Development and Characterization of Microsatellite Markers for Common Buckwheat

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So far, only five microsatellite markers have been developed in common buckwheat (*Fagopyrum esculentum*). The purpose of the present study was to develop a larger number of microsatellite markers in common buckwheat. By sequencing 2785 clones from the libraries, which were enriched for (CT)_n and (GT)_n repeats using magnetic particles, it was shown that 1483 clones contained microsatellites, of which 352 had unique sequences. Primer pairs were designed for 237 of the microsatellite loci, of which 180 primer pairs each amplified PCR products. Fifty-four primer pairs that each amplified a clear PCR product of the expected size were evaluated for their ability to detect variations in common buckwheat populations and to be utilized in seven related *Fagopyrum* species. Forty-eight (88.9%) out of the 54 microsatellite markers tested were found to be highly variable (the average number of alleles was 12.2 and the average polymorphism information content (PIC) was 0.79) in a population of cultivated buckwheat. This PIC value was comparatively large when compared with the average values for microsatellite markers reported for other crops. A high rate of successful amplification of common buckwheat microsatellite markers was observed in closely related species; in particular all the 54 loci were successfully amplified in the wild ancestor of cultivated common buckwheat. The microsatellite markers developed in the present study should contribute to the promotion of molecular breeding in common buckwheat.

Key Words: *Fagopyrum esculentum*, cross-specific amplification, cymosum group, simple sequence repeats (SSRs).

Introduction

The genus *Fagopyrum* consists of two major phylogenetic groups, the cymosum group and the urophyllum group. The cymosum group, characterized by large seeds, contains all the useful *Fagopyrum* species, including the two cultivated species, common buckwheat (*F. esculentum* Moench) and Tartary buckwheat (*F. tataricum* Gaert.) (Ohnishi and Matsuoka 1996, Yasui and Ohnishi 1998). As for the other members of the cymosum group, *F. homotropicum* Ohnishi, a self-compatible wild species, has been successfully crossed with common buckwheat (Campbell 1995, Wang *et al.* 2002), and is now considered to be an important genetic resource for the improvement of cultivated common buckwheat. *Fagopyrum cymosum* Meisn., another wild species, has been utilized as a medicinal plant in China and Southeast

Asia (Anderson 1986).

The origin of cultivated common buckwheat has been studied by the analysis of the diffusion routes of cultivated common buckwheat using RAPD markers (Murai and Ohnishi 1996), by the determination of the allozyme diversity of the cultivated populations (Ohnishi 1998) and by the analysis of the genetic relationships between cultivated populations and natural populations of wild common buckwheat using AFLP markers (Konishi *et al.* 2005). During the course of these studies, several issues that were difficult to solve by the use of allozymes as well as of RAPD and AFLP markers, were identified, for example, the determination of the center of genetic diversity, detailed genetic relationships among cultivated populations, and gene flow between cultivated populations and natural populations of wild common buckwheat. If more powerful (more polymorphic) markers became available, it would be possible to address these issues, so that studies on the origin of cultivated common buckwheat could progress more rapidly.

A microsatellite, also called a simple sequence repeat

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(SSR) which is a sequence consisting of tandem repeats of a short nucleotide motif, is abundantly distributed throughout eukaryotic genomes (Litt and Luty 1989, Weber and May 1989). Microsatellite markers are a powerful tool for the analysis of wide genetic variations within or among populations because of their co-dominance, multiallelism and high variability (Litt and Luty 1989, Tautz 1989). In many crops, they have been developed and used for the study of genetic diversity and population structure, for example in durum wheat (Thuillet *et al.* 2005), maize (Vigouroux *et al.* 2005) and rice (Garris *et al.* 2005).

Iwata *et al.* (2001) who developed microsatellite markers for common buckwheat, observed a significant genetic differentiation among Japanese common buckwheat cultivars (Iwata *et al.* 2005). However, only five microsatellite markers were developed by Iwata *et al.* (2001). In the present study, we developed a large number of microsatellite markers for common buckwheat and evaluated their variation within a common buckwheat population. Since the development of microsatellite markers is expensive and time-consuming, the utility of the markers would be increased if the microsatellite markers developed for common buckwheat could be used for related species. Therefore, we also determined whether the microsatellite markers developed for common buckwheat could be used for other *Fagopyrum* species of the cymosum group.

Materials and Methods

Plant materials and DNA extraction

An individual of *F. esculentum* ssp. *esculentum* from the Boke population (accession number, C9701) in Sichuan province, China was the source of the DNA used to construct a microsatellite-enriched library. An artificial population of common buckwheat consisting of 34 individuals, one individual each from 34 worldwide accessions (19 Chinese landraces and 15 landraces from 15 countries) was used to analyze the microsatellite variation.

To determine whether the microsatellite markers which were developed for cultivated common buckwheat could be utilized in related *Fagopyrum* species, one individual was used for each of the following seven taxa; *F. esculentum* ssp. *ancestrale* (C2009), diploid and tetraploid *F. homotropicum* (C9740, C9737), *F. tataricum* ssp. *tataricum* (C9722), *F. tataricum* ssp. *potanini* (C9022), diploid and tetraploid *F. cymosum* (C8924, B9103). *F. esculentum* ssp. *ancestrale* and *F. tataricum* ssp. *potanini* are the wild ancestors of cultivated common and Tartary buckwheat, respectively (Ohnishi 1998).

The total DNA of each individual plant was extracted from fresh leaf tissue using Plant DNAzol® Reagent (Invitrogen Corp., Carlsbad, CA, USA).

Isolation of microsatellites and primer design

A library enriched for (GT)_n dinucleotide was constructed according to the method of Tani *et al.* (2004) using

magnetic particles. The hybridizing probe used for enrichment consisted of the 5'-biotinylated (GT)₁₅ oligonucleotide. Cloned DNAs from the library were sequenced using an ABI PRISM 3100 Genetic Analyzer with a BigDye Terminator ver. 3 (PE Applied Biosystems, Foster City, CA, USA). In addition, many cloned DNAs from the (CT)_n-enriched library constructed by Iwata *et al.* (2001), of which sequences had not been elucidated, were also sequenced, and used in this study. Microsatellites, according to Weber (1990), were identified by checking nucleotide sequence data. Duplicated sequences among clones were detected by homology search of the clones in the libraries. If homologous sequences were found, only one unique sequence was kept for primer design. For each unique sequence, primer pairs were designed using the computer software OLIGO (National Biosciences, Inc., Plymouth, MN, USA) and Primer3 listed at <http://waldo.wi.mit.edu/cgi-bin/primer/primer3> (Whitehead Institute for Biomedical Research).

