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Fingerprint analysis of *Bombyx mori* local variety resources based on SSR markers

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ABSTRACT: 41 Simple Sequence Repeats (SSR) markers were used to research the fingerprints of 96 silkworm local variety resources, including 83 Chinese univoltine varieties, 11 Chinese bivoltine varieties and 2 Japanese bivoltine varieties. These SSR markers showed rich polymorphism, with the number of alleles reaching 3–35, averaged 12.78, suggesting that there was rich SSR polymorphism among various local varieties and its polymorphism information content ranged from 0.162–0.958, averaged 0.690. The genetic distance between the various varieties was calculated from their fingerprints. By using UPGMA method, unique patterns of clustering among local species of silkworm were revealed. These included the patterns of 5 groups comprising of 54 varieties, while the other 42 species clustered in twos or threes, and as single nodes. In addition, the clustering among varieties did not indicate any obvious groups based on voltinism or moltinism, which seemed to support the theory of multiple origin of silkworm. The results prove that SSR markers can provide a fingerprint analysis of silkworm varietal resources and can be used for their classification and identification.

KEYWORDS: *Bombyx mroi*, simple sequence repeats, local variety resources, fingerprint

INTRODUCTION

Silkworm variety resources are valuable wealth of all human beings, an important component of biodiversity and one of China's most advantageous and distinctive genetic resources. A silkworm local variety is also known as a native variety, referring to a variety formed from long-term natural and artificial selections under local natural conditions. Due to longstanding geographic isolation, there is a difference in ecological character and economic character among various varieties. The Sericulture Institute of Chinese Academy of Agricultural Sciences preserves more than 700 silkworm varieties different in voltinism and moltinism collected from different regions, including local varieties from China and silkworm variety resources from other countries or regions such as Central Asia, Europe, Japan and India[1]. Furthermore, the general characters and special traits of these silkworm variety resources have been researched, providing precious basic materials for sericulture teaching, scientific research and silkworm breeding[2,3].

The study of genetic differences, classification, evolution and genetic relationships of different silkworm cultivars can benefit from the use of tools available from molecular biology for identification and preservation of silkworm germplasm resources. Hence such tools are potentially, of great significance for breeding silkworm cultivars.

Currently, silkworm varieties are still classified mainly based on morphological markers, which are, however, often susceptible to the environment and less stable. Instead, molecular markers, which are genetic markers evolved from the rich polymorphism of the genomic DNA, can directly reflect differences in DNA level among individual organisms[4–6]. Hence it is an accurate, reliable and effective way to construct fingerprints of different silkworm varieties for variety classification and identification.

As 2nd-generation molecular markers were developed in recent years, Simple Sequence Repeats (SSR) markers have been widely used for molecular map construction, genetic diversity analysis, genetic relationship identification and molecular markers-assisted breeding due to its high stability, polymorphism and efficiency and compliance with the Mendelian genetic law[7–13]. Reddy et al[14,15] initiated the research of silkworm varieties with

SSR markers; Miao et al[16] built an SSR markers-based genetic linkage map for silkworm varieties and mapped a series of silkworm genes based on different phenotype traits[17–21]; researchers built fingerprints for Chinese, Japanese and introduced silkworm varieties by using SSR markers[22,23], laying a foundation for the identification and evaluation of silkworm variety resources. However, there remains a lack of related studies on domestic silkworm variety resources with local characteristics. Thus in this study, we built DNA fingerprints for the 96 silkworm local varieties with SSR markers, and clustered the genetic distance between various varieties with UPGMA to analyse the genetic relationship between the varieties, improving the silkworm variety resource bank.

MATERIALS AND METHODS

Silkworm varieties

The tested varieties consist of 83 Chinese univoltine varieties, 11 Chinese bivoltine varieties and 2 Japanese bivoltine varieties. Their main characters are shown in Table 1. The above variety resources are preserved by the Sericulture Institute of Chinese Academy of Agricultural Sciences.

Genomic DNA extraction

Various varieties of silkworms were fed with mulberry leaves for 2 instars until incubation, and 10 silkworms were selected, mixed and put in a precooled mortar. Then the larvae were frozen quickly in liquid nitrogen and ground into powder. Subsequently, extraction buffer, containing 10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, and 0.5% sodium dodecyl sulphate, was added to the powder. The mixture was transferred into Eppendorf tubes and incubated with Proteinase K at 56 °C for 3–5 h. The DNA was then purified by extraction with phenol/chloroform followed by ethanol precipitation. The purified DNA was dissolved in 0.1 × TE buffer, refrigerated at −20 °C using the method from Li et al[24].

