTCTCTCTCTCTA
UBC-826	ACACACACACACACACC
UBC-834	AGAGAGAGAGAGAGAGYT
UBC-835	AGAGAGAGAGAGAGAGYC
UBC-844	CTCTCTCTCTCTCTRC
UBC-846	CACACACACACACACART

UBC-848	CACACACACACACACARG
UBC-856	ACACACACACACACACYA
UBC-873	GACAGACAGACAGACA
UBC-880	GGAGAGGAGAGGAGA
UBC-890	VHVGTTGTGTGTGTGTGT
UBC-891	HVHTGTGTGTGTGTGTGTG

Data analysis

The data of ISSR-PCR amplification bands were statistically analyzed. There were bands ' 1 ' and no bands ' 0 ' in the electrophoresis map(Zhang et al,2014). The inconsistent bands among the samples were considered as variation bands (including deletion and increase).

Product detection

The leaf DNA of wild and tissue culture seedlings of *Rubus sachalinensis* Leveille were amplified by PCR with 9 primers according to the PCR reaction program. The amplified products were electrophoresed in 0.8 % agarose gel. Staining was performed after electrophoresis, and the staining results were observed and photographed in a gel imaging system.

Results

Rapid propagation technique

Establishment of A Disinfection Program for Explants

After the primary culture medium was inoculated with the explant for 5 days, the axillary buds began to germinate and grow (Fig.2). After 15 days, the explant expanded and elongated (Fig.3). According to the result of explant sterilization (Table 4) showed that 75% alcohol processing time has significant effects on germination rate and contamination rate. The germination rate and contamination rate of 20 s were significantly lower and higher than other levels ($P < 0.05$). In 0.1% HgCl_2 with the same treatment time, the germination rate increased with the increase of 75% alcohol treatment time, and the contamination rate decreased with the increase of 75% alcohol treatment time. No. 8 sterilization effect is the best and most significantly, so the axillary buds of *Rubus sachalinensis* Leveille best sterilization conditions of 75% alcohol 40 s, 0.1% HgCl_2 7 min. The germination rate of explants was 78.34% and the contamination rate was 5.527%.

Fig 2 after culture for 5 days, the axillary buds begin to grow



Fig 3 after 15 days of culture, the explant expanded and elongated



Table 4 Effects of different sterilizations on explants sterilizing

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 in the form of mean ± SE

Means with the same letter along the column are not significantly different at $p \leq 0.05$

Effect of Orthogonal Test on Cluster Bud Propagation of *Rubus sachalinensis*

Leveille Shoot Multiplication

The primary cultures were transferred on shoot multiplication medium. New axillary buds came out after 6 d, and after 30 days the proliferation coefficient was 2.06 to 7.71. According to the buds proliferation results (Table 5) showed that the effect on

the proliferation of hook buds on library pages is from 6-BA > KT > NAA > IBA, in which the range of 6-BA is 2.48 and the range of KT is 1.14. No. 1 and No. 2 have strong growth, high buds and fresh leaves. No. 3, No. 4 and No. 5 have weak growth, short buds and yellow leaf tips. No. 9 the proliferation coefficient was 7.71 times, and bud proliferation was significantly higher than other levels ($P < 0.05$) (Fig.4). The optimal proliferation medium is MS + 6-BA 2.0 mgL⁻¹ + KT 0.5 mgL⁻¹ + NAA 0.1 mgL⁻¹ + IBA 0.05 mgL⁻¹.

Fig 4 multiple-buds of multiplication culture after 30 days



Table 5 Effect of different concentrations of PGRs for shoot multiplication of *Rubus sachalinensis* Leveille

Sr.No.	PGRs (mgL ⁻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	the bud proliferation was significantly high, 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 in the form of mean $\pm$ SE

Means with the same letter along the column are not significantly different at $p \leq 0.05$

Effect of GA₃ on Multiple Bud Rejuvenation of *Rubus sachalinensis* Leveille

The seedlings obtained from propagation in multiplication medium grew slow and stayed weak, unfit for rooting induction. The growth culture medium supplemented GA₃ on the basis of multiplication medium. According to the buds proliferation results (Table 6) showed that No. 4 bud proliferation was significantly higher than other levels ($P < 0.05$) (Fig.5). No. 3 bud proliferation was significantly lower than other levels ($P < 0.05$) . The optimal growth medium is MS + 6-BA 2.0 mgL⁻¹ + KT 0.5 mgL⁻¹ + NAA 0.1 mgL⁻¹ + IBA 0.1 mgL⁻¹ + GA₃ 0.1 mgL⁻¹.

Fig 5 multiple-buds grow into vigorous multiple-shoots after 30 days



Table 6 Effect of different concentrations of PGRs for shoot grow of *Rubus sachalinensis* Levl

Sr.No.	PGRs (mgL ⁻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	the bud proliferation was significantly high, 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 in the form of mean ± SE

Means with the same letter along the column are not significantly different at $p \leq 0.05$

Effects of Different Concentrations of IBA and NAA on Rooting of *Rubus sachalinensis* Leveille

According to the root induction results (Table 7) showed that No. 3 root induction was significantly higher than other levels ($P < 0.05$). The rooting rate after 40 days were 97.04%, the average root number 8.05, the average root length being 3.05 cm. The root germination time of NO.1 was the longest and the rooting rate was the lowest. In the absence of the hormone NAA, the rooting rate increased with the increase of the mass concentration of the hormone IBA. This indicates that IBA can effectively promote the root of *Rubus sachalinensis* Leveille.

