RESEARCH NOTE

Genetic relationships among buckwheat (*Fagopyrum*) species from southwest China based on chloroplast and nuclear SSR markers

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Introduction

Buckwheat (*Fagopyrum*, family Polygonaceae), originates from China (Linnaeus 1753), has great value as the green food material and medicinal plant. The genus *Fagopyrum* includes 23 species, three variations and two subspecies (Yang and Xia 2008), which can be divided into small-achene and big-achene buckwheat groups. Phylogenetic relationships and classification of *Fagopyrum* species have been debated ever since Gross (1913) first classified them in China. The origin and classification of buckwheat gradually became the worldwide concern. Studies have provided strong support for the hypothesis that the Jinsha river basin (Chinese western region) is the distribution centre or centre of origin for buckwheat (Ohnishi and Yasui 1998; Wang *et al.* 2008).

Buckwheat resources in Sichuan Pan'xi regions are quite rich (Wang *et al.* 2006) and new wild *Fagopyrum* species are continuously being discovered. We investigated the genus-level phylogenetic relationship and genetic backgrounds of 12 wild and two cultivated accessions representing the main distribution of buckwheat in western Sichuan by using chloroplast simple sequence repeat (cpSSR) and nuclear (nSSR) markers.

Materials and methods

Plant materials

Fourteen accessions representing 10 species of *Fagopyrum* were obtained from western Sichuan, China. Details of the collection sites, natural altitude distributions and accession codes are provided in table 1. Each accession was represented by five individuals. Individual seedlings were collected from autumn 2008 to spring 2009 and planted in the botanical garden of the College of Life and Physical Science, Sichuan Agricultural University. The *Fagopyrum* species were not compared with an outgroup species because the current research aimed at establishing their interspecific relationships.

DNA extraction

DNA was extracted and isolated by Genomic DNA Isolation kit (TaKaRa, Dalian, China). DNA concentrations were determined by comparison with a serial dilution of standard lambda DNA, and the quality of DNA was checked with a DNA–protein instrument (Bio-Rad, California, USA).

CpSSR analysis

Seven pairs of primers for chloroplast microsatellites (table 2), designated as CSU03, CSU05, CCMP02, CCMP07, NTCP9, RC3 and RC6 were used. PCR was carried out in a total volume of 25 μL containing 1× reaction buffer, 100 μm of each dNTP, 100 μm primer (each direction) and about 50 ng DNA as the template on a PTC_200 thermocycler (MJ Research, Massachusetts, USA), following the programme: predenaturing at 94°C for 3 min, 32 cycles of denaturing at 94°C for 1 min, annealing at the appropriate temperature for each primer pair (table 2) for 1 min and extending at 72°C for 1 min with an extra extension at 72°C for 10 min. PCR product was separated on a 6%

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Table 1. Botanical names and sources of the tested materials.

	Fagopyrum species	Common name	Chromosome number	Natural altitude distribution (m)	Code	Origin
1	*F. densovillosum* J. L. Liu	Mimao Yeqiao	16	1700 (1380–1910)	M	Xichang Puge county, Luoji mountain town
2	*F. crispatofolium* J. L. Liu	Zhouye yeqiao	32	ca. 1900	Z	Xichang Puge county, Luoji mountain town
3	*F. gracilipes* var. *odontoperum* (Gross) Samuelss	Hongchi Yeqiao	16	1200	H	Xichang Puge county, Luoji mountain town
4	*F. gracilipes* (Hemsl.) Dammer ex Diels var. *odontoperum* (Gross) Sam	Baichi Yeqiao	32	1200	B	Xichang Puge county, Luoji mountain town
5	*F. leptopodum* (Dieos) Hedberg var. *grossii* (Levl.) Sam	Shusui Yeqiao	16	319–2450	S	Sichuan, A'ba
6	*F. gracilipes* (Hemsl.) Dammer ex Diels	Xibing Yeqiao	32	1200–3100	X	Xichang, Sanwoshu town
7	*F. urophyllum* (Bur. et Tranch) H. Gross	Yingzhi Wannianqiao	16	2500–3600	Y	Sichuan, Xichang
8	*F. cymosum* (Trev.) Meissn	Jingqiao	16	319–2450	J1	Sichuan, Ya'an
9	*F. cymosum* (Trev.) Meissn	Jingqiao	16	319–2450	J2	Xichang Puge county, Luoji mountain town
10	*F. cymosum* (Trev.) Meissn	Jingqiao	16	319–2450	J3	City, Befengxia mountain
11	*F. cymosum* (Trev.) Meissn	Jingqiao	16	1800–2800	J4	A'ba, Sichuan
12	*F. wenchuanense* Ji-Rong Shao	Wenchuan Yeqiao	16	1250–3200	J5	Wenchuan, A'ba
13	*F. esculentum* Moench	Tianqiao	16	510–3100	T	Xichang Puge county, Luoji mountain town
14	*F. tataricum* (L.) Gaertn	Kuqiao	16	914–3100	K	Xichang Puge county, Luoji mountain town

(w/v) denaturing polyacrylamide gel and visualized by silver staining. The range of allele sizes was determined by reference to a 100-bp DNA ladder (Sangon, Shanghai, China) (Panaud *et al.* 1996). In all cases, PCRs were performed at least twice to determine whether the absence of any DNA band was real or due to a failed reaction.

