园 艺 学 报 2007, 34 (2): 311 - 316
Acta Horticulturae Sinica

Development of SSR Markers in Sweet Cherry Using Selectively Amplified Microsatellite

AI Cheng-xiang[1], YU Xian-mei[2], ZHANG Li-si[1], WEI Hai-rong[1], XIN Li[1], YUAN Ke-jun[1], JIN Song-nan[1], SUN Qing-rong[1], and LIU Qing-zhong[1*]

([1] *Shandong Institute of Pomology, Key Laboratory for Fruit Biotechnology Breeding of Shandong, Tai'an, Shandong* 271000, *China*; [2] *Institute of Environment and Plant Protection, Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan* 571737, *China*)

Abstract: A total of 100 sequences were isolated and cloned by SAM (Selectively Amplified Microsatellite) and another one was obtained from the NCBI and EMBL databases. 82 SSR sequences were used to design the special primers at 77 loci from 69 fragments. Thirty-eight pairs of special primers were synthesized, matching with 5′anchored degenerate SSR primer, to detect 36 SSR loci. 27 primer pairs amplified clear and robust DNA fragment, of which nineteen pairs of SSR primers amplified the corresponding SSR sequences and eight amplified the unexpected fragments. 24 polymorphic primer pairs were selected from the 27 primer pairs by using the genomic DNA of 27 sweet cherry germplasm, and 24 locus-specific SSR markers were obtained.

Key words: *Prunus avium* L.; Germplasm; SSR markers; Selectively Amplified Microsatellite

甜樱桃 SSR标记的选择性扩增微卫星 (SAM) 法筛选

艾呈祥[1], 余贤美[2], 张力思[1], 魏海蓉[1], 辛 力[1], 苑克俊[1], 金松南[1], 孙清荣[1], 刘庆忠[1*]

([1] 山东省果树研究所, 山东省果树生物技术育种重点实验室, 山东泰安 271000; [2] 中国热带农业科学院环境与植物保护研究所, 海南儋州 571737)

摘 要: 以甜樱桃‘红灯’为试材, 应用选择性扩增微卫星 (SAM) 法分离、克隆了 100个 SSR序列, 其中 81个非重复, 可用。加上搜索数据库所获得的 1个 SSR序列, 一共 82个序列用于特异引物的设计。仅从 69个序列的 77个基因座设计出特异引物。合成 38对特异引物, 对其中的 36个基因座进行检测。其中 19对引物扩增出相应大小的片段, 另外 8对引物扩增出非预期片段。最后, 以 27个甜樱桃种质的基因组 DNA为模板, 从 27对可扩增出带的引物中, 筛选出多态性引物 24对, 获得了 24个甜樱桃基因座特异性 SSR标记。

关键词: 甜樱桃; 种质; SSR标记; SAM法

中图分类号: S 662.5 **文献标识码**: A **文章编号**: 0513-353X (2007) 02-0311-06

Received: 2006 - 07 - 08; **Accepted:** 2006 - 12 - 26

Supported by the Youth Foundation of Shandong Academy of Agricultural Sciences (2005YQ013), International Science and Technology Cooperation Projects (2006DFA33130), and by the Chinese National Programs for High Technology and Development (2006AA100108)

* Author for correspondence (E-mail: qzliu@sdip.cn)

Prunus avium L. is one of the *Prunus* plants belonging to the family Rosaceae, also called large cherry. It is the earliest precocious deciduous fruit tree in North China (Fang, 1996). The fruit-selection seeds and bud-selection seeds are most commonly used in sweet cherry (Sun et al, 2000). While cross breeding of sweet cherry is just initiated, and the genetic background of sweet cherry is not clear yet. It is necessary to find out an effective method to analyze the germplasms of sweet cherry in an effort to explore and utilize these valuable germplasms (Wiersma et al, 2001; Choi et al, 2002). In recent years, the development of DNA markers makes it possible to identify sweet cherry cultivars and establish their genetic map (Downey & Iezzoni, 2000; Chen et al, 2004; Wang et al, 2005; Cai et al, 2006). DNA markers are fast and highly efficient, but there should be an accurate analysis standard to increase the results repeatability. Comparatively, SSRs (Simple Sequence Repeats) was a more reliable method to study DNA fingerprint for its advantages of high repeatability, rich polymorphism and co-dominant inheritance (Yamamoto et al, 2002).