Table 7 Effect of various concentrations of IBA and NAA on root induction

Sr. No.	PGRs (mgL ⁻¹)		Rooting rate/%	Number of roots/micro cutting	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	Roots little, short and thin, grow fibril
2	1.0	0	46.67 ± 1.82 ^c	4.46 ± 0.22 ^b	1.07 ± 0.17 ^{cd}	Roots little, short and thin
3	1.5	0	97.04 ± 2.77 ^a	8.05 ± 0.60 ^a	3.05 ± 0.19 ^a	Roots grow quickly, long and strong
4	0	0.2	36.67 ± 1.11 ^d	3.19 ± 0.26 ^b	1.78 ± 0.33 ^{bc}	Roots grow slow, short and thin

5	0.1	0.5	72.59 ± 3.78^b	5.14 ± 0.99^b	2.16 ± 0.24^b	Roots are many, long and strong
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Data are in the form of mean $\pm$ SE

Means with the same letter along the column are not significantly different at $p \leq 0.05$

Effects of different soil substrates and acclimation days on survival rate of acclimation seedlings

The robust tissue culture seedlings were selected and transplanted into different soil matrixes after hardening-seedling (3, 5, 7, 10 d). According to the results (Table 8) showed that The survival rate of the three soil matrixes were high When the cap was opened for 7 days (Fig.6). When the cap was opened for 3 days, the survival rate was the lowest, which may be the weak ability to adapt to the external environment due to the short time of hardening-seedling. When the cap was opened for 10 days, a lot of bacteria may be generated in the medium, which is not conducive to plant growth. Sand: vermiculite: perlite (2:1:1, V/V) has a lower survival rate than the other two matrixes. It may be because of its good hydrophobicity and weak water retention ability, reducing the survival rate of hardening-seedling.

Fig 6 transplanting plantlets after 30 days



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

Sr. No.	soil matrixes	Survival rate of 3 days seedlings (%)	Survival rate of 5 days seedlings (%)	Survival rate of 7 days seedlings (%)	Survival rate of 10 days 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 in the form of mean $\pm$ SE

Means with the same letter along the column are not significantly different at $p \leq 0.05$

Establishment of ISSR-PCR Reaction System

DNA Extraction from Different Parts of Wild Hook Seedling

The results of agarose gel electrophoresis for detecting the purity of genomic DNA extracted from different parts of wild *R. leptoneusis* seedlings are shown in Fig. 7.

The results showed that each DNA electrophoresis band had no tailing phenomenon, and the sample holes at each lane were relatively clean(Yang et al,2008) . The band

comparison between stem and leaf showed that the DNA band of leaf was brighter and clearer than that of stem.

Fig 7 Whole genome DNA extraction of different parts of wild *Rubus sachalinensis* Leveille

M: 100 bp DNA ladder Marker 1: Stem 2: Leaf



Effects of DNA from different parts on purity and concentration

The concentration and purity of leaves and stems were detected by UV spectrophotometry(Cao et al,2008) , and the whole genome DNA was extracted. According to the table 9 data, there were differences in the UV absorbance of A260 / A280 extracted from different parts of the whole genome DNA of *L. manshurica*. The average values of A260 / A280 in leaves and stems were 1.8184 and 1.6291, respectively, indicating that the DNA purity of leaves was high. Leaf DNA

concentration was higher than stem DNA concentration. Therefore, the leaf is the best extraction site for the detection of library page hook.

Table 9 Determination of DNA concentration and purity of different parts

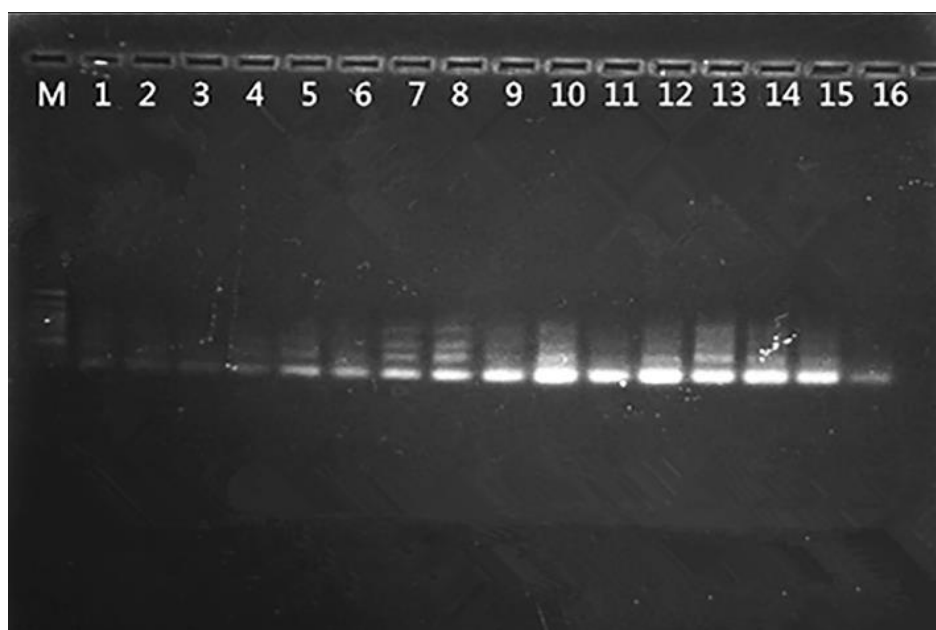
Absorbance extractive fractions	blade				stem			
	X ₁	X ₂	X ₃	$\bar{X}$	X ₁	X ₂	X ₃	$\bar{X}$
A ₂₃₀	0.0877	0.0942	0.0923	0.0914	0.1252	0.1803	0.1791	0.1615
A ₂₆₀	0.2409	0.2593	0.2406	0.2469	0.1383	0.1427	0.1504	0.1438
A ₂₈₀	0.1329	0.1412	0.1332	0.1358	0.0854	0.0871	0.0923	0.0883
A ₂₆₀ /A ₂₃₀	2.7469	2.7527	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	120.45	129.65	120.30	123.45	69.15	71.35	75.2	71.9
(ng/μL)								

Establishment of ISSR-PCR Reaction System

The experimental results were analyzed according to the method of He et al. . According to the number of amplified bands, the degree of separation, brightness and clarity of PCR amplification results, the optimal ISSR-PCR amplification system was obtained.