Nuclear SSR analysis

In a preliminary study, a number of microsatellite DNA (SSR) markers, originally developed for *Fagopyrum esculentum*, were examined. Based on the clarity and reproducibility of the band patterns, 17 primer pairs were selected for further study. The amplification reactions were performed in a volume of 25 μL containing 1× reaction buffer, 0.22 mM dNTPs, 2.0 mM $MgC1_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.3 mM primer (each direction), 1.0 U *Taq* DNA polymerase (TaKaRa) and 20–30 ng genomic DNA. PCR amplifications were performed using the following programme: 94°C for 10 min, 30 cycles of denaturing at 94°C for 1 min, annealing (1 min at 55–64°C), extension (72°C for 1 min) with an extra extension at 72°C for 10 min. The amplified PCR products were visualized by the procedure described for the cpSSR analysis. Each PCR was performed at least twice to ensure reproducibility.

Data analysis

The banding patterns obtained from cpSSR and nSSR markers were analysed to estimate genetic relationships among the 14 buckwheat accessions. Since the chloroplast genome is haploid and does not undergo recombination, each cpSSR

Table 2. Universal chloroplast SSR primer pair sequences applied in this study.

Code	Sequence (5′ → 3′)	Sequence (5′ → 3′)	T_m (°C)	Number of alleles	Source
CCMP02	GATCCCGGACGTAATCCTG	ATCGTACCGAGGGTTCGAAT	55	3	5′-trnS
CCMP07	CAACATATACCACTGTCAAG	ACATCATTATTGTATACTCTTTC	52	2	
CSU05	TGTTCGATAGCAAGTTGATTG	GAGTTAGTTGAACTTATCACTC	52	1	atpB-rbcL
NTCP9	CTTCCAAGCTAACGATGC	CTGTCCTATCCATTAGACAATG	52	2	Tobacco
RC3	TAGGCATAATTCCCAACCCA	CTTATCCATTTGGAGCATAGGG	–	–	Rice
RC6	GAATTTTAGAACTTTGAATTTTTTACCC	AAGCGTACCGAAGACTCGAA	–	–	Rice
CSU03	AAAGTATTCCTGACCCAATCG	ACTAGGACTTATCTTTATCGC	–	–	trnC-trnD

allele was scored as a binary character for presence (1) or absence (0). In the case of diploid nuclear SSRs, the banding patterns were scored as homozygous or heterozygous genotypes. Band scoring was analysed using the Gel Doc 1000TM image analysis system (Bio-Rad). Based on the data, the coefficients were computed to provide similarity matrices (Nei and Li 1979). Dendrograms were constructed using TREECON software with UPGMA cluster analysis (Van de Peer and De Wachter 1994). The bootstrap values were obtained with 1000 replications to evaluate the internal support for the trees. Cophenetic correlation coefficients were calculated by NTSYS-pc ver. 2.1p, to check the goodness fit of the cluster analysis (Rohlf 2000). In addition, the programs DCENTER, EIGEN and MOD3D of NTSYS-pc ver. 2.1p were used to perform a principal coordinate analysis for the nSSR character matrix. To measure the informativeness of the markers, the average polymorphic information content was calculated for each marker system according to the formula given by Powell *et al.* (in Haussmann *et al.* 2002). This value provides an estimate of the discrimination power of a molecular locus by taking into account not only the number of alleles per locus but also their relative frequencies in the studied population (Lubberstedt *et al*. 2000).

Results

The cpSSR analysis was performed in 14 *Fagopyrum* accessions. Three of seven analysed chloroplast loci were polymorphic in all investigated *Fagopyrum* species. Three different allele size variants were detected at the locus CCMP02 (figure 1), and two allele size variants were detected at CCMP07 (figure 2) and NTCP9, respectively (table 2).

Figure 1. The cpSSR-PCR amplification results of the ccmp2.

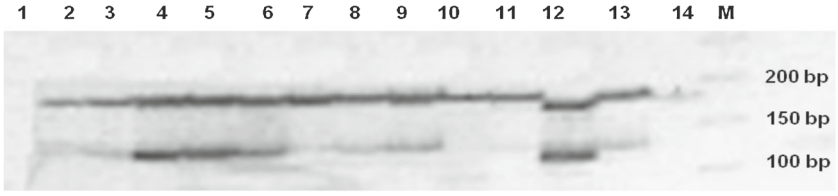

Figure 2. The cpSSR-PCR amplification results of the ccmp7. The DNA samples are showed as follows: 1, *F. densovillosum* J. L. Liu; 2, *F. crispatofolium* J. L. Liu; 3, *F. gracilipes* var. *odontoperum* (Gross) Samuelss; 4, *F. gracilipes* (Hemsl.) Dammer ex Diels var. *odontoperum* (Gross) Sam; 5, *F. leptopodum* (Dieos) Hedberg var. *grossii* (Levl.) Sam; 6, *F. gracilipes* (Hemsl.) Dammer ex Diels; 7, *F. urophyllum* (Bur. et Tranch) H. Gross; 8, *F. cymosum* (Trev.) Meissn; 9, *F. cymosum* (Trev.) Meissn; 10, *F. cymosum* (Trev.) Meissn; 11, *F. cymosum* (Trev.) Meissn; 12, *F. wenchuanense* Ji-Rong Shao; 13, *F. esculentum* Moench; 14, *F. tataricum* (L.) Gaertn; M, DNA marker (50-bp ladder).

