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Analysis of genetic diversity and similarities between different *Lycium* varieties based on ISSR analysis and RAMP-PCR markers

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Abstract. The plant species *Lycium*, commonly known as box-thorn, is a genus of flowering plants of the Solanaceae family. It is commonly used in Traditional Chinese Medicine (TCM) for its fruits. As there are numerous different species *Lycium*, the present study, aimed to analyze the genetic diversity and similarities between some of these species. For this purpose, samples from 16 *Lycium* species were collected, and cluster dendrograms were created using random amplified microsatellite polymorphism (RAMP)-PCR and inter-simple sequence repeat (ISSR) markers. A total of 1,249 bands were produced, where each random amplified polymorphic DNA (RAPD) primer had 2-9 valuable bands with an average of 6, of which 89.05% bands were polymorphic by RAMP-PCR. Genetic distances were observed among different cultivars or species, which had a similarity coefficient (SC) index of 0.37-0.98. Similar to RAMP-PCR, ISSR analysis of the *Lycium* DNA samples yielded an SC index of 0.36-0.98. ISSR markers produced 956 bands with average of 5.9 bands per ISSR primer, and 88.28% of the bands were polymorphic. The SC index between sample 2 and sample 16 was found to be 0.72 by both RAMP-PCR and ISSR, which clustered together, which indicated that these two cultivars have good genetic association, in spite of their vast geographic distance. On the whole, the present study performed a genetic analysis of different *Lycium* species, which may prove to be useful for the conservation of the genetic diversity of different *Lycium* species/cultivars, as well as of other plant species.

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Key words: *Lycium* species, genetic authentication, RAMP-PCR, ISSR, genetic distance, genetic diversity

Introduction

Lycium is a species of plant commonly known as box-thorn, and is a genus of flowering plants of the Solanaceae family. There are numerous species of *Lycium*, such as *Lycium barbarum*, *Lycium chinense*, *Lycium europeaum* [also known as Gouji (pinyin: gǒuqǐ), or Goji (1). Carl Linnaeus, a naturalist, provided the genus name *Lycium* in the year 1753, and gave the species name *barbarum*, while Philip Miller, a botanist, described *Lycium chinense* after 15 years (2). The *Lycium* fruit, known as 'Gou qi zi', 'Gojizi', 'Wolfberry', or 'Goji berry', is the red berry obtained from two closely related species, *Lycium chinense* Miller and *Lycium barbarum* L. of the box-thorn in the family, *Solanaceae*, originating from Northwestern China, mainly in Ningxia, Qinghai, Gansu and Inner Mongolia (3-5). The *Lycium* plant is extensively cultivated in most parts of China, particularly in the Ningxia Hui Autonomous Region; however, it is also cultivated in many other parts of China and worldwide (1,5,6). *Lycium barbarum* is regarded as Ningxia Goji, and its products have a number of medicinal properties; thus, they are a main part of Traditional Chinese Medicine (TCM).

The root bark (Digupi) and the fruit (Goji) of the *Lycium* plant have long been used in TCM. This therapeutic approach including Goji was first promoted by Jingyue Zhang, indicated in his book 'Jingyue Quanshu' (1640 A.D.); he advised using gentle heating and using 'thick' tonic herbs for nurturing the internal organs. During the Ming Dynasty (1368-1644 A.D.), the book 'Bencao Gangmu', written by Shizhen Li reported that the regular consumption of Goji berries generates vital energy, strengthens one's physique and increases longevity. It is well known that the Goji berry has valuable properties

in nourishing the blood, enriching the 'Yin' (opposite to the 'Yang' in TCM), and is beneficial to the kidneys, liver and lungs (4-6). It is used in the treatment of consumptive diseases that are associated with symptoms, such as thirst, dizziness, hypoplasia and chronic cough. Furthermore, recent pharmaceutical investigations have focused on proteoglycans, such as '*Lycium barbarum* polysaccharide (LBP)', which has antioxidant properties and may be effective against age-related diseases (3,7). Currently, based on folk remedies and research studies, Goji berry or LBP is most well-known for the treatment of poor vision (1,8), anemia (3), diabetes mellitus, memory enhancement and liver disease (6,8-11), as well as Alzheimer's disease (12), atherosclerosis (13) and other diseases (14-17). The juice products of Goji, known as Himalayan Goji Juice, are popular in the new food markets in developed countries, such as the USA and UK. In first decade of the 21st century, UK, USA, Canada and some other countries began cultivating Goji commercially to meet potential markets for fresh fruits, juice and other products (2-6).

Random amplified polymorphic DNA (RAPD), as one of the important molecular marker techniques, was first reported in 1990 by Williams *et al* (18). Subsequently, RAPD, alone or in combination with other molecular marker techniques, for example, inter-simple sequence repeat (ISSR), simple sequence repeat (SSR) or variable number tandem repeat (VNTR), oligonucleotide polymorphism (OP), sequence-characterized amplified region (SCAR), single nucleotide polymorphism (SNP) and amplified fragment length polymorphism (AFLP) has been widely utilized in the analysis of genetic or molecular diversity in various organisms, germplasm characterization, genotype identification and fingerprinting, estimating distances between species or offspring, and molecular marker-assisted breeding (19-25).