PCR amplification and detection of PCR products

After optimization of the PCR conditions, PCR amplification was performed in a 15 µl reaction solution containing 0.2 µM of each primer, 200 µM of each dNTP, 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of MgCl₂, 0.5 U of *Taq* DNA polymerase (New England Biolabs, Beverly, MA, USA), and 15 ng of template DNA using an I-Cycler (BIO-RAD, Hercules, CA, USA) under the following conditions: 2 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 46–69°C and 60 sec at 72°C, followed by 3 min at 72°C. The annealing temperature used for each primer pair is listed in Appendix 1. Microsatellite alleles were identified by running the PCR products on a denaturing 5% (w/v) urea-polyacrylamide (19 : 1 acrylamide:Bis, 8 M urea) gel in a TBE buffer at 100 W for 2 hours and visualized by a silver-staining method using SILVER SEQUENCE™ DNA Staining Reagents (Promega Corp., Madison, WI, USA).

Analysis of microsatellite variation

Fifty-four primer pairs that yielded an amplification product of the expected size were analyzed for their variability in the artificial population comprising 34 individuals. For the primer pairs that detected polymorphism, the number of alleles (NA) and the polymorphism information content (PIC) were calculated as follows: $PIC = 1 - \sum p_i^2$, where p_i is the frequency of the i -th allele in the sample examined (Anderson *et al.* 1993).

Amplification in related *Fagopyrum* species

The fifty-four primer pairs which were used for the analysis of microsatellite variability were also analyzed for their use in the seven *Fagopyrum* species or subspecies which are closely related to common buckwheat. PCR amplification was performed using the same protocol as that described above, and repeated at least twice when no amplification was observed on the first attempt. Since the required amount of DNA was not obtained to test all the primer pairs

for diploid and tetraploid *F. cymosum*, only 30 and nine primer pairs were evaluated for diploid and tetraploid *F. cymosum*, respectively.

Results and Discussion

DNA sequences were determined for 1875 and 910 clones from the two libraries, enriched for (CT)_n and (GT)_n dinucleotides, respectively. Of the 1875 clones from the library enriched for (CT)_n, 1079 (57.5%) were found to contain microsatellites. Two hundred and forty-five clones each containing a unique sequence were identified. Although the remaining 834 clones were duplicated more than once in the enriched library, they could be classified into 148 groups based on unique sequences. In total, 393 unique sequences were thus obtained. For the library enriched by (GT)_n, 404 (44.4%) of the 910 clones were found to contain microsatellites. One hundred and six clones contained unique sequences, and the remaining 298 clones were classified into 59 groups based on unique sequences. Therefore, in total, 165 unique sequences were obtained.

Although 393 and 165 unique sequences were obtained from the libraries enriched for (CT)_n and (GT)_n, respectively, many of the sequences were duplicated between the two libraries. By removing all the unnecessary duplicated sequences, 352 unique sequences were thus obtained, of which 237 sequences could be used to design primers. Of the 237 primer pairs, 180 pairs amplified PCR products (see Appendix 1), while the remaining 57 pairs did not amplify any PCR products even at the lower annealing temperature of 46°C. Of the 180 primer pairs which amplified PCR products, 54 pairs each amplified a clear PCR product of the expected size (Fig. 1a). We evaluated these 54 markers for their ability to detect variation in a common buckwheat pop-

ulation, and for their ability to be utilized in the 7 related *Fagopyrum* species. The remaining 126 pairs amplified more than one fragment (Fig. 1b,c). These pairs may amplify several sites other than the target site. The use of these 126 pairs is not convenient for detecting genetic variation because the amplified loci may not be homologous among individuals.

Among the 54 loci at which a clear PCR product with the expected size was amplified, 48 (88.9%) loci exhibited microsatellite variation in the experimental common buckwheat population. Figure 2 shows some examples of the loci displaying variation (Fes1585 and Fes3331). The number of alleles per polymorphic locus, NA, ranged from two to 30 with an average value of 12.2, while the PIC ranged from 0.42 to 0.95 with an average value of 0.79 (Table 1). Many authors have reported that microsatellite loci with a larger number of repeats of motif showed a wider variation than microsatellite loci with a smaller number of repeats of motif for such crops as barley (Ramsay *et al.* 2000), maize (Sharopova *et al.* 2002) and tomato (He *et al.* 2003). In common buckwheat, a significant correlation was detected between the number of (GA/CT) repeats in the microsatellites and NA and PIC (correlation coefficient (r) = 0.654 ($P < 0.001$) and $r = 0.584$ ($P < 0.001$), respectively). Microsatellites with the other types of repeats were not investigated because the number of samples was small. The average PIC value (0.79) was comparatively large when compared with that for other crops; namely 0.37 for tomato (He *et al.* 2003), 0.59 for rice (Temnykh *et al.* 2000), 0.62 for maize (Smith *et al.* 1997) and 0.71 for wheat (Prasad *et al.* 2000). This may be due to the fact that the artificial population consisted of 34 individuals from a world-wide collection of landraces, i.e. the population was expected to contain variation at the species level.