SSRs has many advantages, but it is not easy to acquire the SSR primers. Researchers have developed SSR primers using many methods, such as the classical screening of genomic library (Tokuko et al, 1998), microsatellite enrichment (Huang et al, 1999), 5′-anchor PCR (Fisher et al, 1996), STMP (Sequence-Tagged Microsatellite Profiling) (Hayden et al, 2001a), SAM (Selectively Amplified Microsatellite) (Hayden et al, 2001b) and database blast search (Ramsay et al, 2000). Among them, SAM is a newly-developed method to develop SSR markers. SSRs have the advantages of high polymorphism of alleles, high recovery of useful SSR, and capable of detecting the polymorphic SSR alleles just with one specific primer. Thus, SAM was used to study SSR markers of sweet cherry in this study so as to provide an effective detecting method to study populational genetic structure of sweet cherry.

1 Materials and Methods

1.1 Plant material and DNA isolation

The 28 cultivars, such as Hongdeng, Sunmi, Sweet heart, Lapins, were offered by Xingzhuang Tai'an, Sijiazhuang, Dawangzhuang Feicheng, Lüshun Horticulture Field Dalian and Dalian Agricultural Science Institute. All the samples were conserved in the Tai'an Cherry Repository, Shandong Institute of Pomology. Young leaves of the plant materials were treated with liquid nitrogen and stored at -40℃ until being used.

Genomic DNA was extracted from the young leaves of sweet cherry using Cetyltrimethyl Ammonium Bromide (CTAB) (Ai & Liu, 2006), and the concentration and purity of DNA samples were tested.

1.2 Isolation of SSR sequence

SAM segments were isolated from the genomic DNA of sweet cherry Hongdeng using SAM (Hayden et al, 2001b), after recovery, cloning and sequencing, the fragments were analyzed by the software of Sputnik. SSR sequences were obtained via alignment with other published DNA sequences of sweet cherry blasted from databases such as NCBI, EMI.

1.3 SSR primers design

Locus-specific primers were designed according to Primer Premier 5.0. The primer design parameters were: length of primer (18 - 24 nt), T_m value (51 - 60℃), GC content (45% - 55%), rating value of primer (>90). The primers were synthesized by Beijing SBS Genetech Co., Ltd.

1.4 Selections of polymorphic primers

Genomic DNA of sweet cherry Hongdeng was used to select the polymorphic primers. SSR amplification parameters: 94℃ 3 min; 94℃ 45 s, 51℃ 45 s, 72℃ 1 min, 32 cycles; 72℃ 10 min. After amplification,

the PCR products were tested by 3% agar-gel electrophoresis and 6% denatured polyacrylamide gel electrophoresis, and then the primers producing strong bands were selected. Then the polymorphic SSR primers were selected by testing the amplified efficiency with the genomic DNA of other 27 sweet cherry cultivars as the templates.

2 Results and Analysis

2.1 Isolation, cloning and sequencing of SAM segments

Genomic DNA of sweet cherry Hongdeng was double digested with enzyme *Mse* I and *Pst* I. After being linked to adaptor, the digested products were amplified by suppressed PCR, pre-amplification and SAM, and then SAM products were finally isolated after polyacrylamide gel electrophoresis. The silver-dyed results showed that there were 18 - 25 legible bands in each gel lane and that the bands size ranged from 80 bp to 650 bp. 100 SAM fragments were randomly selected, cloned and sequenced, 11 of them were identical, and 8 did not produce the desired results, so the remaining 81 segments were used for further study.

2.2 Results of Sputnik analysis

The 81 sequenced fragments were analyzed by Sputnik, the results showed that SSR sequence existed in all the fragments, that most of the fragments contained dinucleotide AG/GA and most of the AG/GA repeats were $(AG)_8$, which accounted for 79.2%. There were also $(AG)_9$, $(AG)_{10}$, $(AG)_{11}$, $(AG)_{12}$, $(AG)_{13}$, $(AG)_{14}$, $(AG)_{16}$ and $(AG)_{21}$. No. 69-2 fragment was sequenced and the SSR length was 38 nt. There were 7 fragments containing more than 10 repeats of AG/GA, which accounted for 8.4%. Besides the dinucleotide repeat, there were trinucleotide, tetranucleotide and pentanucleotide repeat in a few fragments. There were even SSR sequence in the middle or double end of few fragments, most of which were simple SSR. Three of the fragments contained compound SSR: No. 85-1 contained $(GA)_6$ $(GAGAG)_4$, No. 32-1 contained $(GA)_5$ $(CTT)_4$ and No. 46-3 contained $(GA)_5$ $(CTTG)_3$.