Although RAPD is popular due to its numerous advantages, it also possesses some drawbacks, such as poor reproducibility and a lower production rate. By using a technique of improved RAPD-PCR or random amplified microsatellite polymorphism (RAMP)-PCR, its production and resolution can be markedly increased, and its ramp time can be prolonged from 0 min (as in regular PCR) to 2-3 min at annealing to the extension stage in the PCR machine (termed RAMP-PCR) (26,27).

There are countless *Lycium* species or cultivars, and confusion arises regarding the naming system and identification process. For example, the same cultivars grown in different soils or climates may produce very dissimilar fruits. Therefore, the medicinal or/and nutritional values of *Lycium* fruits can be significantly variable. Zhongning County in Ningxia, China is the largest producer (approximately 20%) of Goji berries worldwide, where *Lycium barbarum* plantations range between 40 and 400 hectares (100-1,000 acres). As regards the importance of *Lycium* species and the roles of LBP in the new food market and modern medicine, research into the genetic authentication and characterization of TCM, particularly with DNA-based molecular techniques is necessary. In the present study, varieties of their samples were collected from different localities, and they were characterized genetically by RAMP-PCR, and the results were also verified by ISSR markers. Thus, the present study may provide valuable insight into genetic information and biological diversity of these medicinal plants.

Materials and methods

Experimentals. RAPD primers (2.5 μmol/l) are listed in Table I and ISSR primers (2.5 μmol/l) are listed in Table II. Taq Mastermix (2X PCR; TianGen Biotech Co. Ltd.) and the DNA molecular weight marker, DL2000 (Takara Biotechnology Co. Ltd.) were applied for PCR amplification. Other reagents that were used were all of analytical grade and have been previously described (26,27).

Plant sample collection. A total of 16 *Lycium* species or cultivars were collected from 6 different regions of China and the USA: One from Zhongning, Lingxia (ZN), one from Gongzhou, Jiangxi (GZ), one from Panzhihua, Sichuan (PZH), 3 from Haidong, Qinghai (HD), 9 from the Ningxia Academy of Agriculture and Forestry Sciences (YC) and the last one from Houston, TX, USA (TX) (Fig. 1 and Table III).

DNA isolation. The plant genomic DNA was isolated from fresh or dried leaves using the cetyltrimethylammonium bromide (CTAB) method as previously described (27-29). DNA quality and concentration were detected by agarose gel electrophoresis and spectrophotometry (NonoDrop 2000 spectrophotometer, Thermo Fisher Scientific Inc.) (28). DNA samples were adjusted to 10 ng/μl as a final concentration and used for the next step of DNA amplification.

RAMP-PCR marker amplification. In total, 15 different SBS primers were first used to evaluate polymorphic detection using RAMP-PCR marker amplification. Among these, 13 primers amplified products with valuable polymorphic bands for the following data analysis (listed in Table II). Thermal cycling of PCR (10 μl in total) was carried out with the following reaction conditions: 1 μl of primers, 1 μl of DNA templates for *Lycium* cultivars or species (10 ng in total), 5 μl of Master Mix buffer and 3 μl of double-distilled water. The PCR conditions were as follows: i) Pre-denaturation at 95˚C for 1 min and 30 sec; ii) 40 PCR cycles of 40 sec at 94˚C, 1 min at 36˚C, 1 min and 30 sec at 72˚C; and iii) extension for 5 min at 72˚C. PCR amplification was performed in a thermal cycler (Applied Biosystems Veriti® 96-Well Thermal Cycler, Life Technologies; Thermo Fisher Scientific, Inc.). The ramp rate (the stage from annealing to extension) was set from 5% (0.125˚C/sec) to 10% (0.25˚C/sec) or to 40% (1˚C/sec) or to 100% (2.5˚C/sec), respectively for samples using RAMP-PCR to compare the reproducibility in this study. A ramp rate set to 0.125˚C/sec (5% ramp rate) was used to execute in triplicates for each of the 16 samples (27).

ISSR marker amplification. ISSR amplification reactions were also executed in 10 μl reaction volumes containing 1 μl of ISSR primers, 1 μl of templates for DNA of *Lycium* cultivars or species, 5 μl of Master Mix, and 3 μl of double-distilled water. The steps of the PCR reaction were as follows: i) Denaturation at 95˚C for 1 min and 30 sec; ii) 35 cycles of 40 sec at 94˚C, 30 sec at 50˚C, 1 min and 30 sec at 72˚C; and iii) final extension for 5 min at 72˚C (24). The PCR reaction was performed in the aforementioned PCR machine. In total, 17 primers were used initially, among which 10 primers (listed in Table II) amplified products well with high number of polymorphic bands.

Table I. RAPD primer sequences.

Primer	5'-3' sequence	Primer	5'-3' sequence
SBS-A1	CAGGCCCTTC	SBS-A7	GAAACGGGTG
SBS-A11	CAATCGCCGT	SBS-A12	TCGGCGATAG
SBS-A16	ACCTGGACAC	SBS-I8	TTTGCCCGGT
SBS-I10	ACAACGCGAG	SBS-I18	TGCCCAGCCT
SBS-Q3	ACCTCAGCTC	SBS-Q4	AGTGCGCTGA
SBS-Q12	AGTAGGGCAC	SBS-Q16	AAGCGACCTG
SBS-Q18	AGGCTGGGTG		

Table II. ISSR primer sequences.