Of the 54 microsatellite markers which were evaluated for their ability to be utilized in the seven related *Fagopyrum* species, two loci (Fes1693 and Fes2459) could be amplified in all the seven taxa (Table 1). Many authors have reported that the transferability of the microsatellite markers decreased with the increase in the genetic distance between taxa in rapeseed (Lagercrantz *et al.* 1993) and soybean (Peakall *et al.* 1998). In the *Fagopyrum* species, this can be observed by comparing the number of different nucleotides in *rbcL* gene sequences (Yasui and Ohnishi 1998) and the percentage of successful amplification (Table 2). The percentage of successful amplification in *F. esculentum* ssp. *ancestrale*, which showed exactly the same *rbcL* sequence as common buckwheat, was 100%. In a more distantly related species, diploid and tetraploid forms of *F. cymosum* were different from *F. esculentum* ssp. *esculentum* at seven nucleotides. The percentage of successful amplification was 36.7% and 33.3% in diploid and tetraploid forms of *F. cymosum*, respectively. The rate of transferability in tetraploids was slightly lower than that in diploid counterparts, namely 79.6% vs. 68.5% in *F. homotropicum* and 36.7% vs. 33.3% in *F. cymosum* (Table 2), which was consistent with previous reports indicating that the percentage of primer

Fig. 1. PCR products separated on 5% denaturing polyacrylamide gels and visualised by silver staining. (a) PCR products with a single clear band of the expected size, (b) PCR products with more than one band and (c) PCR products with ladder-like bands.

Table 1. Polymorphism parameters (NA and PIC) and transferability of 54 microsatellite markers

Locus	NA ¹⁾	PIC	ANC ²⁾	HOM2x	HOM4x	TAT	POT	CYM2x	CYM4x
Fes1094	20	0.92	○ ³⁾	○	○	○	×	–	–
Fes1284	1	–	○	○	○	○	×	○	–
Fes1286	22	0.93	○	○	×	○	○	×	×
Fes1303	28	0.95	○	○	○	○	×	×	–
Fes1322	24	0.95	○	×	×	×	×	–	–
Fes1346	9	0.84	○	×	×	○	×	×	–
Fes1362	10	0.82	○	×	○	×	×	×	×
Fes1368	15	0.89	○	○	○	○	×	–	–
Fes1394	11	0.75	○	○	○	○	×	–	–
Fes1407	23	0.92	○	○	○	×	×	–	–
Fes1409	8	0.73	○	×	○	×	×	–	–
Fes1475	15	0.83	○	×	×	○	×	×	–
Fes1497	18	0.92	○	×	×	○	×	–	–
Fes1498	6	0.64	○	○	○	×	×	–	–
Fes1501	5	0.65	○	○	○	○	○	–	–
Fes1582	14	0.89	○	×	×	×	×	–	–
Fes1583	5	0.62	○	○	○	○	×	×	–
Fes1585	4	0.63	○	○	○	○	×	–	–
Fes1623	13	0.83	○	○	×	○	×	–	–
Fes1693	1	–	○	○	○	○	○	○	○
Fes1816	13	0.87	○	○	○	○	×	–	–
Fes1822	20	0.85	○	×	×	○	○	○	○
Fes1840	8	0.80	○	○	○	×	×	×	–
Fes1896	1	–	○	×	×	×	×	–	–
Fes2328	11	0.86	○	○	○	×	×	×	–
Fes2459	1	–	○	○	○	○	○	○	○
Fes2468	11	0.80	○	○	○	○	○	–	–
Fes2480	13	0.78	○	○	○	×	×	×	–
Fes2507	7	0.62	○	○	×	○	×	×	×
Fes2644	8	0.80	○	○	×	○	×	×	–
Fes2741	2	0.48	○	○	×	○	×	×	–
Fes2743	4	0.62	○	○	○	○	×	×	–
Fes2751	24	0.93	○	×	×	×	×	×	–
Fes2798	9	0.72	○	○	○	×	×	–	–
Fes2802	4	0.69	○	○	○	×	×	×	–
Fes2809	8	0.52	○	○	○	○	○	×	×
Fes2857	14	0.84	○	○	○	○	×	–	–
Fes2900	11	0.76	○	○	○	○	○	○	–
Fes2976	7	0.67	○	○	×	○	×	–	–
Fes3075	1	–	○	○	○	○	○	○	–
Fes3108	12	0.76	○	○	×	○	×	–	–
Fes3141	9	0.81	○	○	×	×	×	–	–
Fes3164	18	0.88	○	○	○	○	×	–	–
Fes3177	7	0.80	○	○	○	×	×	–	–
Fes3214	15	0.86	○	○	○	○	○	○	–
Fes3265	14	0.90	○	○	○	○	○	○	×
Fes3331	7	0.82	○	○	○	○	○	–	–
Fes3508	19	0.93	○	○	○	×	○	–	–
Fes3663	8	0.75	○	○	○	×	×	○	–
Fes3664	10	0.80	○	○	○	○	○	○	×
Fes3768	7	0.78	○	○	○	×	×	×	–
Fes4036	30	0.94	○	○	○	○	×	×	–
Fes4045	5	0.42	○	○	○	×	○	○	–
Fes4620	1	–	○	×	×	×	×	×	–

¹⁾ NA: number of alleles, PIC: polymorphic information content²⁾ ANC: *F. esculentum* ssp. *ancestrale*, HOM2x: *F. homotropicum* (2x), HOM4x: *F. homotropicum* (4x), TAT: *F. tataricum* ssp. *tataricum*, POT: *F. tataricum* ssp. *potanini*, CYM2x: *F. cymosum* (2x) and CYM4x: *F. cymosum* (4x)³⁾ ○: amplified, ×: not amplified and –: not tested

Fig. 2. Polymorphism detected among 34 common buckwheat accessions at a microsatellite locus, (a) Fes1585 and (b) Fes3331. M stands for the size marker lanes. Lanes 1 to 34, as numbered from left to right, correspond to the 34 individuals examined.