2.3 NCBI, EBI blast and microsatellite sequence analysis

Six sweet-cherry-related gene sequences were obtained by blast in the databases of NCBI and EMBL. After Sputnik analysis, it was found that the 28S rRNA gene sequence (ID No. CG3234545) of Italian sweet cherry contains a GT repeat: dinucleotide 526: 547 - length 22 scores 10 CACACACACAGCACACACACAC. It is a discontinuous microsatellite, intermitted by a C in the middle. It was also used to design the locus-specific primers.

2.4 Specific primers design

Locus-specific primers were designed according to the SSR flanks of 82 DNA fragments by Primer Premier 5.0, among them, 13 fragments were not suitable to design primers for resulting in primer-dipolymer, harboring haipin structure or easy to mismatch. Finally, locus-specific primers were designed according to one or two flanks of 77 SSR loci existing in the 69 fragments, and then, 38 pairs of primers (numbered SC1, SC2 ……SC38) were synthesized for polymorphism analysis.

2.5 Selection of polymorphic primers

The selected 38 pairs of primers were used to amplify the genomic DNA of sweet cherry Hongdeng, the results showed that 19 primer pairs (SC2, SC3, SC6, SC7, SC9, SC10, SC13, SC14, SC16, SC19, SC20, SC23, SC25, SC26, SC29, SC30, SC32, SC36, SC38) produced the desired main bands, and belonged to functional SSR primers; that 8 primer pairs (SC1, SC4, SC11, SC15, SC17, SC22, SC24, SC37) did not produce the desired bands. There might be two reasons: one is primer design is illogical; the other is sequen-

cing allows for some errors. The main bands amplified by these 8 primer pairs were not compatible with the desired, but they could be used to test the polymorphism for their recurrence and good legibility.

The selected 27 pairs of primers were used to amplify the genomic DNA of 27 sweet cherry cultivars, 20 primer pairs (SC1, SC2, SC3, SC4, SC6, SC7, SC9, SC10, SC13, SC16, SC19, SC20, SC22, SC23, SC26, SC29, SC30, SC32, SC36 and SC37) demonstrated distinct polymorphism: each locus had 2 or more alleles; SC4 and SC7 had 6 alleles; SC26 had 5 alleles. 4 primer pairs (SC11, SC14, SC17 and SC24) had only 1 allele, but they demonstrated polymorphism among the studied materials, and produced the desired fragments, so they belonged to polymorphic primers. 3 primer pairs (SC15, SC25 and SC38) were regarded as monomorphic for they produced identical band type in most of the samples and even with no products in few samples. In conclusion, 24 pairs of SSR primers demonstrated polymorphism among the sweet cherry cultivars according to 24 SSR loci. The SSR amplified products of sweet cherry Hongdeng were tested by agar-gel electrophoresis (Fig. 1) and 6% denatured polyacrylamide gel electrophoresis (Fig. 2). From Fig. 1 and Fig. 2, it could be concluded that the latter had higher resolution precision than the former and had the capability to separate the DNA fragments just having the one-base discrepancy.

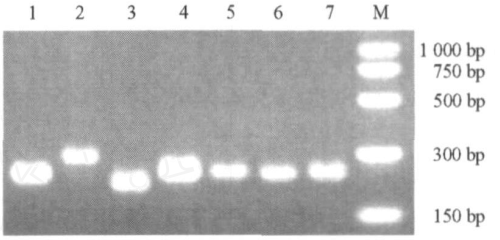

Fig. 1 SSR profile of Hongdeng amplified by SSR with different primers

1: Primer SC1; 2: Primer SC2; 3: Primer SC6; 4: Primer SC9; 5: Primer SC22; 6: Primer SC26; 7: Primer SC29; M: DNA marker.

