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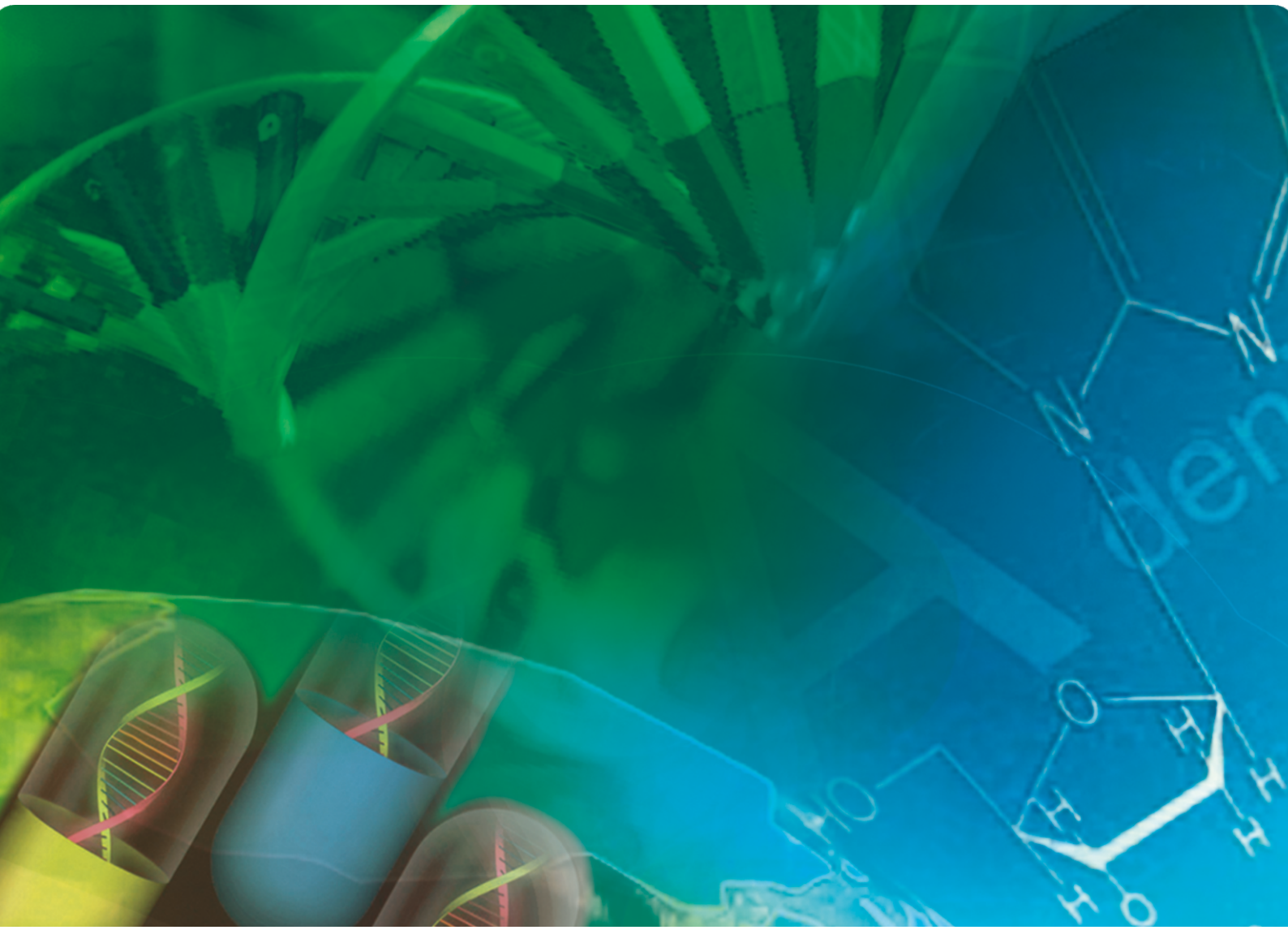

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Efficiency of Triple-SCoT Primer in Characterization of Genetic Diversity and Genotype-Specific Markers against SSR Fingerprint in Some Egyptian Barley Genotypes

