Genetic diversity among mulberry genotypes from seven countries

Zhenjiang Wang¹ · Yufei Zhang¹ · Fanwei Dai¹ · Guoqing Luo¹ · Gengsheng Xiao¹ · Cuiming Tang¹

Received: 15 August 2016 / Revised: 2 March 2017 / Accepted: 6 March 2017 / Published online: 24 March 2017
© Prof. H.S. Srivastava Foundation for Science and Society 2017

Abstract Mulberry (*Morus alba*) is an economically and ecologically important, widespread woody plant. It has served human beings for over hundreds of years, and it is still widely used in pharmaceuticals, food industry and farming nowadays. Using modern techniques, deeper understanding in classification and conservation resources of mulberry leads to higher-efficiency hybrids among populations. Genetic polymorphisms among 42 mulberry genotypes from seven countries over Asia and South America were detected using 17 inter simple sequence repeat (ISSR) primers. A dendrogram was constructed using the similarity matrix among genotypes and a principal component analysis (PCA) was carried out to further identify and cluster the mulberry genotypes. In the 42 genotypes, 175 distinct bands were displayed, among which 169 were polymorphic bands (96.57%). The polymorphic information content of 17 ISSR primers ranged from 0.2921 to 0.3746 with the mean of 0.3494. And Nei's index and Shannon's information index averaged 0.116 and 0.174, respectively, indicating low diversity of mulberry. For further study, cluster analysis and PCA were carried out and the results were similar. 42 genotypes were grouped, showing some hybridized combinations. Additionally, a connection between mulberry diseases and their genotypes was noted, which indicates possible application for ISSR in studying disease resistance of mulberry.

Keywords Genetic diversity · Inter simple sequence repeat · Mulberry · Cluster analysis · Principal component analysis

Introduction

Horticultural plants have been an indispensable part of human life for ages. Ever since ancient times, their fruits, seeds, even roots and branches have been used to meet personal and social needs such as severing food, curing diseases and beautifying the planet. One of the most common horticultural plants, mulberry (*Morus alba*), was first found in Middle China. Because of its high agricultural, medicinal and economic values, it is now cultivated widely. The leaves of mulberry have been used to feed silkworms in China since ancient times, and the fruit is both edible and healthy for human beings (Awasthi et al. 2004). It may also denote macaque habitats (Enari and Sakamaki 2010). Additionally, mulberry has been used as a medicine for a long time. Multiple parts of mulberry, including its root, leaf and branch, have been shown to be efficient for treating diseases. In the last decade, many studies showed that *Morus* has relatively significant effects on chronic diseases, like diabetes, and the nervous system (Yadav and Nade 2007; Yadav et al. 2008; Nakamura et al. 2009). Additionally, mulberry has both the anticancer and the anti-inflammatory properties, which act through different pathways (Kollar et al. 2013; Eo et al. 2014; Kwon et al. 2015; Qian et al. 2015). Some reports have demonstrated the leaf's role in ameliorating obesity-induced reactions, increasing bone density and antiplatelet activity (Ann et al. 2015; Sungkamanee et al. 2014; Kim et al. 2014). Therefore, it is essential and beneficial for us to improve the production and quality of mulberry. Here, we

✉ Cuiming Tang
tangcuiming@21cn.com

¹ Sericulture & Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510610, China

try to achieve this goal using ISSR primers to identify the genotypes, study the genetic polymorphism and look for hybridized parents.

Inter simple sequence repeats (ISSRs) are commonly used as molecular markers, which combine the advantages of random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs). ISSRs are based on microsatellite DNAs, which have high levels of mutations in non-coding regions due to replication slippage, and, therefore, these mutations can accumulate and differ between varieties in different populations (Zietkiewicz et al. 1994). Other than applications to genetic stability and gene variation (Qian et al. 2001; Naz et al. 2016), ISSRs have higher efficiencies in polymorphism analyses in plants (Nagaoka and Ogihara 1997; Devarumath et al. 2002; Zargar et al. 2016). However, AFLPs require known flanking sequence and advanced digestion, respectively. Here, we collected 42 mulberry genotypes from 11 different regions, distributed in Asia and South America. With abundant germplasm resources, we detected their genetic diversity using ISSRs, to determine the differences and similarity in their genomes and provide information for further breeding and hybridization strategies.