Table 2. Percentage of successful amplification and number of different nucleotides in *rbcL* gene sequence between *F. esculentum* ssp. *esculentum* and the related *Fagopyrum* species or subspecies

	Percentage of successful amplification	Number of different nucleotides ¹⁾
<i>F. esculentum</i> ssp. <i>ancestrale</i>	100.0%	0
Diploid <i>F. homotropicum</i>	79.6%	1
Tetraploid <i>F. homotropicum</i>	68.5%	—
<i>F. tataricum</i> ssp. <i>tataricum</i>	63.0%	1
<i>F. tataricum</i> ssp. <i>potanini</i>	25.9%	7
Diploid <i>F. cymosum</i>	36.7%	7
Tetraploid <i>F. cymosum</i>	33.3%	7

¹⁾ Sequence data of *rbcL* gene for six species or subspecies were obtained from DDBJ database. Accession numbers in DDBJ database: *F. esculentum* ssp. *ancestrale*, AB000310; diploid *F. homotropicum*, AB000312; *F. tataricum* ssp. *tataricum*, AB000319; *F. tataricum* ssp. *potanini*, AB000318; diploid *F. cymosum*, AB000307; tetraploid *F. cymosum*, AB000304. (—): Sequence data for tetraploid *F. homotropicum* could not be obtained. A total of 1,428 nucleotides were compared.

pairs that successfully amplified microsatellite loci was lower in the polyploid species than in their diploid counterparts in tetraploid alfalfa (Diwan *et al.* 1997) and hexaploid wheat (Roder *et al.* 1995). The low efficiency of PCR amplification in the tetraploids may be due to the complexity of the polyploid genome (Roder *et al.* 1995).

In conclusion, it was clearly shown that the 54 microsatellite markers displayed a high variability in common buckwheat and high transferability for use in closely related *Fagopyrum* species. We have recently detected and analyzed microsatellite variations in common buckwheat and its wild ancestor populations, and showed that gene flow between cultivated and wild populations was not important for determining the phylogenetic relationships between them (Konishi and Ohnishi, unpublished data). Furthermore, we have recently constructed a linkage map based on the 180 microsatellite markers developed in the present study (54 markers listed above together with the 126 markers that amplified more than one fragment). This may contribute to the promotion of molecular breeding in common buckwheat. The results related to the construction of a linkage map of

microsatellite markers will be published elsewhere.

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Appendix 1. Primer sequence, repeat type, expected fragment size, annealing temperature of 180 microsatellite markers