Primer	5'-3' sequence	Primer	5'-3' sequence
UBC807	AGAGAGAGAGAGAGAGT	UBC811	GAGAGAGAGAGAGAGAC
UBC825	ACACACACACACACA CT	UBC847	CACACACACACACACARC
UBC851	GTGTGTGTGTGTGTGTYG	UBC876	GACAGACAGACAGACA
UBC879	CTTCACTTCACTTCA	UBC880	GGAGAG GAG AGG AGA
UBC885	ACACACACACACACA CYT	UBC886	ACACACACACACACACYA

R, base A or G; Y, base C or T.

Agarose gel analysis and data analysis. The PCR-amplified products were then tested on a 1.8% agarose gel electrophoresis. Ethidium bromide (EtBr) staining was used for visualizing the gels and the images were captured on a Chemi Doc XRS system (Bio-Rad Laboratories, Inc.). Bands visualized by ethidium bromide were selected on scoring for data analysis. '1' was used to mark the presence of a clear band in the gel, and '0' was used to denote that the corresponding band was absent in other sample(s). The similarity index (SI) and similarity matrix (SM) were calculated using the SM coefficient. Based on the unweighted pair group method with arithmetic averages (UPGMA), the sequential, agglomerative, hierarchical, and nested clustering (SAHN) module was used to produce the dendrograms (30).

Results

Technical comparison between regular RAPD amplification and RAMP-PCR. To obtain more specific bands from RAMP-PCR amplification, at first, the primer SBS-I4 was used for amplification with a ramp rate (from annealing to extension) of 5, 10, 40 and 100% (2.5˚C/sec), respectively, from the *Lycium* barbarum 'Ningqi-1' sample (no. 7) (listed in Table III) in a PCR thermocycler. As shown in Fig. 2, the PCR bands were quantitatively increased with ramp rates from 100 to 5%. The 5% ramp rate had the most bands with stronger signals. As a negative control, without a template, no bands were visible in the 5% ramp rate. Specifically, the band numbers were 4 by regular PCR (with 100% ramp rate), and this then increased to 7 in RAMP-PCR (with a 5% ramp rate), and signals that had at least 2 PCR bands with the 5% ramp rate were much stronger than those with the 100% ramp rate

Figure 1. The localities of samples of *Lycium* from different regions in China. Spots in dark blue color indicate cities and lines in green color indicate Yangtze River and Yellow River, respectively. Detailed information of the 16 samples is presented in Table III.

(Fig. 2). This finding indicates that the decreased ramp rate (for example, 5%), markedly increases the band numbers and production. Therefore, the 5% ramp rate has better RAPD amplification, and this optimized RAMP-PCR condition was then used to complete the amplification of all 16 samples of the *Lycium* species or cultivars using RAPD primers.

Amplification of Lycium species or cultivars with RAMP-PCR. To compare whether other samples can also get more and specific bands in this study, either regular RAPD

Table III. *Lycium* species sources for RAPD-ISSR analysis.

No.	Species or cultivars	Sources	Abbreviation
1	*Lycium chinense* Miller	Zhongning, Lingxia	ZN
2	*Lycium chinense* Miller	Gongzhou, Jiangxi	GZ
3	*Lycium chinense* Miller	Panzhihua, Sichuan	PZH
4	*Lycium chinense* Miller (207)	Haidong, Qinghai	HD
5	*Lycium chinense* Miller	Haidong, Qinghai	HD
6	*Lycium barbarum* 'Ningqi-1'	Haidong, Qinghai	HD
7	*Lycium barbarum* 'Ningqi-1'	NAAFS, Yinchuan, Lingxia	YC
8	*Lycium barbarum* 'Ningqi-2'	NAAFS, Yinchuan, Lingxia	YC
9	*Lycium barbarum* 'Ningqi-3'	NAAFS, Yinchuan, Lingxia	YC
10	*Lycium barbarum* 'Ningqi-4'	NAAFS, Yinchuan, Lingxia	YC
11	*Lycium barbarum* 'Ningqi-5'	NAAFS, Yinchuan, Lingxia	YC
12	*Lycium barbarum* 'Ningqi-6'	NAAFS, Yinchuan, Lingxia	YC
13	*Lycium barbarum* L.cv. 'Ningqi-7'	NAAFS, Yinchuan, Lingxia	YC
14	*Lycium chinense* Mill. 'Cai-1'	NAAFS, Yinchuan, Lingxia	YC
15	*Lycium barbarum* 'Ningqi-9'	NAAFS, Yinchuan, Lingxia	YC
16	*Lycium chinense* Miller	Houston, TX, USA	TX

NAAFS, Ningxia Academy of Agriculture and Forestry Sciences.

or RAMP-PCR was applied to amplify the DNA samples by setting the ramp time with a ramp rate of 5 and 100% using *Lycium* samples (shown in Table III). The PCR product amounts and the bands numbers were obviously increased by RAMP-PCR when the ramp rate was set from 100 to 5% (data not shown).

For the estimation of polymorphisms, 15 RAPD primers were applied for RAMP-PCR analysis and 13 of these (shown in Table I) produced polymorphic amplification bands, which were highly reproducible. In Fig. 3, 4 representative primers (SBS-A16, SBS-I1, SBS-I18 and SBS-Q12) from 16 samples are presented. From these 13 primers, a total of 1,249 bands were gained in total, where each primer exhibited 2-9 valuable bands with an average of 6 per primer. The band size was in the range of 200-2,200 bp, and 89.05% of these were polymorphic in the 16 samples.