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Abstract

Ten Egyptian barley genotypes (2 commercial varieties and 8 breeding lines) were cultivated under normal condition at the Experimental Farm of Sakha Agricultural Research station and exposed to salinity stress condition at the Experimental Farm of El-hosainia plain Agricultural Research station, Elsharkia Governorate, Egypt, in an attempt to identify the relative salinity tolerant genotypes. A susceptibility index (SI) was used to estimate the relative stress loss because it accounted for variation in yield potential and stress intensity. Giza 123, Line-1, Line-5, Line-6 and Line-8 genotypes were considered as saline tolerant genotypes on the basis of their highly tolerance indices values. Barley genotypes were characterized by seven SSR markers and three SCoT primers in different combinations to discern the extent of genetic variation and develop a fingerprinting key. Normal SCoT reactions amplify single segments of DNA which are 15- to 19-mer long. A new strategy was used to increase SCoT potential in genetic diversity studies by using two and three different primer combinations per reaction. Amplification products scored a polymorphism percentage of 94.44% for Triple-SCoT and 90.91% for SSR, while the average no. of polymorphic fragments/primer was 17 and 7.14 in the two marker systems, respectively. On the other side, Triple-SCoT exhibited the highest average number of positive and negative genotype-specific markers. The cluster analysis of the studied genotypes using these different marker systems revealed four dendrograms varied in their topology. The dendrogram based on Triple-SCoT data exhibited the closest relationships to those illustrated by SSR dendrogram.

Keywords

Barley, Triple-SCoT, SSR, DNA Barcoding, Genetic Similarity

1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most cultivated crop worldwide. Environmental stresses such as low water availability, salinity and mineral toxicity frequently affect plants in agricultural systems and represent major limitations to the yield and quality of barley and other crops. Salinity is a strong abiotic stress affecting crops in Egypt and worldwide. However, Egypt is one of the countries that suffer from severe salinity problems in some areas. About 30% of the cultivated area in Egypt and is already salinized [1]. Salinity adversely affects the growth and yield of crop plants by decreasing the availability of soil moisture, and also due to the toxicity effects of sodium ions at high concentrations to the plant [2].

Application of DNA fingerprinting was primarily used in plants for identification of genetic diversity, biodiversity protection or germplasm conservation and identifying markers associated with specific traits [3]. Molecular markers based on SSR (Simple Sequence Repeats) are powerful techniques because they are co-dominant, multi-allelic, easily scored and highly polymorphic, which can be used to identify and determine plant genomes or to evaluate the phylogenetic relationship among genotypes of barley [4] [5] [6]. However, a major drawback of microsatellite markers is the time and cost required to characterize them [7]. The molecular markers technologies continue to be improved with development of simpler protocols with greater reliability and lower cost. This makes them increasingly practical for routine applications to tropical or subtropical species for which very limited research resources are available.

Start codon targeted (SCoT) polymorphism; a simple and novel DNA marker technique, performed through PCR using single primer targeting the short consensus conserved region flanking the ATG translation initiation codon in plant genes. This has been validated through study on genetic diversity among rice varieties and marker segregation in rice backcross population [8]. There is an urgent need to develop more suitable and tightly linked markers for improved traits (molecular signature) and its further utilization in plant improvement and breeding programmes for exploitation of genetic resources for the sake of commercial and academic needs. Also, in this investigation we used two and three different primer combinations based SCoT (Double and Triple-SCoT, respectively) per single reaction as a new strategy to enhance SCoT efficiency for genetic diversity assessment and this is the first time for using this method in SCoT marker.

DNA barcoding, a relatively new concept, developed for providing rapid, accurate and automatable species identification using standardized DNA sequences as tags is being recognized as a powerful addition to the tools of the taxonomist [9]. In DNA barcoding, the unique nucleotide sequence patterns of small DNA fragments (400 - 800 bp) are used as specific reference collections to identify specimens and to discover overlooked species[10].

The purpose of this study was the molecular evaluation of some Egyptian barley genotypes (commercial cultivar and improved/selected lines) for salinity to-

lerance by using SCoT and SSR markers. Also, comparison between Triple-SCoT (three primer based SCoT marker) and SSR techniques was an important aim of this study. This may provide rare diagnostic marker(s) and may help in defining genetic relationship among the genotypes.