Locus	Forward primer (5' to 3')	Reverse primer (5' to 3')	Repeat type	Fragment size (bp)	Annealing temp. (°C)	Evaluation test*
Fes1002	CAGGGAGGAGAAGGAAAGAGAT	CCCTTCTCTCAACTCACCTCTA	(GA) ₇	189	64	
Fes1012	CTTCCTTTCTAGCCGTTACA	GAAGGTTGAGTCGCACAGGT	(TC) ₈	100	59	
Fes1051	GAAGGAGCATAAGAGAGGAT	CGTTTCCCGCCTACA	(GA) ₂₂	200	55	
Fes1061	TGCACTACGGTTTGAGTATT	AGGGTTGGCTGACTTATGTT	(GA) ₁₀ GG(GA) ₁₁	201	59	
Fes1094	GAAGCCTTGGAAGAAGTGAAAT	TAAAGTCATCCCAATATGCAA	(AG) ₃₇	160	61	○
Fes1264	GCTGTCTCCAGTCCGCATCC	GCTCTTGACCTTGAATACC	(GA) ₉	186	63	
Fes1284	GAATACACCATTTCGGAACAAGA	ATCATCTCTCTTTGAGTCACCT	(ATG) ₄	114	61	○
Fes1286	CCATATTCTACTTTCCGACC	GAAGCACAAGGAAATGAGGG	(GA) ₂₅	180	62	○
Fes1298	TTGTTGTAGTTGTTGTTTCG	ATTGAAGGAGCTATTGATGG	(TTG) ₆	123	62	
Fes1303	AGGAGACGGGAGAGAAGCAG	GGATGTTTGGGTGATTTCAG	(GA) ₃₁	225	60	○
Fes1307	AGAGGAACAAGAAGCACCAT	CCCTCAGTTGCTTGACCTAT	(GA) ₇	155	63	
Fes1308	ACTCTTGACCCGTGTTTTACCT	TAGCGTGAGAGAGAGAGAGAGA	(CT) ₂₀	92	59	
Fes1309	GTGTTGAATGTATGCACCAG	TTGACAAGTCATGGACAAGG	(GA) ₁₀	155	63	
Fes1322	AAGCATTCAATTCATTCATTC	GAGTTTGTTGTGTTTGGAGG	(CT) ₃₁	177	58	○
Fes1323	TGAAGGAGGGAAGAAGATGAAT	TCCCTCTTATGGATTGGAGACT	(AAG) ₄	94	62	
Fes1326	GACCTTCGTTAGTAGACCA	GGAGGAAGAGAAGAGAAAGT	(TC) ₁₁	146	61	
Fes1346	GGTGCAAACTAACATGAATG	GCTGAGTCTCGGGATTATT	(GA) ₁₇	202	60	○
Fes1347	ATTCCCCAAGAACACCCAC	CCCTTGTTGGAGTATAAGAG	(GA) ₄₁	167	59	
Fes1354	TCCATGGCTTACACAACACT	CTTGACGCTTCTGACCTCT	(GA) ₂₃	253	62	
Fes1356	ACGGAAGTGCAAGGAAGGAA	ATGAGAAACAACCAATCTG	(GA) ₃₀	183	61	
Fes1362	CCCAGCGTGTGATGATTGAC	CGGCATGCGAAACGAAGGAG	(CT) ₈	276	63	○
Fes1367	GTTCAATTCAGCAACTAACA	TCATACCATCACCAACAAG	(CT) ₂₂	175	61	
Fes1368	CAACCACTCAAAGCCTCATC	CTTTCATATCCCTAACACAC	(CT) ₃₄	177	55	○
Fes1393	GCGGCTAGTCTGGGAAGAAA	CTCGAATCCTTGGAATCAC	(GA) ₂₆	168	63	
Fes1394	GCCTTGCTGCTTGACCTTT	GATCACACGGGAGGAGAGGA	(CT) ₁₈	181	60	○
Fes1403	CTAAGGCTCTTACATCTCAC	ACCTTCTGAGCGGAGATGTG	(CT) ₂₅	176	63	
Fes1407	GTGATGAGTAGTTGCCTCTG	CTTGCTTAGACCTCTCGTA	(CT) ₁₃ CA(CT) ₂₁ (CA) ₉	153	62	○
Fes1409	GATCCGGTAGGTGAACGAGA	AATCAACGCTATTGCCTTCC	(GA) ₁₀	166	56	○
Fes1427	AAGAACTGTATCGGATGGA	ATATTGGCTTATTGGAACCT	(CT) ₂₄	183	57	
Fes1429	CGTGCTTACTTGTCTTCTTTG	GTGGTGAGAAGAAGCTCATAACG	(TC) ₁₀	197	63	
Fes1434	TTGCTCAACGGGAGAAAATT	GTGGTGGTGGCGGCACATAG	(GA) ₂₉	181	59	