According to the results of orthogonal test electrophoresis Fig 8, the electrophoresis bands of 16 orthogonal design treatments were clear and no trailing phenomenon. In the 16 treatment combinations, there were significant differences in PCR amplification results due to different combinations of 5 factors and different levels of concentration. The bands amplified by combination 2,3,6,16 were not obvious and the brightness was weak. The bands amplified by combination 1,4,5 were obvious and the brightness was slightly weak. The bands amplified by combination 9,10,11,12,13,14,15 were more, but the background was fuzzy and the bands were difficult to distinguish. The bands amplified by combination No.7 and No.8 were clear and indigenous. Repeated tests showed that combination No.8 had higher stability and reproducibility than combination No.7. Therefore, through the comprehensive analysis of various factors in ISSR-PCR reaction system, No. 8 was finally determined as the optimal reaction system combination, namely, the total reaction volume was 20 μ L, the primer content was 0.3 μ mol / L, the template DNA content was 30 ng, the Mix enzyme content was 11 μ L, the deionformamide content was 0.75 %, and the bovine serum albumin content was 3 μ g / mL. The reaction procedure was 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 extended at 72° C for 30 s, and stored at 4° C.

Fig. 8 Orthogonal test of ISSR-PCR reaction system



ISSR-PCR primer screening

According to the optimum ISSR-PCR Reaction System, primers were selected. As can be seen from Fig. 9、10、11 , 9 out of 14 primers were high in brightness, low in variation and suitable for amplification, 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, UBC-890 and UBC-891 had the largest number of bands, 7,8,9 and 8 respectively.

Fig. 9 Primer UBC-807 ~ Primer UBC-834 amplification

M: 100 bp DNA ladder Marker 1: Primer UBC-834 2: Primer UBC-826
3: Primer UBC-814 4: Primer UBC-811 5: Primer UBC-807

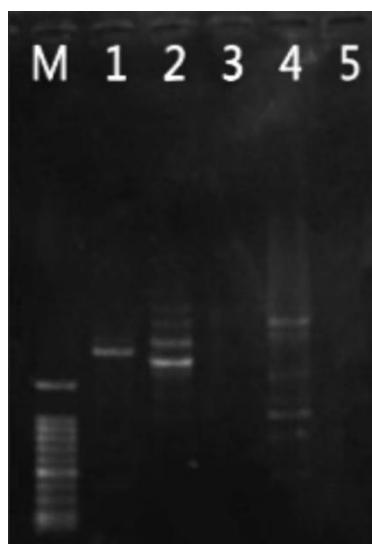


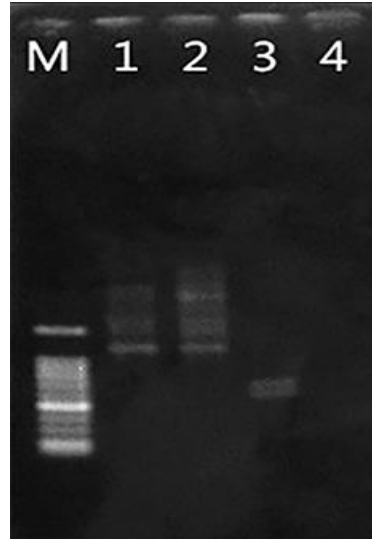
Fig. 10 Primer UBC-835 ~ Primer UBC-856 amplification

M: 100 bp DNA ladder Marker 1: Primer UBC-856 2: Primer UBC-848
3: Primer UBC-846 4: Primer UBC-844 5: Primer UBC-835



Fig. 11 Primer UBC-873 ~ Primer UBC-891 amplification

M: 100 bp DNA ladder Marker 1: Primer UBC-891 2: Primer UBC-890
3: Primer UBC-880 4: Primer UBC-873



Effect of Annealing Temperature on ISSR-PCR

In the reaction system, the specific binding of primers and templates was affected by annealing temperature. According to the optimal combination of reaction system, the annealing temperature of 9 suitable primers was screened. According to the method of Liu et al. , the annealing temperature usually fluctuates around 3 °C based on the T_m value. Eight gradients automatically generated by the PCR instrument are denoted as 1 – 8 for temperature test. Finally, the optimal annealing temperature is determined according to the number and brightness of bands. According to the following gel electrophoresis Fig12、13、14、15、16、17、18、19、20, the number of bands at eight annealing temperatures corresponding to each primer is counted, as shown in Table 10.