2. Material and Methods

2.1. Plant Material

This study was carried out at the laboratory of molecular genetics, Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Egypt and the Experimental Farm of Sakha Agricultural Research Station (ARC), Egypt, (normal condition) and the Experimental Farm at El-hosainia plain Agricultural Research station, Elsharkia Governorate, Egypt (saline soil condition) during the two successive seasons 2013/14 and 2014/15. Ten barley genotypes (two local varieties; Giza 123 and Giza 132 and eight breeding lines named as Line-1 to Line-8) as presented in Table 1 were chosen from Sakha Agricultural Research Station (ARC), Egypt for the study based on their reputed differences in yield performance under normal and saline conditions.

2.2. Tolerance Indices

These indices were calculated as mentioned in previous studies as follow; stress susceptibility index (SSI) [11], mean productivity (MP) [12], stress tolerance index (STI) and geometric mean productivity (GMP) [13], yield index (YI) [14], yield stability index (YSI) [15] and tolerance (TOL) [16].

2.3. DNA Extraction

Total genomic DNA was extracted from fresh young leaves at tillering stage from 100 - 150 mg by using Cetyl trimethyl ammonium bromide (CTAB)-based procedure for plants [17]. Quantity and quality of the extracted DNA samples were estimated by comparing band intensities against standard DNA ladder on 1.0% agarose gel. DNA samples were diluted to a final concentration of 40 ng/µl before PCR amplification.

2.4. Primer Selection

A preliminary experiment on 10 selected barley genotypes was carried out to se-

Table 1. Name and sequence of seven SSR primers used in this study.

Primer name	Forward (5'→3')	Reverse (5'→3')
SSR-1	CCGTCGCCGTAGTAGAGAAG	TCCCGGTTATTTTAAGGCG
SSR-2	CTCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA
SSR-3	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC
SSR-4	GAGCTCCATCAGCCATTCAG	CTGAGTGCTGCTGCGACT
SSR-5	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC
SSR-6	GATCCCCTCCGTCAAACAC	CCCTTCTCCTTTCCTCAACC
SSR-7	CTCTTCACTCACTCACCATGG	ATCCATCTGGAGCAAGCAAC

lect the most suitable primer types for identification of genetic diversity. Three SCoT primers (were used in different combinations as single, double and triple) and seven SSR primers were screened for repeatability, scorability, and their ability to distinguish the different genotypes. These primers were employed for genotypic identification and phylogenetic analysis for studying the relationships.

2.5. Amplification Conditions and Fragment Analysis

2.5.1. SCoT Analysis

DNA samples were subjected to SCoT assay, using 3 (14 - 19 mer) primers and primer combinations (iNtRON Biotechnology, Inc, Korea) with GC content of 60% (**Table 3**). Amplification reactions were performed in 20 µl volume containing 1 µl of template DNA (40 ng/µl), 1.0 µl of primer (10 pmol/ µl), 10 µl 2X PCR Master mix solution [(i-Taq^{TM}) iNtRON Biotechnology] and 8 µl of sterile ddH2O. The reaction mixtures were overlaid with 20 µl of mineral oil per sample. The PCR amplification was performed on a thermal cycler (Perkin Emer Cetus) programmed for 5 min. at 94˚C followed by 35 cycles of 1 min. at 94˚C, 1 min. at 50˚C and 1.5 min. at 72˚C and a final stage of 7 min. at 72˚C (one cycle) then at 4˚C for keeping until use.

2.5.2. Microsatellite (SSR) Analysis

The flanking regions of the microsatellite, alternatively called simple sequence repeats, are generally conserved among genotypes of the same species. Therefore, PCR primers to the flanking regions are used to amplify SSR-containing DNA fragments. A total of 7 microsatellite specific primer pairs (SSR-1 to SSR-7) (iNtRON Biotechnology, Inc, Korea) (**Table 1**) were tested. PCR reaction was performed as previously described [18].

2.6. Detection of PCR Products

Amplification products were separated by horizontal gel electrophoresis unit using 1.5% agarose gel. Bands were detected on Benchtop UV-transilliminator and photographed using photo Doc-It^{TM} imaging system. The molecular size of the amplified products was determined against O'GeneRuler DNA Ladder Mix ready-to-use (Thermo Scientific).