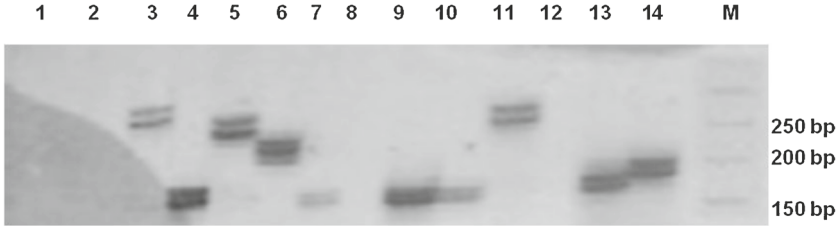

Figure 3. The parts of nSSR-PCR amplification results of the ccmp2. The DNA samples are showed as follows: 1, *F. densovillosum* J. L. Liu; 2, *F. crispatofolium* J. L. Liu; 3, *F. gracilipes* var. *odontoperum* (Gross) Samuelss; 4, *F. gracilipes* (Hemsl.) Dammer ex Diels var. *odontoperum* (Gross) Sam; 5, *F. leptopodum* (Dieos) Hedberg var. *grossii* (Levl.) Sam; 6, *F. gracilipes* (Hemsl.) Dammer ex Diels; 7, *F. urophyllum* (Bur. et Tranch) H. Gross; 8, *F. cymosum* (Trev.) Meissn; 9, *F. cymosum* (Trev.) Meissn; 10, *F. cymosum* (Trev.) Meissn; 11, *F. cymosum* (Trev.) Meissn; 12, *F. wenchuanense* Ji-Rong Shao; 13, *F. esculentum* Moench; 14, *F. tataricum* (L.) Gaertn; M, DNA marker (50-bp ladder).

The genetic variation present among the 14 accessions representing 11 *Fagopyrum* species was also determined by investigating 17 nSSR alleles (figure 3). Fifteen of 17 nSSR markers were polymorphic in at least two accessions. A total of 55 SSR alleles, with fragment sizes ranging from 106 to 454 bp were detected.

A similarity matrix was calculated separately based on cpSSR and nSSR data. According to the cpSSR and nSSR analyses: (i) wild small-achene and big-achene group samples had different cpDNA. The genetic relationship between *F. dibotrys* and the small-achene and big-achene groups was very complex, with this species acting more like a hybrid and excessive species from them; (ii) the genetic variation in cpDNA among small-achene group buckwheat varieties was low, and the intraspecific genetic variation of *F. dibotrys* was also lower than among the big-achene group. However, the nuclear DNA results indicated significant differences for intraspecific and interspecific, and small-achene and big-achene variations; and (iii) according to both cpSSR and nSSR data, *F. crispatofolium* and *F. densovillosum* showed relatively closer affinity; the genetic backgrounds of two subspecies of *F. gracilipes* could not be distinguished by cpSSR analysis, but their genetic variation was clear by nSSR analysis.

Conclusion

Western Sichuan has abundant buckwheat resources. With 16 species, Sichuan alone comprises 70% of the 23 endemic *Fagopyrum* species occurring in China. In fact, western Sichuan is regarded as the natural distribution and variation centre for *Fagopyrum* species in China (Ohnishi and Yasui 1998). The main objective of this study was to assess the genetic relationships among the *Fagopyrum* species on the basis of cpSSR and nuclear SSR markers. A striking feature of buckwheat is the occurrence of multiple ploidies. In phylogenetic studies conducted on groups in which hybridization between lineages played a substantial role in their evolution, the combined use of cpDNA and nDNA markers was crucial to acquiring a comprehensive understanding of their evolutionary history (Paola *et al.* 2012).

In this study, we elucidated the pattern of genetic interrelationships and intraspecific variability among native *Fagopyrum* species in western Sichuan. This new knowledge provides information for understanding the evolution of *Fagopyrum*, and it will help guide future research. Our results demonstrated that the combination of cpSSR and nSSR molecular markers can be practical when monitoring a defined but limited set of genetic changes that may have occurred during the evolutionary process among *Fagopyrum*. However, the range of *Fagopyrum* accessions and species included in this study was limited. Therefore, a more extensive survey of *Fagopyrum* specimens, based on a wide range of markers covering both noncoding and coding regions, and including data based on, for example, AFLPs, DNA sequencing and isozymes, would enable us to obtain a more complete view of the genetic variation, interrelationships and evolution in *Fagopyrum*.

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