SSR markers and PCR amplification

The SSR markers were acquired by the method based on Shen et al[16], and Table 2 shows the characteristics of the 41 SSR markers. The PCR amplifier is Techne flexigene Cycler. The touchdown procedure was adopted for PCR amplification, and the procedure is as follows: pre-denaturation for 3 min at 94 °C, denaturation for 40 s at 94 °C, annealing for 40 s at 63 °C and extension for 1 min at 72 °C. Then, PCR amplification was performed for 15 times, with the annealing temperature lowered to 0.44 °C each time until to 56 °C; the following is the condition for the last 24 times of amplification: denaturation for 40 s at 94 °C, annealing for 40 s at 56 °C, extension for 1 min at 72 °C. PCR reaction system 15 µl, containing 1 × PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 µM dNTPs, 10 pM of 5′ apex primer and 3′ apex primer, 0.5 U Taq polymerase and 20 ng of template DNA.

PIC value calculation

According to the results of amplification of various SSR markers among the 96 silkworm varieties, the polymorphic index content (PIC)[21] of various SSR markers was calculated by the formula below:

$$\mathrm{PIC} = 1 - \sum_i p_i^2,$$

where p_i represents the frequency of occurrence of i allele in the 96 local varieties.

Electrophoresis

PCR products were tested with ABI 377 automatic sequencer. The concentration of denaturing gel was 5% and the length was 36 cm. After PCR products were diluted to 0.4 ng DNA/µl, 1 µl plus 3 µl of buffer (containing 5 mg/ml of dextran blue and 5% deionized formamide) was added, and placed on ice for use after 3-min denaturation at 95 °C. For sample injection, 0.2 µl of fluorescent markers (ABI, rox400, containing 80, 100, 150, 180, 200, 250, 300, 320, 350, 400 bp fluorescently-labelled DNA fragments) first, and then 0.5 µl of denatured sample was added. Electrophoresis was performed for 2 h under the following condition: electrophoresis buffer 1 × TBE, voltage 3000 V, current 50 mA and gel temperature 51 °C.

Data analysis

With the rox400 internal standard as reference, an amplified band of certain size appeared in a particular position after primer electrophoresis, and Gene Scan Analysis Software v.3.1 was used to calculate the size of each amplified fragment. Each observed polymorphic band was regarded as a trait, with a value of '1' and a value of '0' when no polymorphic band was observed. The genetic distance was expressed in absolute distance and calculated with Microsat v.1.5 (E.M inch, Stanford University, USA). Subprograms NEIGHBOR and DRAWGRAM in PHYLIP software package (v.3.57 c. Joe Felsenstein, University of Washington, USA) were used as

Table 1 Characteristics of silkworm strains used in the present study.