2.7. Data Analysis

DNA banding patterns generated from SCoT and SSR techniques were analyzed by GelAnalyzer 3 program. The presence (1) or absence (0) of bands was scored. By comparing the banding patterns of genotypes (cultivars) for a specific primer, genotype-specific bands (positive or negative unique bands) were identified. The binary data generated were used to estimate the levels of polymorphism which calculated by dividing the polymorphic bands/the total number of scored bands. From this matrix, Jacard's similarity coefficient between paris of genotypes was calculated using Nei & Li coefficients [19] by computational package MVSP 3.1. The distance coefficients were used to construct a dendrogram using the unweighted pair grouped method arithmetic average (UPGMA) to determine the

genetic diversity and relationships among the used genotypes.

3. Results

3.1. Salinity Susceptibility Index (SSI)

A susceptibility index (SI), which provides a measure of stress tolerance based on minimization of yield loss under stress as compared to optimum conditions, rather than on yield level under stress, has been used to characterize the relative tolerance of wheat genotypes [11]. This index was used to estimate the relative stress loss because it accounted for variation in yield potential and stress intensity. This index could be estimated based on many traits. Lower stress susceptibility index than unity (SI < 1) is synonymous to high stress tolerance, while higher stress susceptibility index (SI > 1) means higher stress sensitivity. Data in Table 2 indicated that, Giza 123, Line-1, Line-5, Line-6 and Line-8 genotypes were considered as tolerant genotypes based on most tolerance indices for possessed high values for MP, STI, GMP, YI and YSI and also SSI was less than one, in addition to low values of TOL, revealing that these parents were more tolerant to salinity stress.

To assess the genetic variability and relationships among the 10 barley genotypes, 14 different primers and primers combinations were used; they revealed polymorphic patterns which will be used in further analysis.

3.2. Marker Informativeness

3.2.1. Polymorphism as Detected by SCoT Analysis

In the present study, three SCoT primers (SCoT-7, SCoT-8 and SCoT-9) in single, double and triple combinations as shown in Table 3 were used to amplify the ten barley genotypes. These primers yielded a total of 94 bands (38, 38 and 18) in the three marker systems (single, double and triple-SCoT, respectively),

Table 2. Tolerance indices for grain yield characters of the ten barley genotypes as the average of the two seasons under stress conditions.

Genotypes	SSI	MP	STI	GMP	YI	YSI	TOL
Giza 123	0.83	1885.59	1012.42	1875.28	1133.34	0.81	393.82
Giza 132	0.91	1672.44	897.98	1661.26	992.87	0.79	386.12
Line-1	0.96	1735.09	931.62	1721.79	1020.59	0.78	428.82
Line-2	1.04	1767.85	949.21	1751.80	1026.98	0.76	475.30
Line-3	1.32	1777.46	954.37	1749.23	981.16	0.70	631.08
Line-4	0.97	1640.94	881.07	1628.27	964.68	0.78	407.12
Line-5	0.37	1692.60	908.80	1690.94	1085.70	0.92	149.80
Line-6	0.70	1910.09	1025.58	1902.91	1170.93	0.84	330.82
Line-7	1.60	1674.75	899.22	1632.74	873.83	0.64	745.50
Line-8	0.72	1938.09	1040.61	1930.41	1185.02	0.84	344.82

SSI = Salinity susceptibility index, **MP** = mean productivity, **STI** = stress tolerance index, **GMP** = geometric mean productivity, **YI** = yield index, **YSI** = yield stability index, **TOL** = tolerance.

out of them 79 (84.04%) were found to be polymorphic with an average of 11.29 bands/primer combination as shown in Table 3 and Figure 1. While, the total percentages of polymorphism were 73.68, 89.47 and 94.44% in the three SCoT