Table 1 List of the 42 mulberry genotypes

Region	Number	Code
South China	1–7	GD1–GD7
North China	8–16	HB1–HB9
Northeast China	17	X1
Northwest China	18–19	H1–H2
Taiwan	20–21	T1–T2
Japan	22–26	J1–J5
Thailand	27–31	Th1–Th5
Argentina	32–34	A1–A3
Vietnam	35–37	V1–V3
North Korea	38	Nk1
India	39–42	I1–I4

Fig. 1 The ISSR 09 banding patterns for the 42 genotypes

Methodology

Plant material

The 42 mulberry genotypes of *Morus atropurpurea* Roxb. used in the present study were collected from seven countries (Table 1). The fresh leaves were stored in 0 °C ice water mixture temporarily and after returning to the lab, they were wrapped in tinfoil and transferred to –80 °C for long-term storage.

Genomic DNA extraction

Approximately 0.1 g leaf tissue was finely disrupted through bead shaking for 45 s in a 1.5-mL centrifuge tube. The DNAs of these tissues were then isolated using a TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa Biotechnology CO., Dalian, China) and stored at –20 °C. The quantity and quality of DNA were estimated by 1.0% agarose (regular agarose G-10, Biowest SAS, Nuailé, France) gel.

Detection of genetic polymorphisms

Genetic polymorphisms in mulberry were determined using ISSR markers. After testing over 40 ISSR primers, we selected 17 primers that could form clear polymorphic bands for genetic diversity detection.

We amplified ISSR fragments using Applied Biosystems' Veriti™ 96-well thermal cycler in 20 µL reaction system, including 1 µM of selected ISSR primers, 25 ng mulberry genomic DNA, 0.2 mM of each dNTP (GenView Scientific Inc., Jacksonville, Florida, USA), 10 × Taq buffer (20 mM Mg²⁺ plus; Beijing Dingguo Changsheng Biotechnology CO. LTD., Beijing, China), 1 U Taq DNA polymerase (Beijing Dingguo Changsheng Biotechnology CO. LTD.). The polymerase chain reaction was initiated at 94 °C for 2 min, with 35 rounds of 30 s denaturation at 94 °C, 30 s annealing and 45 s

Table 2 Characteristics of 17 ISSR primers

Primers	Sequence	Tm/ °C	PIC	Amplified bands	Polymorphic bands	Polymorphic percentage/ %
ISSR 01	(AG) ⁸ TC	54	0.3721	9	9	100.00
ISSR 02	(AG) ⁸ T	50	0.3599	12	12	100.00
ISSR 03	(CT) ⁶ GC	44	0.3361	9	9	100.00
ISSR 04	GA(GGA) ³ GGC	48	0.3609	13	13	100.00
ISSR 05	(CT) ⁸ G	52	0.3403	13	13	100.00
ISSR 06	(CA) ⁸ A	50	0.3740	13	12	92.30
ISSR 07	(CA) ⁸ G	52	0.3186	7	6	85.71
ISSR 08	(GT) ⁸ C	52	0.3667	6	5	83.33
ISSR 09	(GT) ⁸ T	50	0.3658	7	6	85.71
ISSR 10	(TC) ⁸ G	52	0.3448	11	11	100.00
ISSR 11	(AG) ⁸ YT	53	0.3673	10	9	90.00
ISSR 12	(TA) ⁸ RG	52	0.3746	7	6	85.71
ISSR 13	(CA) ⁸ RT	53	0.3430	11	11	100.00
ISSR 14	(GT) ⁸ YG	55	0.3547	9	9	100.00
ISSR 15	(AC) ⁸ YA	53	0.2921	11	11	100.00
ISSR 16	(GAA) ⁶	48	0.3538	13	13	100.00
ISSR 17	(GGGTG) ³	54	0.3153	14	14	100.00
Mean			0.3494	10.294	9.882	96.57
Total			–	175	169	–

Table 3 Parameters of genetic variation among 11 populations of 42 mulberry genotypes