Figure 12 Amplification effect of annealing temperature on Primer UBC-811

1~8 Annealing temperature 43.1℃、43.4℃、44.1℃、45.3℃、48.8℃、50.0℃、50.7℃、

51.0℃

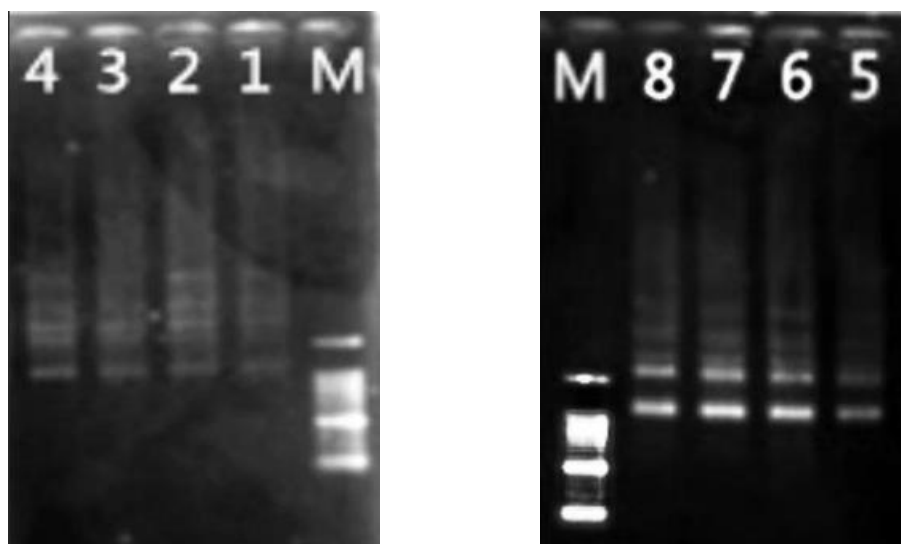


Figure 13 Amplification effect of annealing temperature on Primer

UBC-826

1~8 Annealing temperature 49.1℃、49.4℃、50.1℃、51.3℃、52.8℃、54.0℃、54.7℃、

55.1℃

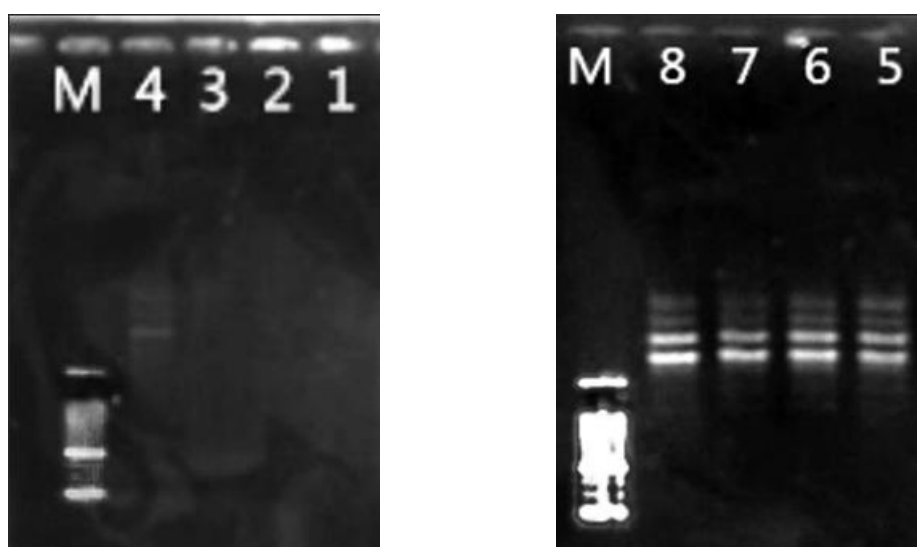


Figure 14 Amplification effect of annealing temperature on Primer UBC-834

1~8 Annealing temperature 45.1℃、45.4℃、56.1℃、47.3℃、48.8℃、50.0℃、50.7℃、

51.0℃

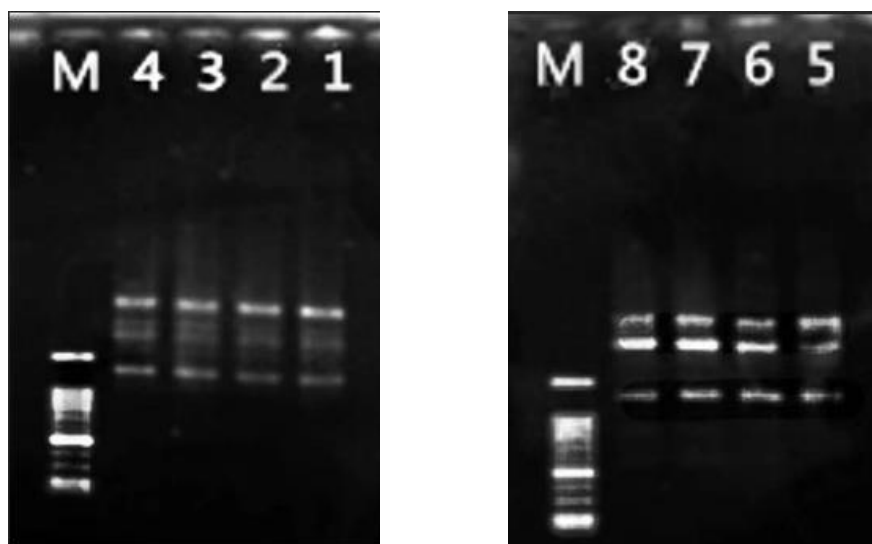


Figure 15 Amplification effect of annealing temperature on Primer UBC-835

1~8 Annealing temperature 47.1℃、47.4℃、48.4℃、49.3℃、50.8℃、52.0℃、52.7℃、

53.1℃

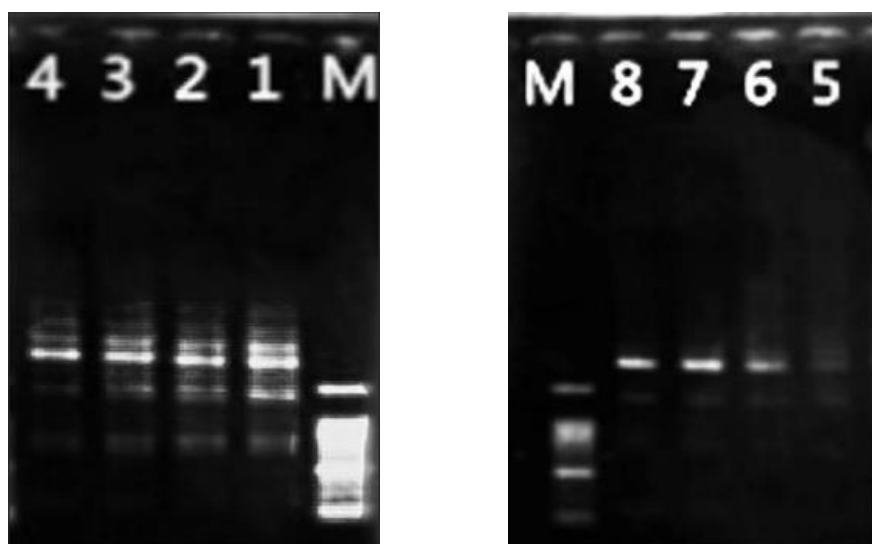


Figure 16 Amplification effect of annealing temperature on Primer UBC-844

1~8 Annealing temperature 45.1℃、45.4℃、46.1℃、47.3℃、48.8℃、50.0℃、50.7℃、

51.0℃

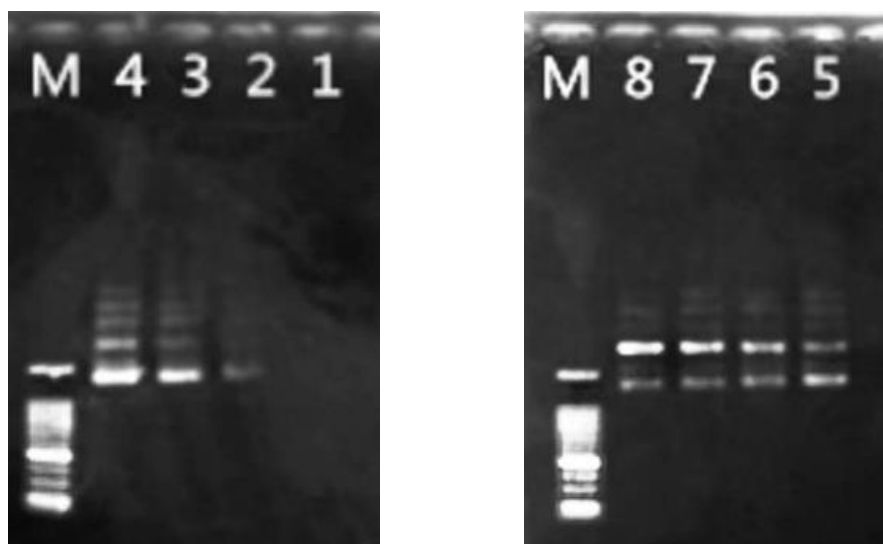