Fes1445	AGAAACTGAGCCCTTAGAAC	CTACGCCTCAGTAAAGAAGA	(CT) ₁₉	151	62	
Fes1455	CAAGAGCGAAGTGGGAACAT	GGAAGGTCATTCTAGGGTC	(CT) ₃₆	153	57	
Fes1459	GATCAACCACCTCAGAAAATGA	CCCTCCCTTTAGTCCGTAAAAT	(GA) ₄₀	138	54	
Fes1461	CTAAATGAGCAGCCCTCTCTCT	GAGAAGATGCCAAGAGTTCAAA	(CT) ₁₈	121	61	
Fes1466	ATGCCAGCTCGTATCTCTTC	GATTGCGCTAAGTAACAAAG	(CT) ₂₅	116	54	
Fes1472	GGGCCACCAACAGTATTAGC	TATCAAAGGAAATCGATGAC	(CT) ₃₂ TT(CT) ₁₁	215	49	
Fes1475	GCAGCAACATTGACAAACTA	GCGGTGCCCTGGTGGTGGTG	(AG) ₂₈	205	52	○
Fes1497	GTTGGCTGACGAAGACCGAC	AAAGAGAGCGAGAGGCACTG	(CT) ₃₃	153	60	○
Fes1498	GACACTTCCTTGCCCTACAT	GATCCAACAGTTACGTTCTG	(CT) ₁₈	147	61	○
Fes1501	AGGAAGTAAATGGGGTTTACA	CGTATTTTCTCTCCCATCTC	(GA) ₆	63	52	○
Fes1526	GCTCTTGACCCTTGAACACC	TTACTCTGCAGCCCGATTCT	(CT) ₈	211	64	
Fes1533	GAAGTAGCACCATGAGAGAA	CCCTCACCTAGAGTATGACC	(GA) ₈	163	57	
Fes1550	AGACCACTTCTCGGAAAGAAAG	ACAATTTACACAGGAAACAGC	(GA) ₂₈	228	53	
Fes1571	GGAGAAGATGTACGTAGGGA	CCTTTGACCCGTGCTTTACT	(GA) ₂₄	167	59	
Fes1575	GAATTAGACGATGCCCTTGA	TAGCGGATTGGTGGTCTTCT	(GA) ₈ AA(GA) ₇ CA(GA) ₇	243	61	
Fes1577	GAGGGAGAAGATGAAGTTGT	CTGCGGATTAGGGTGTGTTG	(GA) ₂₅	172	59	
Fes1582	TCTGGAGAAGCTAAACCCAC	CCGCAGTTTGTAGGGAGGGA	(GA) ₂₄	143	57	○
Fes1583	CTCCAAGTCAGCCCAAGAAA	GGTCCAAGGGAGAAGCGTAG	(CT) ₁₂	210	62	○
Fes1585	GCCTCTACTAGCCCTTCCA	AGCTGACCCCTGTGTCTCCC	(GA) ₇	169	60	○
Fes1589	TCGGAACCGCCTAAATGAAA	CTCAAGCCCTAGCGAACCAC	(GA) ₂₁	232	62	
Fes1592	ACATCTAAAAGAGCGGAGAGG	TAGCGATTAAAGGTGATTGACG	(CT) ₃₃	235	61	
Fes1623	CGACCGAGATTGGTTTGAA	CGTGGTGAATTAGGGTTGTG	(CT) ₁₃ AT(CT) ₃₆	178	55	○
Fes1627	AAAGTATTCGTCCCCAAATC	ATCGTCTAATCCCAGCCATC	(GA) ₇	189	55	
Fes1630	GCGTTGTTTGCTTCATCTTT	GCTCGCTACAACATGAGGAA	(CA) ₈ (GA) ₃₁	207	61	
Fes1636	GATTGCATGCAGATTACAGGT	ACTGACTCGGTCGAGGTAATA	(A) ₁₅	106	61	
Fes1647	TTTATATCTTGCCTGAATGA	GTGGCGACAAGACTGTGAAG	(GA) ₁₀ A(GA) ₁₄	142	55	
Fes1663	GGGAAGAGAGAAACAAGGAGAA	TTATCCAAAAAGGAGCCTATGA	(GA) ₅ GC(GA) ₁₁	123	55	
Fes1667	GGAGCCTATGAGGGTTGTTG	GGGAAGTGAAATATGTGAAT	(CT) ₃₂	142	57	
Fes1689	AATTGCCGTTGTTATTCTCTC	CAGTAATAATGCATAGTGCC	(CT) ₂₄	229	59	
Fes1693	GCATAGAAGTCTGTGGTGGTGT	CCAGCAGTGACTTGCTTCTAGT	(CTG) ₄	223	69	○
Fes1694	GCTGGAAGAAAGCAAACCTC	GCACCGCCTTCTTGAAGTGT	(AG) ₈ G(GA) ₉	115	63	
Fes1709	CAACTAGGCTCTTACATCTC	GTTATGGCCTTCTGAGTGGA	(GA) ₂₀ GG(GA) ₇	186	59	

Appendix 1. (continued)