Genetic distance analysis based on RAMP-PCR results. The cluster dendrogram based on the RAMP-PCR amplified bands is presented in Fig. 4. The dendrogram results presented a similarity coefficient (SC) index ranging from 0.37 to 0.98. The SC index between sample 1 (*Lycium chinense* Miller from Zhongning, Linxia) and sample 2 (from Gongzhou, Jiangxi) was the lowest (0.37), while that between samples 6 and 7 (*Lycium barbarum* 'Ningqi-1' from Haidong, Qinghai and form the Ningxia Academy of Agriculture and Forestry Sciences) was the highest (0.98) (Fig. 4B). Samples 6 and 7, where were obtained from different localities, were identified with the highes SC index 0.98, indicating that they indeed originated from the same cultivar.

Amplification of Lycium species or cultivars DNAs by ISSR. In ISSR-PCR, 10 primers amplified well, which generated 956 reproducible bands from 16 samples. Each reproducible

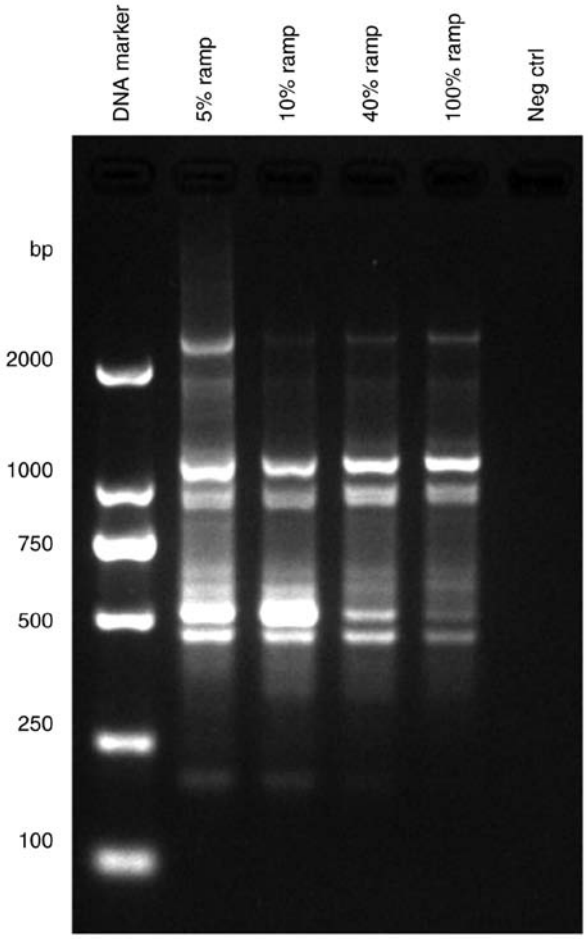


Figure 2. PCR by RAPD amplification with different ramp rates. The sample of *Lycium barbarum* 'Ningqi-1' sample (no. 7) from Yinchuan, Lingxia was used for RAMP-PCR using SBS-I4 primer at ramp rates of 5, 10, 40 and 100%, respectively. The lane labeled 'DNA Marker' indicates the molecular weight size in bp. Neg ctrl, negative control (no template DNA).

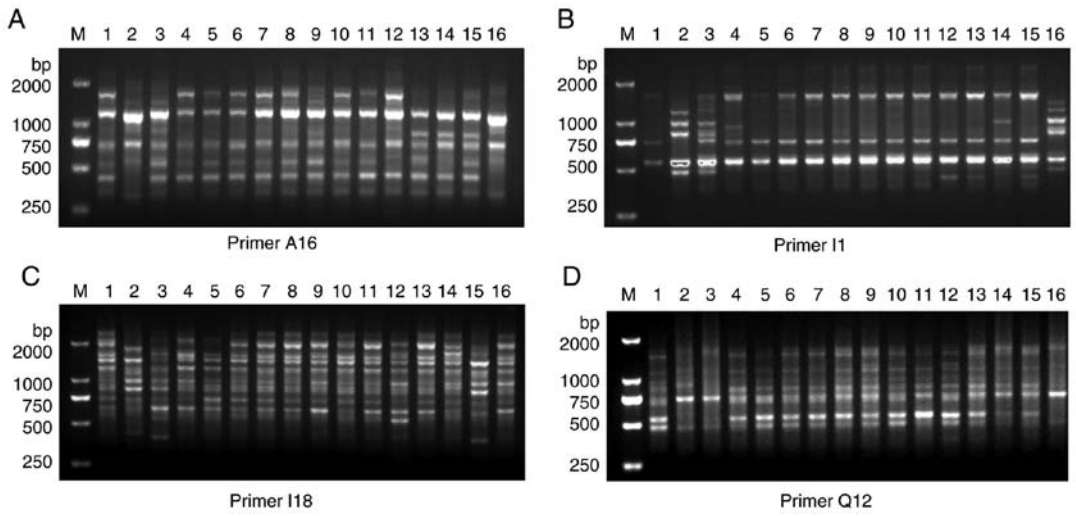
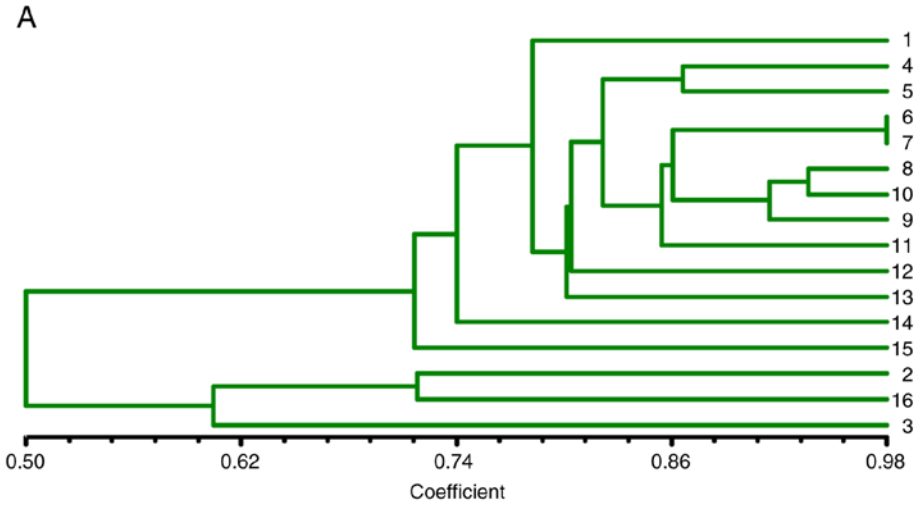