No.	Strain name	System	Voltinism	Moltinism	No.	Strain name	System	Voltinism	Moltinism
pop1	Huiseluan	Chinese	V1	M4	pop49	Tongshan No. 24	Chinese	V1	M4
pop2	3011(D)	Chinese	V1	M4	pop50	Xinyi No. 19	Chinese	V1	M4
pop3	Sanmianbai	Chinese	V1	M3	pop51	Sihong No. 15	Chinese	V1	M4
pop4	Sanmianbailuan	Chinese	V1	M3	pop52	Peixian No. 1	Chinese	V1	M4
pop5	Duodueibanyueban	Chinese	V2	M4	pop53	Fengxian No. 8	Chinese	V1	M4
pop6	Xinlongjiao	Chinese	V2	M4	pop54	Yancheng No. 2	Chinese	V1	M4
pop7	207	Chinese	V2	M4	pop55	Mianyanghong	Chinese	V1	M4
pop8	C2 zhe	Chinese	V2	M4	pop56	Xupuzhong	Chinese	V1	M4
pop9	C110 B	Chinese	V2	M4	pop57	Changdejinhuang	Chinese	V1	M4
pop10	Yingwenpiban	Japanese	V2	M4	pop58	Youxianzhong	Chinese	V1	M4
pop11	HehuiYinghan	Japanese	V2	M4	pop59	Handanzhong	Chinese	V1	M4
pop12	3011(Oxin3)	Chinese	V2	M4	pop60	Datuanyuan	Chinese	V1	M4
pop13	3011(B4xin2)	Chinese	V2	M4	pop61	Jinhuang	Chinese	V1	M4
pop14	3011(5xin1)	Chinese	V2	M4	pop62	Jinguang	Chinese	V1	M4
pop15	Hehuihuaba	Chinese	V2	M4	pop63	Bilian	Chinese	V1	M4
pop16	Songhuaxingwu	Chinese	V2	M4	pop64	Zhong No. 11	Chinese	V1	M4
pop17	Heyuanlongjiao	Chinese	V2	M4	pop65	Zhong No. 14	Chinese	V1	M4
pop18	Fenshui No. 1	Chinese	V1	M3	pop66	Balinghuang	Chinese	V1	M4
pop19	Xiushui No. 2	Chinese	V1	M3	pop67	Jiaxingyoucan	Chinese	V1	M4
pop20	Suqian No. 1	Chinese	V1	M3	pop68	Yuhangbaipi	Chinese	V1	M4
pop21	Ermao	Chinese	V1	M3	pop69	Jilisi	Chinese	V1	M4
pop22	Zhugui	Chinese	V1	M3	pop70	Longwangtang	Chinese	V1	M4
pop23	Tangxisanmian	Chinese	V1	M3	pop71	Zhuji	Chinese	V1	M4
pop24	Zunyi No. 1	Chinese	V1	M3	pop72	Xiaobaiyuan	Chinese	V1	M4
pop25	Zunyi No. 2	Chinese	V1	M3	pop73	Xunba	Chinese	V1	M4
pop26	Zhengan No. 1	Chinese	V1	M3	pop74	Taihuyucan	Chinese	V1	M4
pop27	Yanhe No. 1	Chinese	V1	M3	pop75	Songhuacanji	Chinese	V1	M4
pop28	SanmianA	Chinese	V1	M3	pop76	Huayuan	Chinese	V1	M4
pop29	SanmianB	Chinese	V1	M3	pop77	Wulong3030	Chinese	V1	M4
pop30	Sanguang	Chinese	V1	M3	pop78	357	Chinese	V1	M4
pop31	Lianyuen No. 1	Chinese	V1	M3	pop79	C17(zhuwu)	Chinese	V1	M4
pop32	Shangqiu No. 1	Chinese	V1	M3	pop80	9008	Chinese	V1	M4
pop33	Mengzi No. 1	Chinese	V1	M3	pop81	3042(18)	Chinese	V1	M4
pop34	Luhuang1	Chinese	V1	M3	pop82	Gong No. 2	Chinese	V1	M4
pop35	43xin	Chinese	V1	M4	pop83	Chuan35(1–4)	Chinese	V1	M4
pop36	47xin	Chinese	V1	M4	pop84	Linchengzhong	Chinese	V1	M4
pop37	47qiao	Chinese	V1	M4	pop85	Zhecan (hang)	Chinese	V1	M4
pop38	Chaoxianzhong	Chinese	V1	M4	pop86	Zhenze	Chinese	V1	M4
pop39	Xiaofeng No17	Chinese	V1	M4	pop87	Lu108	Chinese	V1	M4
pop40	Anji No. 7	Chinese	V1	M4	pop88	Zhong20xi	Chinese	V1	M4
pop41	Yuhang11	Chinese	V1	M4	pop89	Zhong21yu	Chinese	V1	M4
pop42	Yuhang24	Chinese	V1	M4	pop90	Yanjizhong	Chinese	V1	M4
pop43	Yiwu No. 10	Chinese	V1	M4	pop91	Hangui(wu)	Chinese	V1	M4
pop44	Xinchang No. 12	Chinese	V1	M4	pop92	CAzhe	Chinese	V1	M4
pop45	Linhai No. 39	Chinese	V1	M4	pop93	Xugui(wu)	Chinese	V1	M4
pop46	Ninghai No. 20	Chinese	V1	M4	pop94	Xingshan No. 1	Chinese	V1	M3
pop47	Fenghua No. 7	Chinese	V1	M4	pop95	Xingshan No. 2	Chinese	V1	M4
pop48	Pixian No. 3	Chinese	V1	M4	pop96	Lanxi2	Chinese	V1	M4

Voltinism: the number of generations that occur in one year. *Bombyx mori* can be divided into three types: univoltine (V1), bivoltine (V2) and polyvoltinism (V3). Moltinism: A characteristic number of times that silkworms molt three (M3) or four-times (M4).

analysis software, and UPGMA was used to cluster the genetic distance between all the varieties, obtaining a tree diagram.