Locus	Forward primer (5' to 3')	Reverse primer (5' to 3')	Repeat type	Fragment size (bp)	Annealing temp. (°C)	Evaluation test*
Fes1721	TAGGGTTGAAAGAGGGAAGAAG	GAGACAACAAATCCGAGCTATG	(GAA) ₉	82	55	
Fes1759	GCGCACCACCTCCATCTTCT	TGGCGCTTACCTCATCTAAC	(CT) ₃₁	168	63	
Fes1776	GGTTATAACGACATGCCTTCAA	TGTTCCATCTTCAGCTATGACC	(GA) ₁₁	127	63	
Fes1805	CGCCTTGTTTGACCGTCTTG	AGAAAGTAAACCCATCATCAC	(CT) ₂₄	125	59	
Fes1810	CGTCTTGCTTTTCGTTCTTCT	AGCTAAACCCACCTCAAAGGA	(CT) ₁₇	88	61	
Fes1816	ACCGGAAGTGTAGTTGATAA	GCAACTCGTAGAGTCCCACA	(GA) ₁₈ AA(GA) ₁₀	166	55	○
Fes1822	ATTGGAACAACCTGCGCCTCA	GGCAGGAACCTGGGCTCTTAG	(GA) ₂₂	156	57	○
Fes1830	AGGAGAGAAAAGGGGAGAGAGTT	ATTGTTAGCGAATTACCGACCT	(GA) ₆	216	64	
Fes1832	TCTTCCAAGAACACCCACAA	CCCAGGACCACCCACGACAT	(GA) ₂₃	180	61	
Fes1838	GAGGAAGCACAAGGAAATGA	CCCATCGTTCCTAGCTTGAG	(GA) ₇ AA(GA) ₂₃	176	59	
Fes1840	ACGACGAAGACAAATGAGGA	ATATGGACGGCCTGGATTAT	(GA) ₈	245	62	○
Fes1852	ATGTGCTTTCAGGCTGACT	AATGCGAAATGTTCTAATCT	(CT) ₂₇	194	55	
Fes1858	ACGTGAGAGTGAAATGAGAGGA	AAAGTGAGAGGATGGGTGAGTT	(GA) ₈	96	63	
Fes1865	GAGGGAGATGATGTGAAGACAA	CTTAGTTGACTCCCATTTGCAC	(GA) ₆	199	59	
Fes1889	GAATGTACCAAAGTCTGCTCA	AGTGGCTTCTGAGTATTCCAT	(TCC) ₄	185	63	
Fes1896	CGAACCCTCTATTTGTTTACG	ACTCTCCACCCTCCATTAGT	(TGG) ₄	186	64	○
Fes2008	CCGAGGGCTTCCGACATAG	TACAACAAGACACCGACAAA	(GTT) ₇	173	63	
Fes2017	GAAGCATGATGGGTGTTATTC	CACTCAGTTGCTGGTCTAACCT	(TG) ₇	133	64	
Fes2222	AGGGCAAAAGACGAACCTACCT	GCTAAGCAACAAGTCCTTGACA	(GGT) ₄	207	55	
Fes2239	AACCCCTATTGTATTTTACGC	CTAACAAACCATTCCCCAACTC	(TGG) ₄	241	61	
Fes2279	CCCCTCTCTCACAAATATCAA	CACCTTTCTGGCGTTGTTGG	(CCA) ₄	123	59	
Fes2280	ACTTGGAGATGAAGTCTCCTG	TTAATTCATCCATATCGGCTTG	(GA) ₇	87	53	
Fes2328	TTACCAACCGAATCTGAATC	TTGTCTTGCTTTCCGACC	(GA) ₈ GG(GA) ₂₁	242	60	○
Fes2420	AGAAAACCTCAAATCGGGATGAT	GCGTATCAGTCGACCTTACTCA	(GA) ₆	206	59	
Fes2456	AGGGTCCAATGAGAGAGAGAGA	ACAGAACGCAAATAACACATCC	(CT) ₃₈	245	55	
Fes2459	CGGGATGAGCAACTCTGACT	TTATACGCTCCACGCTTCCT	(GA) ₂₂	230	49	○
Fes2468	GTAGTGGAAGGCTCGTAAA	ATGATTGAGGATGAACGTCT	(GA) ₃₃	183	55	○
Fes2473	GTGACCTCGGTTGACCATCT	TCCTTGTTGATTTCGGTTTA	(CT) ₁₉	231	54	
Fes2476	TCCATTCTTCACGGCTAATC	TGCCAGGCTAACTTCTTGTA	(GA) ₄₆	205	47	
Fes2480	TTCGTAGTTATTGTTGGTTG	TTGCATGGTAAGAACTGTTT	(CA) ₇ GA(CA) ₉	236	56	○
Fes2481	AGTCCTTTGGGCTTACTTAG	TTATACCAATTTTGATATGTTT	(GT) ₂₄	123	61	
Fes2489	ATTTATATTTTGCATTGTCC	GATCGACCACAAGAAGTAGG	(GA) ₄₉	180	49	
Fes2490	TTTGTACCAAGTTCTTGAA	ATCCAGCTACAACAAACAT	(AT) ₈ AGA(TG) ₁₄	196	59	
Fes2507	AGGCAAGATTTAGTTTAGG	CTTGTGCGATTATTTGAGTC	(CT) ₁₄	129	55	○
Fes2509	AGACTTTGAGATGAAGCTCT	GGTTGCTTGCTTGATTCT	(GA) ₂₃	326	57	
Fes2513	GATCAAGCATCATACCTTTA	AGCCTAACTCAGCACACTCA	(AT) ₁₀ (GA) ₁₆	217	47	
Fes2530	GAGAGAACGAGAAAGAGAAGCA	ATGAGAATGGCTGGTGATAGTG	(GA) ₂₀	189	63	
Fes2534	GTAATGTCCCAGTTCTGAG	GGGAAAGATTGAATCTTGTA	(CT) ₁₉	138	50	
Fes2537	AGAGAGTGCTAGGCAAACTTC	ATTACTTGCAGGGGAGAGAGAG	(CT) ₁₂	138	62	
Fes2542	GATCCGAATGTGTTAAGTAA	ATAAATCCATTGCTTCCACC	(CTT) ₃₀	146	51	
Fes2604	GCGATATGCTAGTCTTGACGG	GGTATTAGCTCTTGCTGAGTGA	(CA) ₁₄	99	63	
Fes2644	GGAGTAAGACCTCCACAAAGGT	GCAAGTGATAACCCCTCTCTCT	(GA) ₁₃	186	50	○
Fes2647	AAGGCTTACAGTTGGTCATC	GTCCCAATAGGCATACAAA	(GA) ₃₅	236	59	
Fes2649	GATCCTGATTCTGAAGAAG	CGCTTCTATTCTTTGTCC	(GAA) ₂₇	234	57	
Fes2666	CCCGAGTTCGCGAGATTTGG	TCTTTGGTCGGGCTCAATAG	(GA) ₁₄	264	62	
Fes2675	TTTGGGATAGAAATGTACGAG	CCGTCTACAGCAGAATAAG	(CA) ₁₅ (TA) ₈ (GA) ₇	156	59	
Fes2695	ACCGAGATAGAGACCGAGAT	CAAACCTAACATACCCAAAGA	(CT) ₂₀	212	57	
Fes2696	GTCACCCAAGCTAAGAAGAT	AACGGATACAATTTGTTCTC	(GA) ₃₃	209	60	
Fes2713	AGTCTCCGGACTTACTTATT	CCTATTACCGGTTTAGTTAT	(GT) ₈	112	57	
Fes2741	CGGTTTAATCGACATCACAT	AGATTGAGGTAAGTCCGTAG	(AC) ₁₀	124	50	○
Fes2742	ATGCGGAGGAAATGTGAGAC	TCTTCCTCAATCTTATCCC	(AG) ₁₃ TGT(GA) ₆	200	55	
Fes2743	AATGCGCTGCGGTAACCTA	TTGGAGTATTCGCTGGTGTG	(GT) ₁₀	263	55	○
Fes2748	TATGCTGCGGCTAATAAACACT	CATCCATTAGAGAGAGAGAGAG	(CT) ₂₀	77	47	
Fes2751	AAGATTGAGGGATGAGGTGAT	CTCCCAACCGATATAAACACAA	(GA) ₁₈	210	61	○
Fes2798	TTCACATATATTCGGCGTAT	TGAAATAACCGGGAGTAGAT	(GT) ₁₀	170	55	○
Fes2802	ACCGCGATTATGAGTTTGA	CTAGTGGGAAGCGATTGGTA	(GA) ₂₅	262	62	○
Fes2809	ATCAACACAAACAATCAAGC	TAGAATTCCCCTCCTTAGGT	(CA) ₁₃	277	58	○
Fes2819	AGGTATGAGGCGAAGAGAC	CCGCGAATATTTCTTCCAC	(CT) ₂₀	280	54	
Fes2822	CTCCACCTTGATGTAAAGTA	AGGGACATATCTTGGTGTG	(AT) ₇ (GT) ₇ GC(GT) ₂₂	297	55	
Fes2832	CCAGAACCTAGAAATCGCAGTA	TGGTGAGTATTAAGGGTCGAGA	(GA) ₂₃	179	63	
Fes2842	AGGGCATCTACAAAATCGGTAT	TGATGTCTATGTGTGTGTGTG	(CA) ₁₁	97	59	
Fes2857	CGTGAATTGTGGGATATGGG	TGCCACCTCAACTCACTCTC	(GA) ₂₄	197	55	○
Fes2864	TGAACAACACATGATAGCCT	CGGGTCCATGTACTCATTAT	(CA) ₉ AA(CA) ₁₇ (TA) ₆	167	61	