Figure 3. Representative results of RAMP-PCR with a ramp rate of 5% using RAPD primers (A) SBS-A6, (B) SBS-I1, (C) SBS-I18 and (D) SBS-Q12. Lanes 1-16 represent different *Lycium* samples listed in Table III, whereas lane 'M' indicates the DL2000 DNA marker.

A



B

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.00															
2	0.37	1.00														
3	0.52	0.66	1.00													
4	0.77	0.47	0.57	1.00												
5	0.79	0.48	0.61	0.87	1.00											
6	0.80	0.42	0.55	0.83	0.87	1.00										
7	0.80	0.43	0.57	0.83	0.87	0.98	1.00									
8	0.80	0.43	0.57	0.81	0.85	0.88	0.90	1.00								
9	0.77	0.43	0.59	0.78	0.77	0.82	0.83	0.90	1.00							
10	0.81	0.44	0.59	0.82	0.84	0.87	0.89	0.94	0.93	1.00						
11	0.80	0.42	0.58	0.79	0.83	0.84	0.86	0.86	0.83	0.89	1.00					
12	0.77	0.46	0.56	0.78	0.83	0.80	0.80	0.83	0.77	0.81	0.83	1.00				
13	0.75	0.42	0.58	0.76	0.78	0.79	0.81	0.81	0.82	0.83	0.84	0.80	1.00			
14	0.67	0.48	0.59	0.77	0.76	0.73	0.73	0.77	0.76	0.77	0.77	0.72	0.75	1.00		
15	0.63	0.46	0.56	0.71	0.72	0.70	0.70	0.77	0.76	0.70	0.73	0.76	0.73	0.74	1.00	
16	0.48	0.72	0.56	0.52	0.53	0.50	0.49	0.50	0.50	0.51	0.47	0.50	0.50	0.58	0.46	1.00

Figure 4. Dendrogram of *Lycium* varieties by the RAMP-PCR marker technique. (A) Dendrogram of *Lycium* varieties based on RAMP-PCR profiles. Bars on the bottom section indicate the SC index. (B) Dendrogram of genetic distance for *Lycium* varieties using RAMP-PCR amplification. SC, similarity coefficient.