Region	Pp	Na	Ne	H	I
South China	75.00	1.697	1.354	0.216	0.333
North China	68.33	1.634	1.333	0.199	0.304
Northeast China	35.56	1.000	1.000	0.000	0.000
Northwest China	44.45	1.205	1.125	0.085	0.124
Taiwan	53.3	1.331	1.234	0.137	0.201
Japan	58.33	1.457	1.278	0.163	0.244
Thailand	53.89	1.445	1.273	0.158	0.237
Argentina	42.78	1.251	1.176	0.100	0.147
Vietnam	40.56	1.251	1.170	0.098	0.144
North Korea	31.12	1.000	1.000	0.000	0.000
India	47.24	1.337	1.211	0.123	0.184
Average	50.05	1.328	1.196	0.116	0.174
Population	96.57	1.966	1.386	0.241	0.379

Pp percentage of polymorphic loci, *Na* number of alleles observed, *Ne* effective number of alleles, *H* Nei's index; *I* Shannon's information index

elongation at 72 °C, followed by a final elongation step at 72 °C for 5 min. The annealing temperatures were based on the Tm values of the different primers, provided by BGI Tech (Beijing Genomics Institution, Shenzhen, China).

We detected genetic diversity in mulberry by agarose gel electrophoresis. The samples were separated in 1.0% agarose (regular agarose G-10, Biowest SAS) gel, stained with 0.5 µg/ml ethidium bromide, containing in 1 × TAE buffer (0.1 mol/L Tris base, 0.5 mol/L acetic acid, 2 mmol/L EDTA). All bands detected were amplified and screened for at least three times. If results were not identical, more repeated tests were done.

Genetic diversity and cluster analysis

Each amplified band generated by ISSR was scored '1' for its presence and '0' for its absence. Based on observed bands, we calculate the polymorphism information content (PIC) using the formula: $PIC = 1 - \sum p_{ij}^2$, in which P_{ij} was the gene frequency shown by POPGENE version 1.32 (Yeh and Boyle 1997). And genetic parameters of the 11 populations, including percentage of polymorphic loci (Pp), the number of alleles observed (Na), effective number of alleles (Ne), Nei's gene diversity (H) and Shannon's information index (I) were calculated using NTSYS-pc version 2.10 (Rohlf 2008).

Cluster analysis was based on a similarity matrix of polymorphic bands, calculated using the UN4 coefficient method. After we constructed the dendrogram using UPGMA by NTSYS program, the genetic distances between different clusters were calculated. Furthermore, a

Fig. 2 The dendrogram based on UPGMA, depicting the genetic relationship among mulberry genotypes

Table 4 Nei's genetic distance between six clusters based on the similarity matrix

Clusters	Genotypes (number)	I	II	III	IV	V	VI
I	GD1, GD3, H1, H2 (1, 3, 18, 19)	-					
II	GD2 (2)	0.1843	-				
III	X1 (17)	0.1700	0.4199	-			
IV	GD4, GD6, GD7, J1–J5, Th2–Th5, A1–A3, V1–V3 (4, 6, 7, 22–26, 28–37)	0.0499	0.2638	0.1985	-		
V	GD5, HB1–HB9, T1, T2, Th1 (5, 8–16, 20, 21, 27)	0.0337	0.2567	0.1648	0.0287	-	
VI	Nk1, I1–I4 (38–42)	0.1264	0.3266	0.3353	0.1064	0.1255	-

principal component analysis (PCA) was carried out using NTSYS program in order to have a deeper understanding of the diversity of mulberry.

Result and discussion

Genetic polymorphism analysis

A total of 17 ISSRs were used to detect polymorphic bands, and 175 bands were identified (Fig. 1), including 169 (96.6%) polymorphic bands. The number of polymorphic bands per primer ranged from 5 (ISSR 08) to 14 (ISSR 17) with an average of 9.882. The minimum of polymorphic percentages was 83.33% (ISSR 08) with an average of 96.57%. Among 17 primers, 11 of them had the

polymorphic percentages of 100%. The PIC was between 0.2921 (ISSR 15) and 0.3746 (ISSR 12) with an average of 0.3494, close to the results in common bean and chrysanthemum (Table 2; Kumar et al. 2015; Zargar et al. 2016).