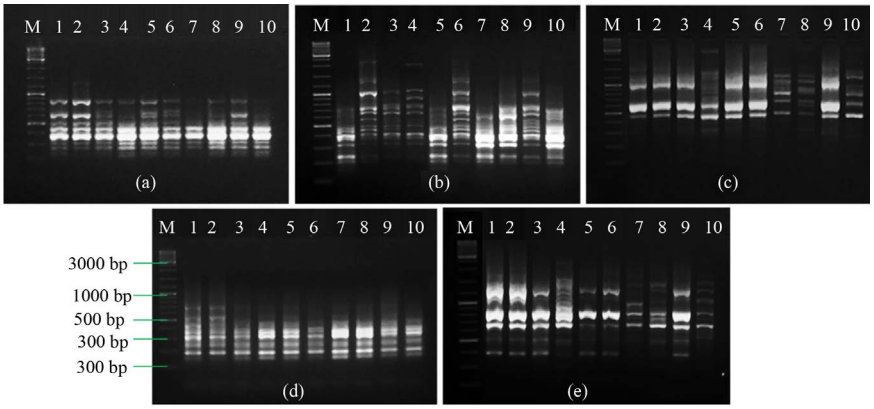

Figure 1. SCoT fingerprints of ten barley genotypes generated by (a) SCoT-8, (b) SCoT-9, (c) Double SCoT (SCoT-7 + SCoT-9), (d) Double SCoT (SCoT-8 + SCoT-9) and (e) Triple-SCoT (SCoT-7 + SCoT-8 + SCoT-9). M is 100 bp DNA Ladder, 1: Giza 123, 2: Giza 132, 3: Line-1, 4: Line-2, 5: Line-3, 6: Line-4, 7: Line-5, 8: Line-6, 9: Line-7 and 10: Line-8.

Table 3. Primer name, sequence, molecular size range, bands number and percentage of polymorphism as detected by SCoT markers.

Molecular marker technique	Primer Name	Primer sequence (5′→ 3′)	Molecular size range (bp)	TAB	PB	P (%)	TSM	
							No.	Genotype
Single-SCoT	SCoT-7	ACAATGGCTACCACTGAC	275-1346	9	5	55.56	3	**Giza 123 (1) Giza 132 (2)**
	SCoT-8	ACAATGGCTACCACTGAG	233-1279	10	5	50.00	1	**Giza 132**
	SCoT-9	ACAATGGCTACCACTGCC	164-2350	19	18	94.74	4	**Giza 132 (1) Line-3 (1) Line-7 (2)**
Total				**38**	**28**	**73.68**	**8**	
Double-SCoT	SCoT-7 + SCoT-8	ACAATGGCTACCACTGAC ACAATGGCTACCACTGAG	223-787	9	9	100.00	6	**Giza 132 (5) Line-7 (1)**
	SCoT-7 + SCoT-9	ACAATGGCTACCACTGAC ACAATGGCTACCACTGCC	259-3004	20	20	100.00	6	**Line-2 (2) Line-5 (1) Line-6 (1) Line-7 (2)**
	SCoT-8 + SCoT-9	ACAATGGCTACCACTGAG ACAATGGCTACCACTGCC	159-720	9	5	55.56	1	**Line-4**
Total				**38**	**34**	**89.47**	**13**	
Triple-SCoT	SCoT-7 + SCoT-8 + SCoT-9	ACAATGGCTACCACTGAC ACAATGGCTACCACTGAG ACAATGGCTACCACTGCC	431-3043	**18**	**17**	**94.44**	**8**	**Line-2 (3) Line-5 (1) Line-8 (4)**
Grand total				**94**	**79**		**29**	

TAB = Total number of amplified bands, **PB** = number of polymorphic bands, **P (%)** = polymorphism percentage and **TSM** = Total no. of genotype-specific markers including presence or absence of a band in a specific genotype.

systems (single, double and triple-SCoT, respectively) indicating that Triple-SCoT showed the highest percentage of polymorphism (94.44%).

From the results summarized in Table 3, it was noticed that Triple-SCoT technique proved to be the best one in comparison with the other two SCoT combination techniques (single and double-SCoT) while it gave the highest polymorphism percentage value (94.44%) and also the highest total genotype-specific markers (TSM) value (8) as presented in Table 3.