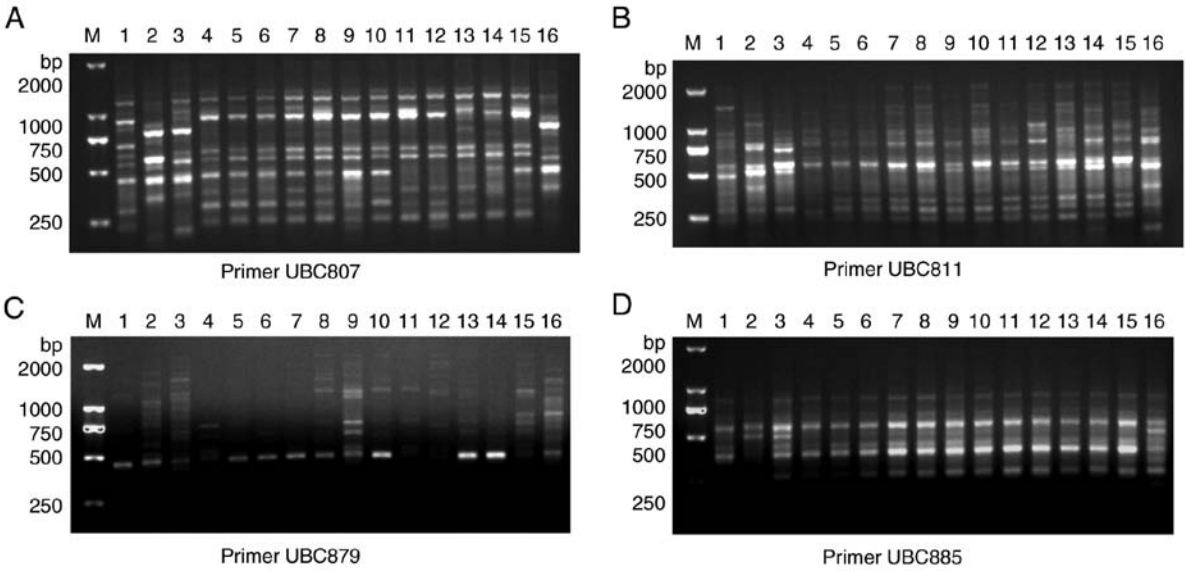
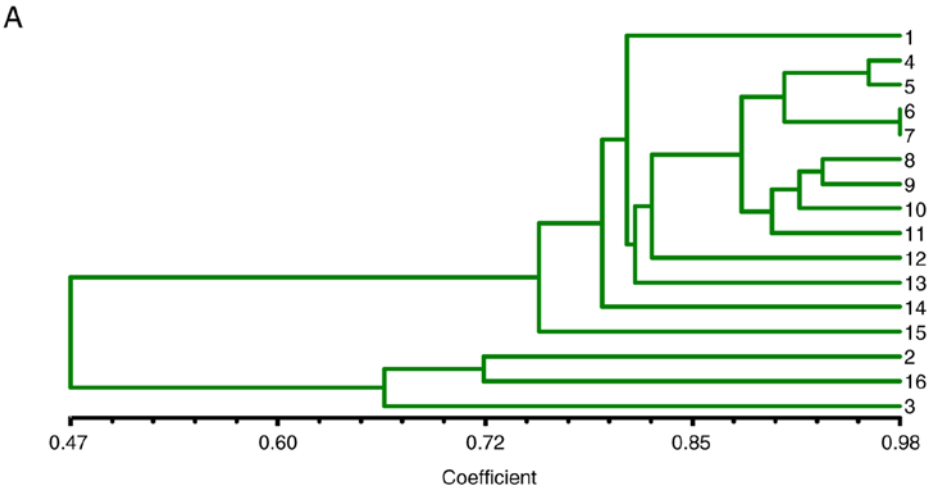


Figure 5. Representative results by ISSR makers in *Lycium* varieties gained by ISSR primers for (A) UBC807, (B) UBC811, (C) UBC879 and (D) UBC885. Lanes 1-16 represent different *Lycium* samples listed in Table III, whereas lane 'M' indicates the DL2000 DNA marker.



B

No. 1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1.00															
2	0.36	1.00														
3	0.59	0.71	1.00													
4	0.86	0.39	0.58	1.00												
5	0.86	0.37	0.58	0.96	1.00											
6	0.87	0.36	0.61	0.90	0.93	1.00										
7	0.87	0.36	0.61	0.90	0.91	0.98	1.00									
8	0.81	0.38	0.57	0.90	0.90	0.90	0.92	1.00								
9	0.76	0.41	0.60	0.85	0.85	0.86	0.88	0.93	1.00							
10	0.82	0.37	0.58	0.89	0.89	0.90	0.91	0.93	0.90	1.00						
11	0.78	0.35	0.54	0.87	0.89	0.88	0.88	0.93	0.87	0.90	1.00					
12	0.72	0.39	0.54	0.83	0.85	0.82	0.82	0.82	0.79	0.81	0.89	1.00				
13	0.78	0.37	0.58	0.79	0.81	0.84	0.84	0.84	0.77	0.83	0.85	0.79	1.00			
14	0.75	0.42	0.59	0.82	0.80	0.81	0.83	0.81	0.76	0.84	0.78	0.78	0.78	1.00		
15	0.74	0.43	0.52	0.77	0.75	0.72	0.74	0.78	0.73	0.77	0.79	0.71	0.79	0.78	1.00	
16	0.37	0.72	0.61	0.42	0.42	0.43	0.43	0.45	0.42	0.46	0.44	0.50	0.50	0.50	0.46	1.00

Figure 6. Dendrogram of *Lycium* varieties of ISSR markers. (A) Dendrogram of *Lycium* varieties using ISSR makers. Bars on the bottom sectoin indicate the SC index. (B) Dendrogram of genetic distance for *Lycium* varieties using ISSR markers. SC, similarity coefficient.

primer amplified 1-10 bands and revealed an average number of 5.9 bands per ISSR primer. The band size ranged between 200-2,000 bp, and 88.28% of the bands were revealed as polymorphic in these 16 samples. The representative results of the primers UBC807, UBC811, UBC879 and UBC885 are presented in Fig. 5. These findings provided a very clear detection of DNA polymorphisms in *Lycium* species or cultivars.

Results of genetic distance and cluster analysis in ISSR amplification. A cluster dendrogram was also obtained from ISSR amplification profiles. It revealed similar results to those produced by RAMP-PCR as regards the SC index among *Lycium* samples (Fig. 6). The dendrogram illustrated that the SC index among the samples ranged from 0.36 to 0.98. The SC index between sample 1 and sample 2 (*Lycium chinense* Miller from Zhongning, Linxia and from Gongzhou, Jiangxi) was the lowest (0.36), and that between samples 6 and 7 of *Lycium barbarum* ('Ningqi-1' from Haidong, Qinghai and from the Ningxia Academy of Agriculture and Forestry Sciences), was the highest (0.98) (Fig. 6B), indicating that they are the same cultivar. All these data are consistent with the RAMP-PCR results and genetic analysis.