RESULTS

The number of alleles in silkworm varieties and the PIC of SSR markers

525 different DNA fragments (data unlisted) were amplified from the 41 SSR markers in the 96 varieties, with 1 SSR marker corresponding to 12.80 alleles averagely (min 3, max 35). The mean PIC value of the 41 SSR loci was 0.69 (min 0.162, max 0.958) (Table 2).

Fingerprint construction

After amplification with 41 SSR primers, a series of specific amplifications emerged at the corresponding loci for each local variety. The size of each amplified band could be read off after electrophoresis with ABI 377 sequencer (Fig. 1). The polymorphic bands amplified by each SSR primer were sorted, and typical SSR marker-based fingerprint of each variety was determined. As shown in Fig. 1, it is the SSR electrophoresis map of 23 varieties of pop1–pop23 with primer FL0547, and the number and the size of bands can be read out. As can be seen from Fig. 1, no polymorphic bands of pop9, pop13,

Table 2 Microsatellite loci, repeat motif, forward and reverse flanking primer sequences, number of alleles, allelic size range, and PIC values in *Bombyx mori*.

Locus	Repeat	Primer sequence (3′–5′)	No.	Size range	PIC
FL0308	CT	gcgacgcactccatcaagc tgctttcgtcctttcttatccct	8	104–148	0.776
FL0315	CA	tcgccgagttggctggt cgtgtaaacatgtatcccgagttg	5	150–160	0.715
FL0317	CT	cccaccaggtaaggttggtaca ggaaatggtagtgcaaggtagagg	17	148–182	0.735
FL0542	CT	tgaaatcagttgaggcggaaaa cttgctaatcgaattcatgattgtaa	18	234–282	0.895
FL0547	CT	cacaggatgacttggtaaaacgg gctttcagtaaaattattttgaatttga	6	288–308	0.257
FL0612	CA	cagatttcgccaggactacacttt gagaagtgcagagtgcccatatt	14	220–264	0.841
FL0617	CT	aagttcttgagtggcgaccg caagaaccgcagacactccaa	14	232–312	0.817
FL0910	CT	acagacttaacttaaaacggattgaaa cgttgtagatgtctatgggctcc	4	234–246	0.478
FL0915	CT	tgctgaaggacaaaagggaatg cattgtggatgtctacgggctc	25	172–300	0.893
FL0932	CT	cagtccttgtcagttgccattgt aaccgttaaatgagacgtgcg	8	224–300	0.566
FL0939	CT	ggtgtattcgtaacaagtagggcg tcttctttgtctcctacttatcccact	6	290–310	0.423
FL1003	CT	aaagttttctggagcgaagcg ccctgcagttgctcgtatgtc	35	94–196	0.958
FL1019	CA	cacctagttttacttcacggaccat cccttgggcttaagtcggttt	15	220–258	0.778
FL1027	CT	actcaccaacaaaccgcaaga caccgcaacatgcctgctata	22	132–282	0.834
FL1028	CT	agctgtggtcggcatatgaagt gccagttttgaagggtcgg	31	250–330	0.808
FL1148	CA	gcctttatctttgaacgatcattga ccccttattacgaaaAttgcgc	19	322–400	0.888
FL1160	CA	ggagtcccgcatcccgataa atcatgtcagacctaagcactcaaa	14	304–346	0.820
FL1162	CA	tttgttgttcataaggtccgca gattccgacgatagatttgacga	8	349–367	0.762
FL1163	CA	cagtggtttatttgtacggcagaa atggagtgcttctagatgcctagtt	4	334–342	0.621