Table 1 Polymorphic SSR markers (loci) and their primers

Name of locus	Primer sequence (5′→3′)	Repeat type	Fragment size (bp)	No. alleles
SC1	ATGGTGTGTATGGACATGATGA/CCTCAACCTAAGACACCTTCACT	$(AG)_8$	224	3
SC2	ATTCGGGTCGAACTCCCT/ACGAGCACTAGAGTAACCCTCTC	$(AG/GA)_8$	304	4
SC3	ACCCACAAATCAAGCATATCC/AGCTTCAGCCACCAAGC	$(AC)_4TT(TA)_5$	175	2
SC4	ATGGAAGGGAAGAGAAATCG/GTCATCTCAGTCAACTTTTCCG	$(AG)_8$	120	6
SC6	TGATACCACCATCCAATCTAGC/TTGCTGGGACATGGTCAG	$(GA/AG)_8$	195	2
SC7	TGCATGAGAAACTTGTGGC/CCAAGAGCCTGACAAAGC	$(AG)_8$	208	6
SC9	GGACGGACAGAAATGAAGGT/CCTTAACCCACGCAACTCC	$(AG)_9$	286	4
SC10	TGAAGGATGGCTCTGATACC/AATTCATCTACTTCTTCCTCAAGC	$(GA)_8$	207	3
SC11	CAATTTTAAGCACAGGGATC/CAATGTGAATTGGCCATCAC	$(AG)_{10}$	225	1
SC13	CACTATTTTATCATGGACGG/CGAATTGAGAGTTCATACTC	$(AG)_8$	195	2
SC14	AATAAGGGAGGAGAGAAAGGGTGC/TCTAGCATTGTCCATCACGTCT	$(AG/GA)_8$	220	1
SC16	GCTAATATCAAATCCCAGCTCTC/TGAAGAAGTATGGCTTCTGTGG	$(AG)_8$	116	2
SC17	ACCACTTTGAGGAACTTGGG/CTGCCTGGAAGAGCAATAAC	$(AG)_{11}$	214	1
SC19	TGTGCTAATGCCAAAAATACC/ACATGCATTTCAACCCACTC	$(AG/GA)_8$	176	3
SC20	ACGTAAAAAGCCCTCAAACC/TTGCTTACGCGTGGACTAAC	$(AG/GA)_8$	180	2
SC22	CTCCTTGACTTTGAAGTTGC/CTGATCGAGAGTAATAAAG	$(GA)_8$	264	4
SC23	TTGGAAACGGCCATAACACAAGCC/ATGTGCGAATCTCGGGTCGAT	$(AG)_{14}$	104	3
SC24	GGAACAACTAGAGAGAACCAAGT/TTGCCTATCCTGCCCCGTATCAC	$(AG)_9$	180	1
SC26	AAGTCAGCAACACCATATGC/CCCACTGTTCATGAGTTTCT	$(AG)_{13}$	246	5
SC29	TTCTGCGACCTCGAAACCGA/GCTAGGGTTTTCATTTCTAG	$(GA)_8$	282	3
SC30	CGAGGTTGTTGTTCATCATTAC/GATCTCAAGTCAAAAGGTGTC	$(CA)_7AA(CA)_5$	108	4
SC32	GAACATGATGATTGGCCTC/CCAAACATGACATATGTCCC	$(AG)_{12}$	172	2
SC36	AAGCTCAATTGGCGTTGCTA/CTTGCCTCGACGGTATGGTA	$(AG)_8$	306	3
SC37	AAGCGGAAAGCACAGGTAG/TTGCTAGCATAGAAAAGAATTGTAG	$(GA)_8$	220	4