Figure 17 Amplification effect of annealing temperature on Primer UBC-856

1~8 Annealing temperature 49.1℃、49.4℃、50.1℃、51.3℃、52.8℃、54.0℃、54.7℃、

55.1℃

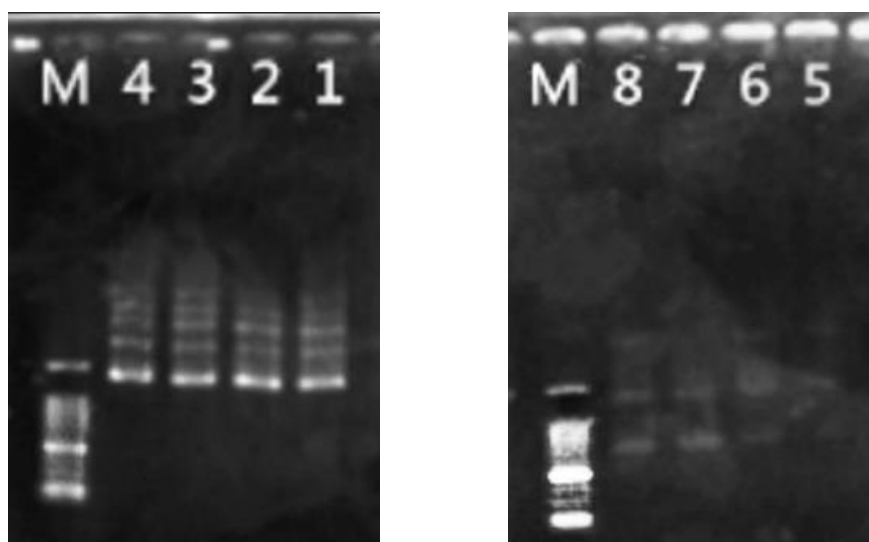


Figure 18 Amplification effect of annealing temperature on Primer UBC-880

1~8 Annealing temperature 44.1℃、44.4℃、45.1℃、46.3℃、47.8℃、49.0℃、49.7℃、

50.0℃

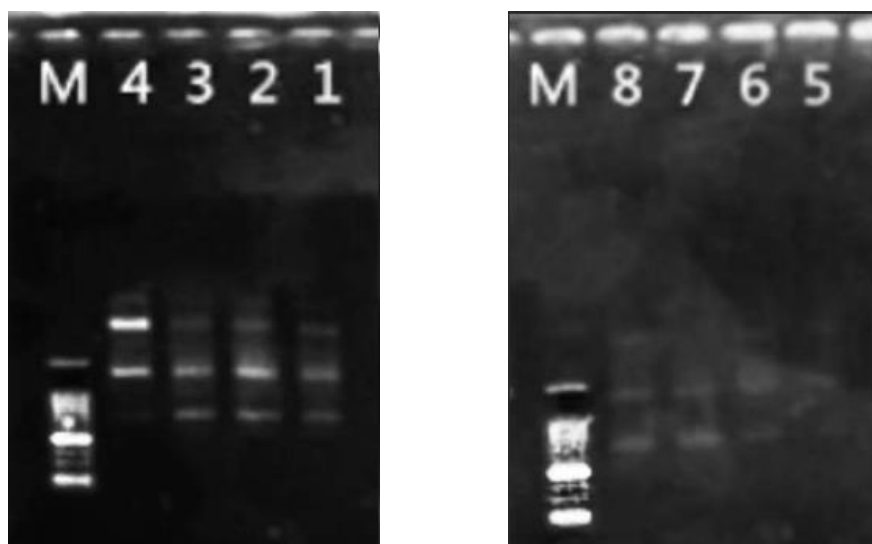


Figure 19 Amplification effect of annealing temperature on Primer UBC-890

1~8 Annealing temperature 48.1℃、48.4℃、49.1℃、50.3℃、51.8℃、53.0℃、53.7℃、

54.1℃

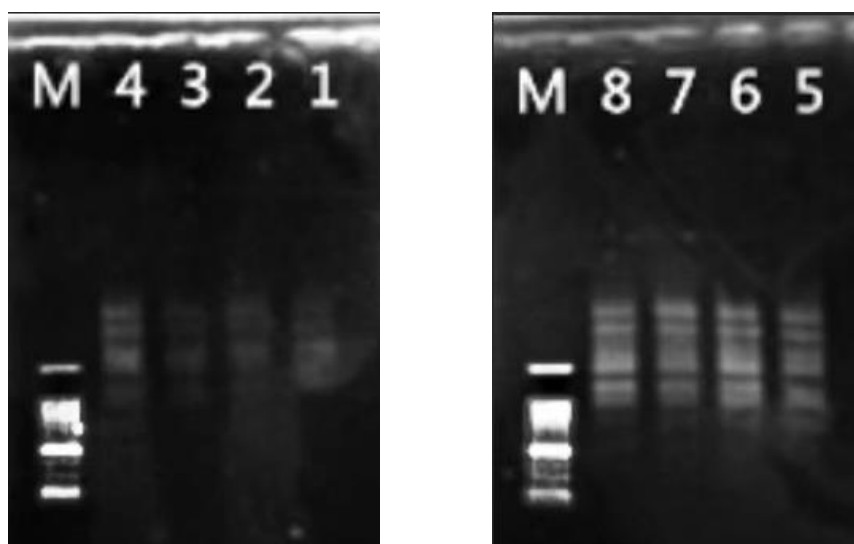
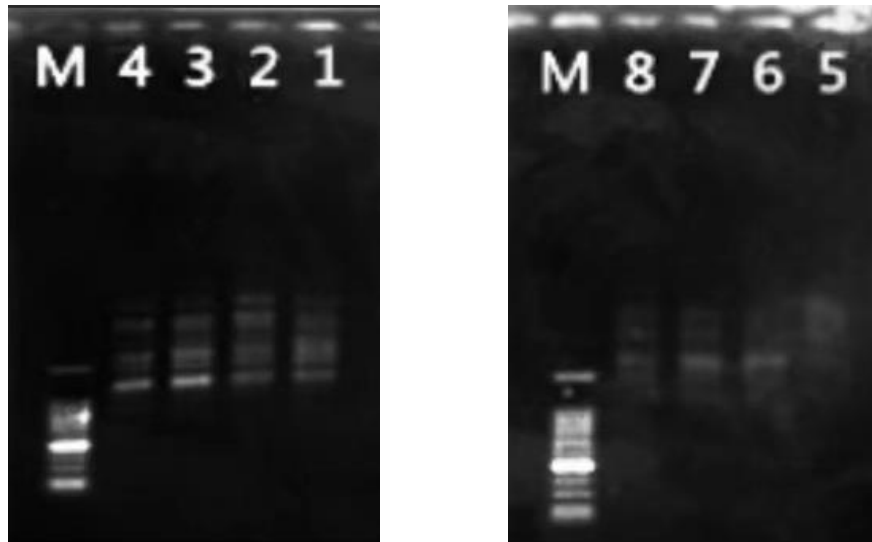


Figure 20 Amplification effect of annealing temperature on Primer UBC-891

1~8 Annealing temperature 450.1℃、50.4℃、51.1℃、52.3℃、53.8℃、55.0℃、55.7℃、

56.1℃



The annealing temperature is an important factor affecting the specificity of PCR. According to the intuitive analysis of the gel electrophoresis map, 8 annealing temperatures were automatically generated by the PCR instrument. There are differences in the number of bands and brightness at different temperatures. The primers with the largest number of bands were UBC-890, and the annealing temperature was 54.1 °C, which was 11 bands. The background was fuzzy, and the bands were difficult to identify. Followed by primers UBC-835, UBC-891, annealing temperature were 48.4 °C, 51.1 °C, 10 bands were clear ; six bands with good clarity were generated at least, with primer UBC-834 and annealing temperature of 48.8 °C. When the number of bands at different annealing temperatures is the same, the optimal annealing temperature can be determined by the brightness, clarity and the degree of separation between bands. For example, the primer UBC-835, when the

annealing temperatures are 47.1 °C, 47.4 °C and 48.4 °C, the bands are all 10, and the bands at 48.4 °C are divided than those at the other two temperatures.