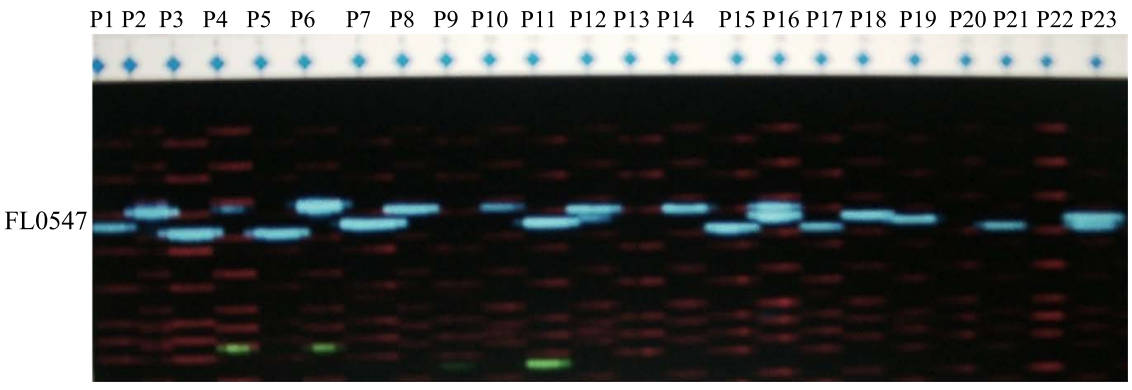

Fig. 1 Amplification results of FL0547 in silkworm variety pop1–pop23.

pop20 and pop23 appeared after the amplification of FL0547, but one or more bands of different lengths were amplified for the other 19 varieties, respectively. By analogy, the SSR polymorphism bands of all local varieties amplified by each SSR primer were sorted out, and the SSR amplification bands of each local variety were obtained, that is, the SSR marker fingerprint of each variety.

Genetic distance analysis and cluster analysis

The genetic distance between the varieties was calculated according to the results of amplification with SSR markers. The genetic distance between Jinhuang (pop61) and Jinguang (pop62) was the smallest (0.1101), and hence, the first to cluster; followed by SanmianA (pop28) and SanmianB (pop29) group, (genetic distance = 0.1152), and then by Minayanghong (pop55) and Youxianzhong (pop58) with a genetic distance estimate of 0.1203. The furthest distances was observed between Xingshan No. 1 (pop94) and other varieties, reaching values of up to 0.2381, indicating that Xingshan No. 1 was least closely related to other varieties. The mean genetic distance among the varieties was 0.1621.

According to the genetic distance among the varieties, UPGMA was used to draw a cluster map for the 96 silkworm varieties, as shown in Fig. 2 There were relatively complex clusters among the silkworm varieties, that could be broadly assigned into the following groups: the first group consisted of 6 univoltine trimolters, 6 bivoltine tetramolters and 8 univoltine tetramolters, involving 20 varieties; the second group consisted of 7 univoltine trimolters and 7 univoltine tetramolters, involving 14 varieties; the third group consisted of 9 varieties, including 3 bivoltine tetramolters, 2 univoltine trimolters and 4 univoltine tetramolters; the fourth group consisted of 8 varieties, including 1 univoltine trimolter and 7 univoltine tetramolters; the fifth group, a relatively centralized group, consisted of 6 univoltine tetramolters and 1 univoltine trimolter. It could be seen from the above results that the varieties in each group included different geographical systems (Chinese or Japanese), different voltinism and moltinism types of behaviour, and that these five groups were clustered with other subgroups based on distances. The remaining 42 varieties were not clustered in any obvious manner. Mostly, 2 or 3 varieties could be clustered together, while some others, such as Huiseluan (pop1), Jilisi (pop69), Peixian No. 1 (pop52), Xiushui No. 2 (pop19), Wulong 3030 (pop77) and Xingshan No. 2 (pop95), were on lone branches. Xingshan No. 1 (pop94) was clustered separately alone from the remaining 95 varieties.

DISCUSSION

The core structure of McRoatellite DNA is a sequence of dozens of nucleotides composed of 1–6 nucleotides in series, with the variation of the number of repeating units forming the polymorphism of SSR markers. It is generally recognized that the number of repeating units is positively correlated with the number of alleles at the locus. But in silkworm genomes, Reddy et al[15] and Hou et al[22], discovered that the length of repeated SSR fragments has nothing to do with polymorphism. In this study, the FL0910 and FL1163 amplified fragments of 4 alleles were 234–246 bp and 334–342 bp, respectively. Although the same number of fragments was amplified from the 2 primers, there was a big difference in the size of the amplified fragments; the FL1003 amplified fragment of 35 alleles was just 94–196 bp. This shows that the results of this study support the previous viewpoints, and the length of the SSR amplified fragments in silkworm genomes has nothing to do with SSR polymorphism.