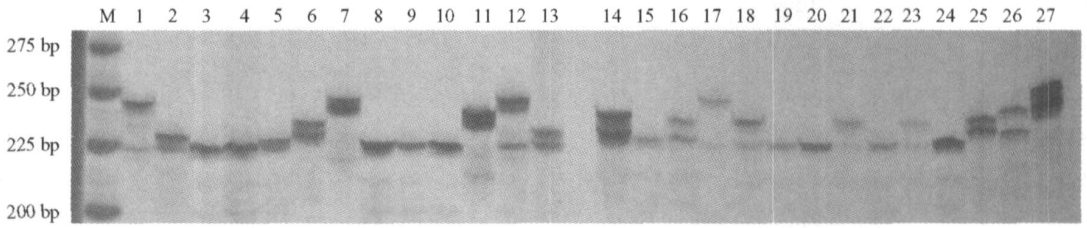

Fig. 2 Polymorphic electrophoresis pattern of special SSR primer SC26

M: DNA marker; 1: Zaodaguo; 2: Tieton; 3: Black tartarian; 4: Bing; 5: Sunmit; 6: Lapins; 7: Youyi; 8: Sweet Heart; 9: Stella; 10: Dragons Yellow; 11: Zuotengjin; 12: Hongfeng; 13: Changbahong; 14: Elton; 15: Red Robin; 16: Jiahong; 17: Van; 18: Santina; 19: Burlat; 20: Laiyang Short Cherry; 21: Oregon; 22: Early Ruby; 23: Jueze; 24: Hongyan; 25: Bigarreau Moreau; 26: Rainier; 27: Napoleon.

3 Discussions

Microsatellites were traditionally isolated by constructing genomic library and then hybridized with SSR radioactive-isotope-labeled or DIG-labeled probes, it is not only a waste of manpower and money, but also low efficient, and not easy to obtain positive clones (only 1% - 3% of possibility) (Tokuko et al., 1998; Hayden et al., 2001b). While the possibility of obtaining positive clones increased to 50% by microsatellite enrichment. In this study, SSR sequences were isolated efficiently using SAM, and 100 DNA fragments were isolated and cloned totally. After sequencing, all the fragments were confirmed to harbor SSR sequences, including the 8 fragments producing non-ideal sequence results. The actual possibility of positive clones was 89% except 11 identical fragments.

Generally, functional SSR primers were those which can amplify desired fragments, if the size of amplified fragments was mismatched, or the products were dispersed and even nothing, the primers were regards as non-functional SSR primers. Fragments amplified by non-functional SSR primers were usually monomorphic (Röder et al., 1998). In this study, 24 primer pairs were obtained, and among them, 19 pairs produced the desired bands, so they belonged to functional SSR primers. The fragments amplified by primer SC1, SC11, SC17, SC22, SC37 and so on, were not the desired, while they demonstrated polymorphism and their electrophoresis pattern has the typical characters of SSR markers, so they should not be classified into non-functional SSR primers. It might resulted from the amplification process, where the desired fragments of gene locus failed to amplify, while the homologous fragments of nonhomologous gene locus were amplified. However, SSR sequences existed in these fragments, so they could be used as locus-specific SSR markers.

SAM is a newly-developed method to study SSR markers, it could produce SSR fingerprint of multilocus and has a high recovery of useful SSRs. Bread wheat was used to clone 20 SAM sequences using SAM to develop locus-specific SSR markers. 20 pairs of SSR primers were designed totally, the results showed that 12 pairs of primers produced the desired fragments, and among the 12 primer pairs, 6 pairs demonstrated allele variation. The possibility of obtaining polymorphic SSR markers was 30% according to the sequenced fragments (Hayden et al., 2001a). In this study, 100 SAM sequences were isolated and cloned using SAM, and the SSR primers were designed according to 77 loci, of which, 38 primer pairs were used for the following study and 19 pairs produced the desired fragments, 8 pairs failed to produce the desired fragments. 24 out of 38 pairs demonstrated polymorphism and were used as locus-specific markers. The possibility of obtaining polymorphic SSR markers in this study was 63% according to the sequenced fragments. The development of SSR markers will simplify the evaluation of sweet cherry family, the study of gene flow and populational varia-

tion, establishment of DNA fingerprint and genetic map, acquiring of purpose-gene-linked SSR markers of sweet cherry and molecular-assistant breeding

References

Ai Cheng-xiang, Liu Qing-zhong 2006. Rapid isolation of sweet cherry DNA. Deciduous Fruits, (2): 4 - 5. (in Chinese)
艾呈祥, 刘庆忠. 2006. 甜樱桃 DNA 的快速提取法. 落叶果树, (2): 4 - 5.

Cai Yu-liang, Li Shan, Cao Dong-wei, Qian Zeng-qiang, Zhao Gui-fang, Han Ming-yu. 2006. Use of amplified DNA sequences for the genetic analysis of the cherry germplasm. Acta Horticulturae Sinica, 33 (2): 249 - 254. (in Chinese)
蔡宇良, 李 珊, 曹东伟, 钱增强, 赵桂仿, 韩明玉. 2006. 利用 DNA 扩增片段序列对樱桃种质资源的遗传分析. 园艺学报, 33 (2): 249 - 254.