Discussion

The present study presented the first overview, to the best of our knowledge, of the genetic variability of *Lycium barbarum* cultivars or species, using both developed RAMP-PCR and ISSR molecular markers. Notably, the Standardization Administration of China (SAC), implemented in July 1, 2015, has approved 'Rules for agricultural seed testing-Verification of genuineness and cultivar' (GB/T3543.5-1995; https://www.codeofchina.com/standard/GBT3543.5-1995.html) as the national standard, no. 1 modification item, in which the regulation for variety authenticity or identity allows DNA molecular detection methods, including SSR and SNP molecular markers, and provides a strong basis for combating various illegal acts of counterfeit rapidly and accurately. The classic markers used by genetic ecologists are DNA sequencing which has also been required to develop SNPs, another codominant and highly polymorphic marker. Based on direct DNA sequencing, such as next generation sequencing (NGS), short tandem repeat (STR) and SNP markers can provide large genome coverage, and exhibit a high level of variability, and thus can be used for phylogenetic studies (31). STR analysis has been successfully and widely used to evaluate genotypes in humans in judicial authentication and forensic sciences (32). However, due to plant genomic complexity, the genomic information remains largely unknown; STR and SNP markers cannot be used for the genetic authentication of plants or herbs.

Given the advantages and disadvantages of different molecular genetic marker techniques, it is important to design the most effective method in order to address particular ecological questions. In RAMP-PCR, where the ramp time is prolonged at the stage from annealing to extension, the resolution and production are greatly increased compared to regular RAPD (25,26,33). The present study, using RAMP-PCR and ISSR marker techniques, successfully characterized and authenticated *Lycium* varieties from different geographic regions indicating that samples 6 and 7 were the same cultivar. Moreover, the similarity coefficient index between samples 2 (*Lycium chinense* Miller from Gongzhou, Jiangxi) and 16 (*Lycium chinense* Miller from Houston, TX, USA) was found to be 0.72 by RAMP-PCR and 0.72 by ISSR, which clustered together, indicating that these two cultivars have a good genetic association, although a diverse geographic distance. The results of RAMP-PCR and ISSR were both mutually consistent. RAMP is a PCR-based technique, which combines ISSR and RAPD analysis, which can generate useful molecular markers, investigate DNA polymorphisms, and can be used to elucidate the genetic associations among accessions, including *Lycium* varieties (34).

In conclusion, the present study combined RAMP-PCR and ISSR to analyze the genetic association and distance of *Lycium* varieties comprehensively. To the best of our knowledge, the present study is the first to genetically characterize the molecular diversity of *Lycium* varieties by combining these two methods, and this molecular characterization may prove to be useful for the conservation and preservation of DNA diversity of *Lycium* species.

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Availability of data and materials