SSR is widespread throughout various eukaryotic genomes, and there are many reports[25–27] on the use of SSR to research species revolution and classification. As can be seen from dozens of species of animals and plants concerned, the SSR technique can quicken material classification and identification accurately. Our team applied SSR to SSR fingerprint construction for different geographic silkworm varieties and to genetic relationship studies[17, 22, 23] concluding that SSR could accurately classify silkworm varieties systematically and even analyse the difference in one variety caused by egg colour[23]. Thus it can be seen that the SSR marker technique is an effective method for researching the genetic differences between silkworm varieties. As shown by the results of this study, the varieties from the same place, such as Xingshan No1 and Xingshan No. 2, Yuhang No. 11 and Yuhang No. 24, are separated far from each other in terms of genetic distance, suggesting that gene swapping didn't occur when they were collected, and their primary characteristics were retained. Thankfully, these varieties have been strictly protected against admixture in the past decades due to preservation, with their local characteristics retained, ensuring the diversity of silkworm variety resources.

It is recognized that domestic silkworms are tamed wild silkworms, but there are differences in varietal evolution and classification among the

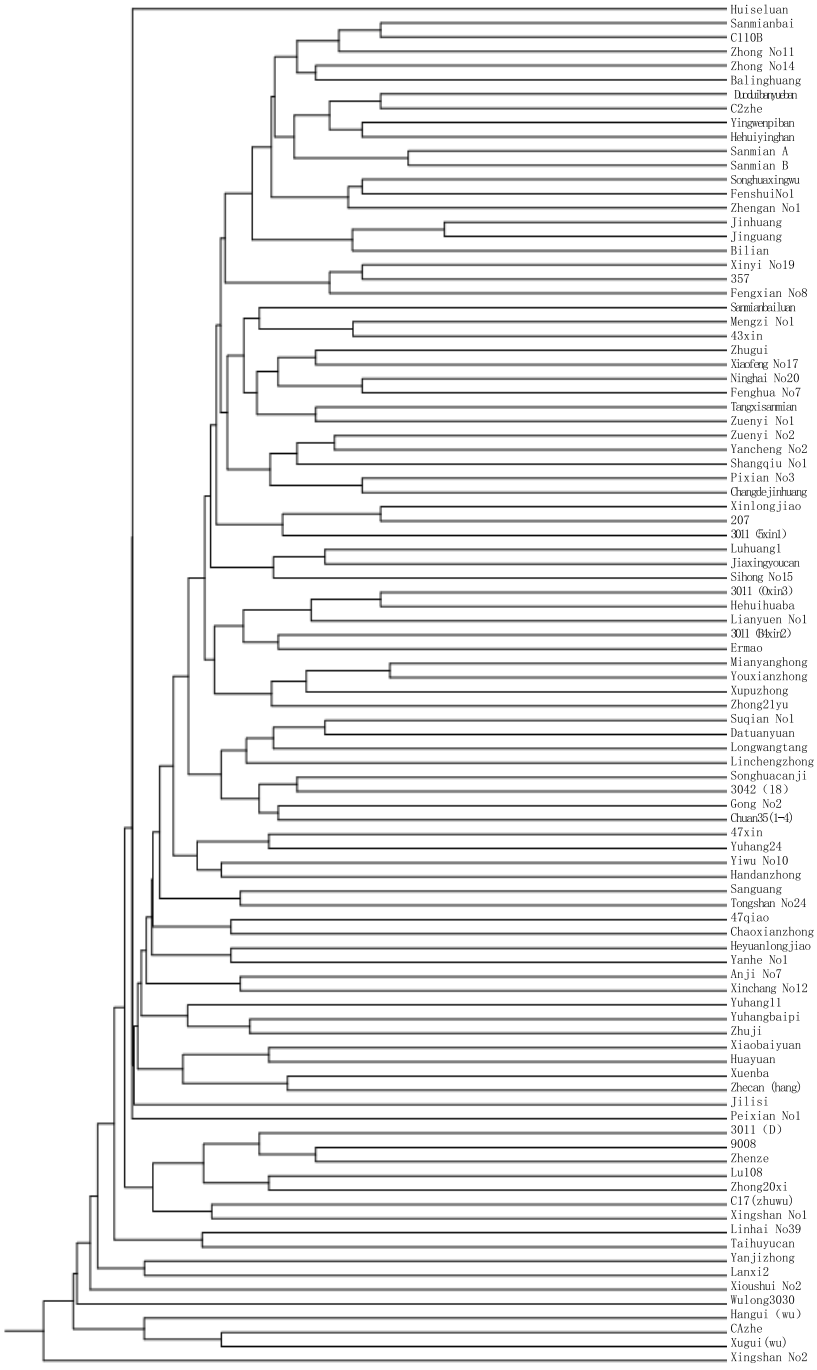

Fig. 2 Phylogenetic tree of silkworm races.