Five genetic parameters were measured (Table 3). Pp ranged from 31.12 to 75.00 with a mean of 50.05. The average of Na and Ne were 1.328 and 1.196, respectively. Nei's gene diversity is between 0.000 and 0.216 with a mean of 0.116, and Shanon's information index is between 0.000 and 0.333 with a mean of 0.174. All the maximums of these genetic parameters appeared in the population from South China, while the minimum of them appeared in the population from North Korea. Compared to several dicotyledons, including chickpea, sea buckthorn and *Jatropha curcas*, the values of these parameters were relatively low, revealing the conservation of mulberry in these

Fig. 3 Two-dimensional principal component analysis plot grouping the 42 genotypes **a** was a plot of component 1 and component 3, and **b** was a plot of component 2 and component 3

regions (Ratnaparkhe et al. 1998; Gupta et al. 2008; Ruan et al. 2009). However, among all 42 genotypes, the Pp, Na, Ne, H and I were 96.57, 1.966, 1.386, 0.241 and 0.379, respectively, which were much larger than the ones of the 11 populations. This indicates that despite the low diversity within populations, the genotypes from different populations vary from each other, which can lead to suitable hybridized combinations, improving the quality of mulberry manually.

Cluster analysis

A dendrogram was constructed based on a similarity matrix, ranged from 0.5162 (I3, HB4) to 0.9372 (J4, J5)

with an average of 0.6667 (Fig. 2). Six major clusters were found, and the genetic distance of the six clusters ranged from 0.0287 (cluster IV and V) to 0.4199 (cluster II and III). Most genotypes from the same region were clustered into the same group, while seven genotypes (GD1 – GD7), coming from South China, were separated in cluster I, II, IV and V, which is a similar result with the analysis of genetic parameters, showing the high diversity of mulberry in South China (Table 4).

Furthermore, similar results were obtained using PCA. Four groups can be distinguished from other genotypes (groups covered in gray) in 2D figures of PCA. Group I was identical to cluster VI, which was also clear in 3D figure of PCA (Fig. 3 and 4). And group II included cluster

Fig. 4 Three-dimensional PCA plot of 42 genotypes based on ISSR markers

I, II, III except H1, which was in group III. Group III and group IV together combined into cluster IV. However, cluster V was not easy to find in the results of PCA.

To sum up, mulberry from these 11 regions has relatively low diversity which limits the chances of hybridization. However, combining the results of cluster analysis and PCA, we noticed that genotypes GD2 and X1 had the largest genetic distances from others, and Nk1, I1 to I4 can be distinguished from other groups easily. All of them can be a good choice for hybridization, improving the cultivation of mulberry.

Additionally, combining the variety information, we noticed that in some clusters, like cluster V, the genotypes were resistant to black wilt disease, while the other clusters showed no resistance. This indicated that there might exist a connection between the mulberry genotype and the resistance level against black wilt disease. In the last decade, the amount of researches on mulberry diseases has increased, but most of them focused on biochemistry and protein expression (Gai et al. 2014a, b). There have been some researches using ISSR marker to map the resistant genes in chickpea and sea buckthorn (Ratnaparkhe et al. 1998; Ruan et al. 2009). We believe that, ISSRs could also be useful in studying mulberry resistance, thereby, improving mulberry breeding and conservation.