The data and material that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

XL, JD, CW and ZM were involved in the study methodology and in performing the experiments. JC was involved in data analysis. JF was involved in the conception of the study, and in the writing and preparation of the original draft. MK and JF were involved in the conception of the study, and in the editing and revising of the manuscript. HC, TH and JF designed and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dong JZ, Yang JJ and Wang Y: Resources of Lycium species and related research progress. Zhongguo Zhong Yao Za Zhi 33: 2020-2027, 2008 (In Chinese).
2. Ram VJ: Herbal preparations as a source of hepatoprotective agents. Drug News Perspect 14: 353-363, 2001.
3. Potterat O: Goji (*Lycium barbarum* and *L. chinense*): Phytochemistry, pharmacology and safety in the perspective of traditional uses and recent popularity. Planta Med 76: 7-19, 2010.
4. Ulbricht C, Bryan JK, Costa D, Culwell S, Giese N, Isaac R, Nummy K, Pham T, Rapp C, Rusie E, *et al*: An evidence-based systematic review of Goji (*Lycium* spp.) by the natural standard research collaboration. J Diet Suppl 12: 184-240, 2015.
5. Jin M, Huang Q, Zhao K and Shang P: Biological activities and potential health benefit effects of polysaccharides isolated from Lycium barbarum L. Int J Biol Macromol 54: 16-23, 2013.
6. Chang RC and So KF: Use of anti-aging herbal medicine, *Lycium barbarum*, against aging-associated diseases. What do we know so far? Cell Mol Neurobiol 28: 643-652, 2008.
7. Ma ZF, Zhang H, The SS, Wang CW, Zhang Y, Hayford F, Wang L, Ma T, Dong Z, Zhang Y and Zhu Y: Goji berries as a potential natural antioxidant medicine: An insight into their molecular mechanisms of action. Oxid Med Cell Longev 2019: 2437397, 2019.
8. Liu L, Lao W, Ji QS, Yang ZH, Yu GC and Zhong JX: *Lycium barbarum* polysaccharides protected human retinal pigment epithelial cells against oxidative stress-induced apoptosis. Int J Ophthalmol 8: 11-16, 2015.
9. Lam CS, Tipoe GL, So KF and Fung ML: Neuroprotective mechanism of *Lycium barbarum* polysaccharides against hippocampal-dependent spatial memory deficits in a rat model of obstructive sleep apnea. PLoS One 10: e0117990, 2015.
10. Yu H, Wark L, Ji H, Willard L, Jaing Y, Han J, He H, Ortiz E, Zhang Y: Medeiros DM and Lin D: Dietary wolfberry upregulates carotenoid metabolic genes and enhances mitochondrial biogenesis in the retina of db/db diabetic mice. Mol Nutr Food Res 57: 1158-1169, 2013.
11. Lin J, Zhang Y, Wang X and Wang W: *Lycium ruthenicum* extract alleviates high-fat diet-induced nonalcoholic fatty liver disease via enhancing the AMPK signaling pathway. Mol Med Rep 12: 3835-3840, 2015
12. Ye M, Moon J, Yang J, Hwa Lim H, Bin Hong S, Shim I and Bae H: The standardized *Lycium chinense* fruit extract protects against Alzheimer's disease in 3xTg-AD mice. J Ethnopharmacol 172: 85-90, 2015.
13. Lin L, Li J, Lv H, Ma Y and Qian Y: Effect of *Lycium ruthenicum* anthocyanins on atherosclerosis in mice. Zhongguo Zhong Yao Za Zhi 37: 1460-1466, 2012 (In Chinese).
14. Zhao R, Cai Y, Shao X and Ma B: Improving the activity of *Lycium barbarum* polysaccharide on sub-health mice. Food Funct 6: 2033-2040, 2015.
15. Dursun R, Zengin Y, Gündüz E, İçer M, Durgun HM, Dağgulli M, Kaplan İ, Alabalık U and Güloğlu C: The protective effect of goji berry extract in ischemic reperfusion in testis torsion. Int J Clin Exp Med 8: 2727-2733, 2015.
16. Telang N, Li G, Sepkovic D, Bradlow HL and Wong GY: Comparative efficacy of extracts from *Lycium barbarum* bark and fruit on estrogen receptor positive human mammary carcinoma MCF-7 cells. Nutr Cancer 66: 278-284, 2014.
17. Chao JC, Chiang SW, Wang CC, Tsai YH and Wu MS: Hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells. World J Gastroenterol 12: 4478-4484, 2006.
18. Williams JG, Kubelik AR, Livak KJ, Rafalski JA and Tingey SV: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18: 6531-6535, 1990.
19. Hess J, Kadereit JW and Vargas P: The colonization history of Olea europaea L. In Macaronesia based on internal transcribed spacer 1 (ITS-1) sequences, randomly amplified polymorphic DNAs (RAPD), and inter simple sequence repeats (ISSR). Mol Ecol 9: 857-868, 2000.
20. Agarwal M, Shrivastava N and Padh H: Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Rep 27: 617-631, 2008.
21. Varela ES, Lima JP, Galdino AS, Pinto Lda S, Bezerra WM, Nunes EP, Alves MA and Grangeiro TB: Relationships in subtribe Diocleinae (Leguminosae; Papilionoideae) inferred from internal transcribed spacer sequences from nuclear ribosomal DNA. Phytochemistry 65: 59-69, 2004.
22. Taški-Ajduković K, Nagl N, Ćurčić Z and Zorić M: Estimation of genetic diversity and relationship in sugar beet pollinators based on SSR markers. Electronic J Biotechnol 27: 1-7, 2017.
23. Pachuau L, Atom AD and Thangjam R: Genome classification of musa cultivars from northeast india as revealed by ITS and IRAP markers. Appl Biochem Biotechnol 172: 3939-3948, 2014.
24. Mei Z, Zhang X, Khan MA, Imani S, Liu X, Zou H, Wei C and Fu J: Genetic analysis of *Penthorum chinense* Pursh. By improved-RAPD and ISSR in China. Electron J Biotechnol 30: 6-11, 2017.
25. Fu S, Cheng J, Wei C, Yang L, Xiao X, Zhang D, Stewart MD and Fu J: Development of diagnostic SCAR markers for genomic DNA amplifications in breast carcinoma by DNA cloning of high-GC RAMP-PCR fragments. Oncotarget 8: 43866-43877, 2017.
26. Fu JJ, Li LY, Xu X, Wang Z, Tang G, Yin C and Lu G: An improved method for increasing the efficiency of the technique of random amplified polymorphic DNA (RAPD). Hereditas 22: 251-252, 1999.
27. Fu J, Yang L, Khan MA and Mei Z: Genetic characterization and authentication of Lonicera japonica Thunb. By using improved RAPD analysis. Mol Biol Rep 40: 5993-5999, 2013.
28. Fu J: Short protocols in medical molecular biology. China Medical Science Press, 2012.
29. Mei Z, Khan MA, Zhang X and Fu J: Rapid and accurate genetic authentication of *Penthorum chinense* by improved RAPD-derived species-specific SCAR markers. Biodiversitas 18: 1243-1249, 2017.
30. Rohlf FJ: NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, version 2.1. Exeter Publishing, Setauket, NY, 2000.
31. Robertson M and Richards C: Opportunities and challenges of next-generation sequencing applications in ecological epigenetics. Mol Ecol 24: 3799-37801, 2015.
32. Fu J, Cheng J, Liu X, Li J, Wei C, Zheng X, He T and Fu J: Evaluation genotypes of cancer cell lines HCC1954 and SiHa by short tandem repeat (STR) analysis and DNA sequencing. Mol Biol Rep 45: 2689-2695, 2018.
33. Mei Z, Zhang X, Liu X, Imani S and Fu J: Genetic analysis of Canarium album in the different areas of China by improved-RAPD and ISSR. CR Biol 340: 558-564, 2017.
34. Liang HY, Liu XJ and Yang MS: Analysis of *Lycium chinense* variety revealed by RAMP-PCR method. Chinese Agric Sci Bull 26: 61-64, 2011.