domestic silkworms. Some scholars also argue that multivoltine silkworms differentiated early and have many centres of origin. However, Xiang et al[1] suggested Chinese univoltine variety represents the original centre of silkworm. In recent years, molecular-genetic techniques have opened up a new way for research into the origin and evolution of species. This has, enabled people to analyse genetic differences between species and varieties through genome-based information, giving a big push to research on the origin and evolution of silkworms. Based on results using the RAPD technology, Xia et al[28] showed that the Chinese univoltine tetramolter was the earliest tamed wild silkworm,

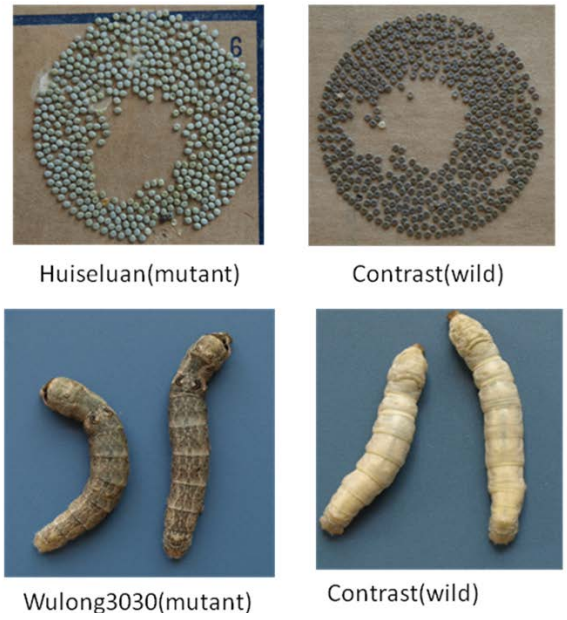

Fig. 3 Some mutant varieties used in this study.

and that the Chinese univoltine trimolter and univoltine tetramolter belonged to two groups with a significant difference in evolution. Lu et al[29] researched silkworm evolution using AFLP, the results confirming that domestic silkworms were evolved from Chinese wild silkworms, and pointed that there were already univoltine, bivoltine and multivoltine silkworms in the early days of domestication, and those silkworms grew into different varieties; Hou et al[22] analysed the evolution of the European univoltine, Japanese univoltine and Chinese bivoltine silkworms with the SSR technique, verifying the theory of mixed origin put forward by Lu et al[29]. Of the 96 objects of this study, there are 63 Chinese univoltine tetramolter varieties, 20 Chinese univoltine trimolter varieties, 11 Chinese bivoltine tetramolter varieties and 2 Japanese bivoltine tetramolter varieties. But in the molecular cluster map, the varieties of the same voltinism or moltinism were not clustered together, whereas the varieties of different voltinism and moltinism are clustered in an interpenetrating way. The research results seem to support the conclusion that silkworms have multiple original centres.

In addition, if a mutation occurs in a trait of a variety, it may also lead to the special alienation of the variety on the molecular evolutionary tree. For example, Huiseluan (pop1) in this study collected from wuxian county, jiangsu province, and its egg colour was mutated with grey and deep egg stripes. Wulong 3030 (pop77) is a local species collected in yuyao county, Zhejiang province, while the larva's markings and body colour were mutated to show dark brown markings (Fig. 3). These research results seem to support the conclusion that mutations of these traits may be related to SSR loci involved in this study, so these varieties do not cluster into groups with other varieties.

CONCLUSIONS

SSR markers have rich polymorphism in silkworm variety resources. With high repeatability, usability (PCR markers) and testability (by polyacrylamide gel electrophoresis), SSR markers can be used to build fingerprints of silkworm variety resources, analyse the genetic relationship between silkworm variety resources and set up a core variety bank of silkworms.

All the 96 silkworm varieties in this study are fully distinguished from one another with SSR markers, suggesting that SSR markers are suitable for variety identification, especially providing evidence about DNA levels for the identification of the varieties involved in intellectual property disputes.

The results of this study basically accord with the theory of mixed origin put forward by Takeyoshi Yoshinari and Lu Cheng that different silkworm local varieties are not clustered together by the same voltinism or moltinism, but more evidence is needed.

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