Appendix 1. (continued)

Locus	Forward primer (5' to 3')	Reverse primer (5' to 3')	Repeat type	Fragment size (bp)	Annealing temp. (°C)	Evaluation test*
Fes2868	TGGAACACACATGATAGCCT	GAGTCTTCCATTCCGGCATAAC	(CA) ₂₉ (TA) ₆	139	61	
Fes2900	CTAATTGCGGGTTCCTTTAAC	GATTGGAGGGAGAGAGAGAGA	(CT) ₁₈	75	52	○
Fes2953	CGGCAGCTTAGTTTATTGGATT	AAGTCCCATCATGCAACTACAA	(AT) ₅ (GT) ₁₂	105	63	
Fes2960	CCCAATGTGAGAGAGAGAGAGA	TCGATCAATACCTACCTACCTACC	(CT) ₁₂	82	55	
Fes2976	AGAAAGAATCGACGTGAGTG	CCCGTTCCAACCCCTTCGTTT	(GA) ₁₈	191	50	○
Fes2977	TTCAAGGGAGGCATTGGAAC	CACGATTACTTTGCCCGACC	(GA) ₃₉	200	62	
Fes2980	ATTTGCATTGTCCCTAATGAGA	ATCGACCACAAGAAGTAGGTCA	(GA) ₅₀	174	62	
Fes3075	AGTTGAAACATGCTTTTCAGGA	TAATTTGTGAACCAGAGCGAGT	(TTC) ₄	249	63	○
Fes3095	GCGACTCTGATAGCGAGGGT	GGGTTCGGTTGACTAGAAATG	(GA) ₂₄	114	57	
Fes3108	GACCTTGACTTCTTTGACCG	GTAAGCATTCTAGGCATTTG	(AT) ₇ (GT) ₁₁ CT(GT) ₁₁	147	58	○
Fes3123	AGAGTTCACGGACGAGATTT	GCTCCACTTAGAGGTTCCCTT	(GAA) ₃₂	159	63	
Fes3141	ACTTCGACGCCAACATTTCT	CCCTAAGATGTGCTAAGATT	(GT) ₉ (GA) ₂₂	192	60	○
Fes3148	ATGTTCTAATCTAATCTCCC	TTGCCTCCGGTTGAGTATTT	(GA) ₃₆	168	57	
Fes3164	CATTGGCTAAAGGTACAGGA	CAACACATGCTATTAGACGG	(GA) ₁₇	195	55	○
Fes3167	CCTCGTGTACCAAATAAGT	TTGTCTAACCTGCGGTAAA	(GT) ₇ CT(GT) ₇	207	63	
Fes3177	CTGTCCACACTGCTGCTCTT	GTCCCAACCATACAAATCAG	(AC) ₆ G(CA) ₆	181	63	○
Fes3214	CGTACTCAGCAGGAGATGAC	TTGACCGCAATTTCCAGAAT	(GA) ₁₉	208	59	○
Fes3237	AGATGACTGAGATTAGACGA	AAAGCATTACACTTCAAACA	(CA) ₄₉	194	47	
Fes3239	ACTCGGATATTATCTCGTTTGT	TGATGAAATCTTTTCAAAACACC	(AT) ₅ (GT) ₈ GC(GT) ₄ GC(GT) ₄ (GC) ₅	175	57	
Fes3265	CTTTTCTTTCAACCATGTACGC	GATAGGCAATTAGAGCGTGAGTT	(CT) ₁₆	145	50	○
Fes3309	AAACACCTAACTCCGAACAACC	TATTGATGCACACACACACACA	(GT) ₁₄	156	63	
Fes3331	CCATGAAAGAACACTTTGAACA	TTAAATCCACACCAGGAATTTG	(AG) ₁₄	145	50	○
Fes3340	CATACACCACAACAGAACGATG	TTGGTTTTGCATTAGAGAGCAC	(GA) ₁₈	114	61	
Fes3393	TAAATGAAGCGACAAGAGAACG	TGTCTATCCCAAGCAATCATCT	(CTT) ₇	117	59	
Fes3443	ACCGCGATTATGAGTTTAGACA	TTTGGTGTGGTGACTCTCTCTC	(GA) ₁₇	113	57	
Fes3508	GAGAAGAAGTGTTAGCGGTGTG	ACTCTTAAACGCAATGATGGTG	(GT) ₁₂ (GA) ₁₀	133	62	○
Fes3531	ACATCACCACCATAACCTCAAG	ATTCTCTCTCTGCTCCATCTC	(CAA) ₄	183	63	
Fes3593	TCCGTTCCATCGTTAGTTAGTG	TCAAGATATCTCCCAAGCAGAA	(TC) ₇	184	59	
Fes3663	TTTTCCGCTTTTCACTCTATGA	AGTTTAGGTAGCGAGGCTTGAG	(CT) ₁₃	249	59	○
Fes3664	ACTCGGTCAAAGTGTCTTCGT	GAGGTCTTAATCTGTTGCATCC	(CT) ₁₄	242	61	○
Fes3684	ATTATCAAGGGCAAAGGAACAC	ATTGTGTAGTTCGGTGTGTG	(AAC) ₅	80	63	
Fes3714	TCATATTGTGTGTTTTGATGTCTT	AACATGCAGGGAACTACAACA	(GT) ₇	117	63	
Fes3768	AACCTCAAACCCGTCCTATAAA	TTGGACTTTTGTGTAAGCATGA	(AC) ₁₀	210	60	○
Fes4011	ACTCGTGGGCCAAGTAATAAGT	GATATATTGGCGAGTGCTTGAG	(GT) ₁₁	117	61	
Fes4014	ATCCAAGCTCTTCAATGCTACA	GTGCAGCTCACTTTTCACTCTT	(GA) ₂₂	179	63	
Fes4021	TTTTGCTCCTGTCACGAACAT	GAATCTTACCCGATTCCAAGAC	(CT) ₁₅	152	65	
Fes4034	AGAGAAGTGAAAACCAACGTGA	TTCTAGCTTGAGGTGACAAGA	(GA) ₁₅	125	63	
Fes4036	ATAACAAGCATCGTCATTGGAG	CCAAATTGAAACCGAACTTACA	(GA) ₄₀ GGA(G) ₁₀	184	60	○
Fes4037	CACGTAAAAGTGAAATGAGAGGA	GGGGCAATGAGTAACAAGATT	(GA) ₆	180	50	
Fes4045	GTACACAGCCGTTACAGACGTT	ACAATTTACACAGGAAACAGC	(CT) ₁₁	296	46	○
Fes4050	AGGTGTGACCCCTTTAATGAAT	TACAACCAAGGCATACAAGCTC	(GT) ₁₉ CT(GT) ₇	220	55	
Fes4068	ATATTCGACAAGCGAATGAAGA	GATATCCACAAACGGGTCAACT	(GA) ₃₇	190	61	
Fes4109	TCGAGTCTAAAGTCAGACCAA	TGGACCAAGTATTGGTGACTAT	(GA) ₃₃	224	63	
Fes4111	CAAGGGAGAAGAGGAGAAgAAA	CTTCATCCCTTACCTCTGATGA	(GA) ₁₅	103	61	
Fes4113	CAGAGGAGTTGTTGCTCCATA	ACCACCAACAAATTGCTTTTC	(GA) ₂₁	113	59	
Fes4123	TCGAGTCTAAAGTCAGACCAA	TGGACCAAGTATTGGTGACTAT	(GA) ₃₅	228	63	
Fes4125	TGAGGGAGAAGATGAAGCTGTA	GTTTCTGAGATGAGTGCTGAGG	(GA) ₂₁	235	63	
Fes4138	TGAAGGAGAGGAAGTGAAATGA	TGTTGAGTGACGGGATTTATC	(GA) ₂₀	235	61	
Fes4150	TGTTGGATTTCCCAACAAAG	AAGTTGTCTTGATTGCAACTGG	(GGA) ₅	111	49	
Fes4151	CACAACAGCAACTCAACAACAT	CCGACTTCCTTATGGTTTTCTT	(GA) ₁₈	220	62	
Fes4175	TGTGTTAACCAAGTGAGTTTGG	CCATAGTGATATGTGATGGTCCT	(CA) ₁₅ CG(CA) ₆	248	60	
Fes4617	AGGGAAGGGGAAAGATTGAA	GGCACTAGACCACCAACATAA	(GA) ₁₀	188	51	
Fes4620	GCTAAGCGTGATATTGGAGTTG	TGCCTGGAATAGAGCTAATGAA	(ATG) ₄	235	62	○
Fes4636	GCCTCAGAAAATGACACAAAT	GATCTAGGAGAGGATGAACGTC	(GA) ₄₅	187	45	

* ○: 54 primer pairs used for the evaluation of their ability to detect microsatellite variation in common buckwheat populations and to be utilized in seven related *Fagopyrum* species