Chen Xiao-liu, Chen Xue-sheng, Shu Huai-rui, Xu Heng 2004. RAPD anlaysis of 15 cherry cultivars Journal of Fruit Science, 21 (6): 556 - 559. (in Chinese)
陈晓流, 陈学森, 束怀瑞, 许 衡. 2004. 15 个樱桃品种的 RAPD 分析. 果树学报, 21 (6): 556 - 559.

Choi C, Tao R, Andersen R L. 2002. Identification of self-incompatibility alleles controlled pollination. Euphytica, 123: 9 - 20.

Downey S L, Iezzoni A F. 2000. Polymorphic DNA markers in black cherry (*Prunus serotina*) are identified using sequences from sweet cherry, peach, and sour cherry. J. Am. Soc. Hort Sci, 125: 76 - 80.

Fang Zhong-da. 1996. Plant pathology research. Beijing: China Agricultural Press (in Chinese)
方中达. 1996. 植病研究方法. 北京: 农业出版社.

Fisher P J, Gardner R C, Richardson T E. 1996. Single locus microsatellites isolated using 5′anchored PCR. Nucleic Acids Research, 24 (21): 4369 - 4371.

Hayden M J, Sharp P J. 2001a. Sequence-tagged microsatellite profiling (STMP): a rapid technique for developing SSR markers Nucleic Acids Research, 29 (8): e43.

Hayden M J, Sharp P J. 2001b. Tagged development of informative microsatellite (SSR) markers Nucleic Acids Research, 29 (8): e44.

Huang H B, Song Y Q, Hsei M, Zahorchak R, Chiu J, Teuscher C, Smith E J. 1999. Development and characterization of genetic mapping resources for the Turkey (*Meieagris gallopavo*). The Journal of Heredity, 90 (1): 240 - 242.

Ramsay L, Macaulay M, degli Ivanissevich S, MacLean K, Cardle L, Fuller J, Edwards K J, Tuvesson S, Morgante M, Massari A, Maestri E, Marmiroli N, Sjakste T, Ganal M, Powell W, Waugh R. 2000. A simple sequence repeat-based linkage map of barley. Genetics, 156: 1997 - 2005.

Röder S Marion, Victor Korzun, Katja Wendehake, Jens Plaschke, Marie-Helene Tixier, Philippe Leroy, Martin W Ganal 1998. A microsatellite map of wheat Genetics, 149: 2007 - 2023.

Sun Qing-rong, Sun Hong-yan, Shi Yin-ping, Yang Jian-ming 2000. Study of inducing shoot regeneration from matured cotyledons of Laiyang dwarf cherry. Journal of Shandong Agricultural University (Natural Science), 31 (3): 291 - 293. (in Chinese)
孙清荣, 孙洪雁, 石荫坪, 杨建明. 2000. 莱阳矮樱桃成熟胚子叶不定梢诱导研究. 山东农业大学学报 (自然科学版), 31 (3): 291 - 293.

Tokuko Ujino, Takayuki Kawahara, Yoshihiko Tsumura, Teruyoshi Nagamitsu, Hiroshi Yoshimaru, Wickneswari Ratnam. 1998. Development and polymorphism of simple sequence repeat DNA markers for *Shorea curtisii* and other dipterocarpaceae species Heredity, 81: 422 - 428.

Wang Cai-hong, Tian Yi-ke, Zhao Jing, Dai Hong-yi, Wang Dong 2005. RAPD analysis of the genetic differences among several cherry varieties Acta Bot Boreal -Occident Sin., 25 (12): 2431 - 2435. (in Chinese)
王彩虹, 田义轲, 赵 静, 戴洪义, 王 东. 2005. 樱桃品种资源间遗传差异的 RAPD 分析. 西北植物学报, 25 (12): 2431 - 2435.

Wiersma P A, Wu Z, Zhou L, Hampson C, Kappel F. 2001. Identification of new self-incompatibility alleles in sweet cherry (*Prunus avium* L.) and clarification of incompatibility groups by PCR and sequencing analysis Theor. Appl. Genet, 102: 700 - 708.

Yamamoto T, Kimura T, Sawamura Y, Manabe T, Kotobuki K, Hayashi T. 2002. Simple sequence repeat for genetic analysis in pear. Euphytica, 124: 129 - 137.