Table 10 The number of bands at 8 Annealing temperatures corresponding to 9 Primers

	primer	Tm (°C)	annealing template concentration grads (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

Study on genetic stability

DNA Extraction from Different Parts of *Rubus sachalinensis* Leveille Wild 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* Leveille. The results are shown in Fig.21. The results showed that there

was no tailing in the electrophoretic bands and the lane holes were clean. Comparing the bands of tissue culture seedlings with those of wild seedlings, it was found that the DNA bands of leaves of tissue culture seedlings and wild seedlings were brighter and clearer than those of stems. The stem DNA of tissue culture seedlings was brighter and clearer than that of wild seedlings. The leaf DNA of tissue culture seedlings was equivalent to the brightness and clarity of wild seedlings.

Fig. 21 Whole genome DNA extraction of different parts of wild bramble and tissue culture seedling plants

M: 100 bp DNA ladder Marker 1: Stem segment of Tissue culture seedlings 2: Leaves of Tissue culture seedlings 3: Stem segment of Wild seedlings 4: Leaves of Wild seedlings



Genetic stability analysis of tissue culture seedlings

The selected 9 primers were used for PCR amplification of the extracted female parent and samples. The amplification results are shown in the Fig22、23、24、25、26、27、28、29、30. Through statistical analysis of 9 maps shown Table 11, a total of 475 clearly identifiable bands were obtained by PCR amplification. Among the tested samples and female parents, sample 1 and 4 in primer UBC-826 each had a variant band, sample 1 in primer UBC-834 had a variant band, and the others were consistent with the parents. Therefore, only 0.63 % of the DNA bands of the test tube seedlings were mutated and maintained high genetic stability.

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

[illegible]