References

- Ann J, Eo H, Lim Y (2015) Mulberry leaves (*Morus alba* L.) ameliorate obesity-induced hepatic lipogenesis, fibrosis, and oxidative stress in high-fat diet-fed mice. *Genes Nutr* 10(6):46
- Awasthi AK, Nagaraja GM, Naik GV, Kanginakudru S, Thangavelu K, Nagaraju J (2004) Genetic diversity and relationships in mulberry (genus *Morus*) as revealed by RAPD and ISSR marker assays. *BMC Genet* 5:1
- Devarumath RM, Nandy S, Rani V, Marimuthu S, Muraleedharan N, Raina SN (2002) RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (assam-India type). *Plant Cell Rep* 21(2):166–173
- Enari H, Sakamaki H (2010) Abundance and morphology of Japanese mulberry trees in response to the distribution of Japanese macaques in snowy areas. *Int J Primatol* 31(5):904–919
- Eo HJ, Park JH, Park GH, Lee MH, Lee JR, Koo JS, Jeong JB (2014) Anti-inflammatory and anti-cancer activity of mulberry (*Morus alba* L.) root bark. *BMC Complement Altern Med* 14:200
- Gai YP, Han XJ, Li YQ, Yuan CZ, Mo YY, Guo FY, Liu QX, Ji XL (2014a) Metabolomic analysis reveals the potential metabolites and pathogenesis involved in mulberry yellow dwarf disease. *Plant Cell Environ* 37(6):1474–1490
- Gai YP, Li YQ, Guo FY, Yuan CZ, Mo YY, Zhang HL, Wang H, Ji XL (2014b) Analysis of phytoplasma-responsive sRNAs provide insight into the pathogenic mechanisms of mulberry yellow dwarf disease. *Sci Rep* 4:5378
- Gupta S, Srivastava M, Mishra GP, Naik PK, Chauhan RS, Tiwari SK, Kumar M, Singh R (2008) Analogy of ISSR and RAPD markers for comparative analysis of genetic diversity among different *Jatropha curcas* genotypes. *Afr J Biotechnol* 7(23):4230–4243
- Kim D, Ji HD, Rhee MH, Sung Y, Yang W, Kim SH, Kim H (2014) Antiplatelet activity of *Morus alba* leaves extract, mediated via inhibiting granule secretion and blocking the phosphorylation of extracellular-signal-regulated kinase and akt. *Evid Based Complement Altern Med* 2014:1–11
- Kollar P, Bárta T, Hošek J et al (2013) Prenylated flavonoids from *Morus alba* L. cause inhibition of G1/S transition in THP-1 human leukemia cells and prevent the lipopolysaccharide-induced inflammatory response. *Evid Based Complement Altern Med* 2013:1–13
- Kumar G, Singh KP, Prasad KV, Verma N, Namita Panwar S (2015) Characterization of chrysanthemum (*Chrysanthemum grandiflorum*) varieties using ISSR markers. *Indian J Agric Sci* 85(4):566–570
- Kwon YH, Bishayee K, Rahman A, Hong JS, Lim S, Huh S (2015) *Morus alba* accumulates reactive oxygen species to initiate apoptosis via foxo-caspase 3-dependent pathway in neuroblastoma cells. *Mol Cells* 38(7):630–637
- Nagaoka T, Ogiwara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theor Appl Genet* 94(5):597–602
- Nakamura M, Nakamura S, Oku T (2009) Suppressive response of confections containing the extract from leaves of *Morus alba* on postprandial blood glucose and insulin in healthy human subjects. *Nutr Metab (Lond)* 6:29
- Naz R, Anis M, Alatar AA (2016) ISSR marker-based detection of genomic stability in *Cassia occidentalis* L. plantlets derived from somatic embryogenesis. *Eng Life Sci* 16(1):17–24
- Qian W, Ge S, Hong DY (2001) Genetic variation within and among populations of a wild rice *Oryza granulata* from china detected by RAPD and ISSR markers. *Theor Appl Genet* 102(2–3):440–449
- Qian Z, Wu Z, Huang L et al (2015) Mulberry fruit prevents LPS-induced NF- κ B/pERK/MAPK signals in macrophages and suppresses acute colitis and colorectal tumorigenesis in mice. *Sci Rep* 5:17348
- Ratnaparkhe MB, Santra DK, Tullu A, Muehlbauer FJ (1998) Inheritance of inter-simple-sequence-repeat polymorphisms and

- linkage with a fusarium wilt resistance gene in chickpea. *Theor Appl Genet* 96(3–4):348–353
- Rohlf FJ (2008) NTSYSpc: Numerical Taxonomy System, ver. 2.20. Exeter Publishing, Ltd.: Setauket, NY
- Ruan CJ, Li H, Mopper S (2009) Characterization and identification of ISSR markers associated with resistance to dried-shrink disease in sea buckthorn. *Mol Breed* 24:225–268
- Sungkamanee S, Wattanathorn J, Muchimapura S, Thukham-mee W (2014) Antiosteoporotic effect of combined extract of *Morus alba* and *Polygonum odoratum*. *Oxid Med Cell Longev* 2014:1–9
- Yadav AV, Nade VS (2007) Anti-dopaminergic effect of the methanolic extract of *Morus alba* L. leaves. *Indian J Pharmacol* 40(5):221–226
- Yadav A, Kawale L, Nade V (2008) Effect of *Morus alba* L. (Mulberry) leaves on anxiety in mice. *Indian J Pharmacol* 40(1):32–36
- Yeh FC, Boyle TJB (1997) Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belg J Bot* 129:157
- Zargar SM, Farhat S, Mahajan R, Bhakhri A, Sharma A (2016) Unraveling the efficiency of RAPD and SSR markers in diversity analysis and population structure estimation in common bean. *Saudi J Biol Sci* 23(1):139–149
- Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20(2):176–183