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
summation							475	3	0.63

Fig. 22 Primer UBC-811

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings



Fig. 23 Primer UBC-826

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings

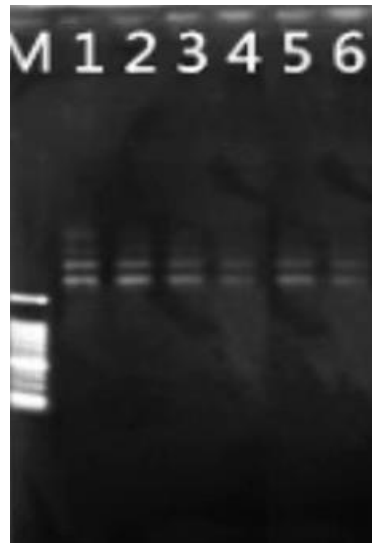


Fig. 24 Primer UBC-834

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings

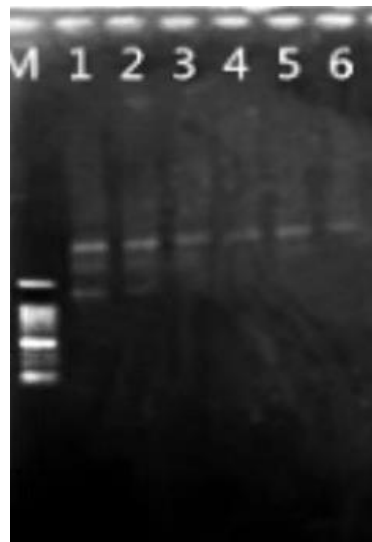


Fig. 25 Primer UBC-835

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings

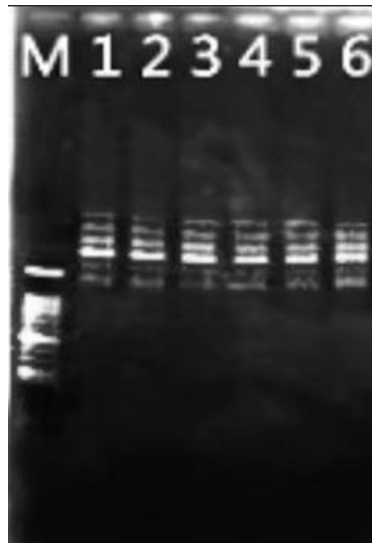


Fig. 26 Primer UBC-844

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings



Fig. 27 Primer UBC-856

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings



Fig. 28 Primer UBC-880

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings

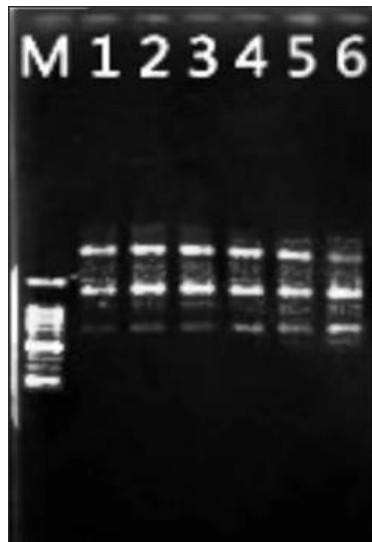


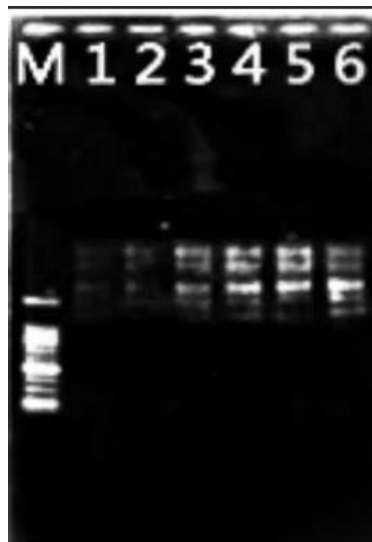
Fig. 29 Primer UBC-890

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings



Fig. 30 Primer UBC-891

M: 100 bp DNA ladder Marker 1~5: Tissue culture seedlings 6: Wild seedlings



Discussion

Establishment of Rapid Propagation System of *Rubus sachalinensis*

Leveille

In this study, the axillary buds of *Rubus sachalinensis* Leveille were used as explants without callus tissue, and the seedlings with suitable genetic properties were obtained in a short period of time. In the process of multiplication culture, the differentiated buds are weak and thin, which is extremely detrimental to the further rooting culture. Therefore, it is necessary to improve the hormone concentration and increase the induction rate of adventitious buds (Wei ying et al. 2015). The phytohormone gibberellin (GA₃) plays a promoting role in stem elongation and cell division in plants (Zhou et al. 2016; Peter Hedden and Valerie Sponsel 2015). GA₃ can promote plant growth and increase crop yield, but there is no inhibition over the optimum concentration. Even if GA₃ concentration is high, it can still show the greatest promotion effect (Li et al. 2011). In this study, the concentration of 0.5mgL⁻¹ GA₃ was added on the basis of the multiplication medium. Although the proliferation coefficient was slightly decreased, the leaves of adventitious buds more widely and the stems more stronger. Therefore, adding GA₃ can significantly increase the rooting rate and the survival rate of seedlings in tissue culture of *Rubus sachalinensis* Leveille.

Acclimatization and transplanting plays an important role in whether aseptic seedlings can be used in production (Zhu et al,2022). The aseptic seedlings grown in high humidity, constant temperature and weak light(Zhou et al,2021). Hardening-seedling will gradually change their growth conditions to adapt to the external environment. In this study, The aseptic seedlings were cultured with water for some time. The most suitable conditions for hardening-seedling refining were selected, which greatly improved its survival rate.

In the study, for the first time, axillary buds were used as explants in tissue culture. This study effectively overcomes the problem of lack of *Rubus sachalinensis* Leveille resources and protects the ecological environment. It not only opens a new way for the technology of tissue culture of *Rubus sachalinensis* Leveille, but also lays a foundation for its large-scale planting.

Establishment of ISSR-PCR Reaction System

In this experiment, the whole genome DNA of wild seedlings was extracted by centrifugal column method. The results showed that the quality of leaf DNA was higher than that of stem DNA. The possible reason was that the stem was too hard to be grinded into powder during grinding, and DNA could not be released sufficiently, resulting in the decrease of its quality. Ultraviolet spectrophotometry was used to detect the purity and concentration of DNA. When DNA samples were contaminated by protein, phenol or small molecules, the absorbance of DNA would be affected. Laboratory testing A_{260} / A_{280} UV absorbance value should be $1.6 \sim 1.9$, A_{260} / A_{230} value should be greater than 2.0. If the A_{260} / A_{280} value is greater than 1.9, it indicates RNA contamination, and less than 1.6, it indicates protein or phenol contamination in the sample ; if the A_{260} / A_{230} value is less than 2.0, it indicates that there is residual salt and small molecular impurities in the solution, such as nucleotides, amino acids, phenols, etc. (mu et al,2007). According to three parallel experiments, the DNA purity and concentration of the leaves were higher than those of the stems. Therefore, the leaves were the best extraction part for detecting the

Rubus sachalinensis Leveille.

Orthogonal experimental design is widely used in practical work. Each test number is representative, which can reduce the number of tests and reduce the workload (Tang et al,2014). In this experiment, 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. Finally, the ISSR-PCR reaction system suitable for *R.papyrifera* was established, that is, the total reaction volume was 20 μ L, the primer content was 0.3 μ mol / L, the template DNA content was 30 ng, the Mix enzyme content was 11 μ L, the deionized formamide content was 0.75 %, and the bovine serum albumin content was 3 μ g / mL. In the ISSR-PCR reaction system, 9 primers with high brightness, good variation and suitable for amplification were obtained from 14 primers. Among them, UBC-826, UBC-856, UBC-890 and UBC-891 had the largest number of amplified bands, which were 7,8,9 and 8, respectively. The annealing temperature of different primers is different, and the annealing temperature of the primers has a great influence on the reaction. Therefore, according to the optimal reaction system level combination, the optimal annealing temperature of each primer is screened to obtain more reliable test results.

Study on Genetic Stability of Tissue Culture Seedlings of *Rubus sachalinensis* Leveille

ISSR molecular marker technology is widely used in genetic, identification and other

aspects of research, in which the genetic stability and diversity of the most common, such as *Gentiana straminea* ultra-low temperature regeneration plant genetic stability (Gao et al,2015), Guangxi sweet tea germplasm genetic diversity (Chen et al,2017).

In this experiment, 9 primers with clear amplification bands were screened from 14 primers by 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 were variant bands. The mutation rate was 0.63 %, and the mutation rate was low, which was similar to the study of Gao et al. It has high genetic stability and provides reference for the maintenance of excellent traits and the protection of resources.

Supplementary Information

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.

Declarations

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.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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