3.2.2. Polymorphism as Detected by SSR Analysis

Data in Table 4 and Figure 2 were obtained from 7 microsatellite primer pairs which were screened against the ten barley genotypes in an attempt to detect polymorphic markers. The seven SSR primer pairs exhibited 55 major SSR alleles and the number of polymorphic alleles was 50, representing a polymorphism percentage of 90.91%. The total number of alleles per primer ranged from six (SSR-2, SSR-5 and SSR-7) to eleven (SSR-4 and SSR-6). The number of polymorphic alleles generated by individual primer pair ranged from six to ten. The average of total alleles per primer was 7.86, while it was 7.14 for polymorphic al leles. Also, the seven SSR primer pairs exhibited 28 loci, ranged from 2 in SSR-7

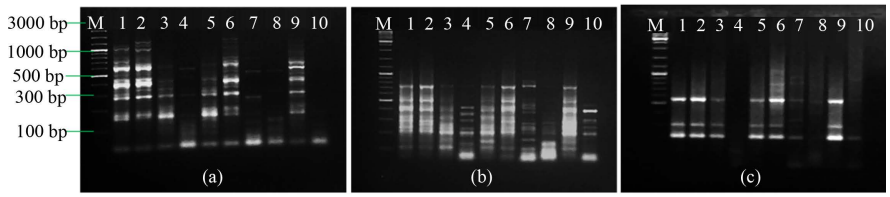

Figure 2. SSR fingerprints of the ten barley genotypes generated by (a) SSR-4, (b) SSR-6 and (c) SSR-7. M is 100 bp DNA Ladder, 1: Giza 123, 2: Giza 132, 3: Line-1, 4: Line-2, 5: Line-3, 6: Line-4, 7: Line-5, 8: Line-6, 9: Line-7 and 10: Line-8.

Table 4. Total number of allels, polymorphic allels, percentage of polymorphism, loci number and size detected by SSR markers.

Primer Name	Repeat motif	Number of alleles		P (%)	No. of loci	Size of loci (bp)	TSM	
		T	P				No.	Genotype
SSR-1	(GA)17	8	7	87.5	4	82 & 346 & 513 & 791	0	--
SSR-2	(CT)17	6	6	100	4	152 & 231 & 366 & 537	0	--
SSR-3	(CA)6(GA)36	7	6	85.7	4	145 & 258 & 576 & 889	2	**Line-8**
SSR-4	(CT)20	11	9	81.82	6	87 & 215 & 356 & 457 & 743 & 1322	1	**Line-8**
SSR-5	C9(CT)8	6	6	100	3	232 & 462 & 1151	3	**Giza 132**
SSR-6	(GAT)8	11	10	90.91	5	140 & 219 & 308 & 482 & 737	4	**Line-5 (1)** **Line-6 (2)** **Line-8 (1)**
SSR-7	(TC)15	6	6	100	2	277 & 1034	2	**Line-4 (1)** **Line-5 (1)**
Total		**55**	**50**	**90.91**	**28**		**12**	
Average		**7.86**	**7.14**				**1.7**	

T = Total number of allels, **P** = number of polymorphic allels, **P (%)** = polymorphism percentage and **TSM** = Total no. of genotype-specific markers including presence or absence of a band in a specific genotype.

to 6 in SSR-4. The total number of genotype-specific markers per primer ranged from 0 to 4.

From the above mentioned results, it was of great interest to make comparison between Triple-SCoT technique and the specific powerful technique (SSR) to estimate the phylogenetic relationships among the used barley genotypes.

3.3. Suitability of SCoT and SSR Markers in Barcoding and Identification of Genotype-Specific Markers

The randomly primer PCR approach beside specific primers (SSR) facilitated molecular distinction of barley genotypes as well as provided some genotype-specific markers too. The average number of amplified fragments and genotype specific markers are presented in Table 5. As presented in Figure 3 eight genotype-specific markers were amplified using Triple-SCoT marker (3) positives in Line-2, one positive in Line-5 and 3 positives and one negative in Line-8 genotypes), the highest number (4) was recorded by Line-8 genotype (Figure 3). On the other hand, the seven SSR primers scored 55 amplified fragments, out of them 12 fragments were genotype-specific markers with an average of 1.71 fragments per primer (Table 4). Data of DNA barcoding shown in Figure 3 showed also that the highest number of genotype-specific markers (4) (one positive marker by SSR-3 and 3 negatives recorded by SSR-3, SSR-4 and SSR-6) were also recorded by Line-8 genotype which generated the highest number of genotype-specific markers in the Triple-SCoT analysis.

3.4. Phylogenetic Relationships among Ten Barley Genotype

In Triple-SCoT analysis the highest genetic similarity value (1.0) was recorded

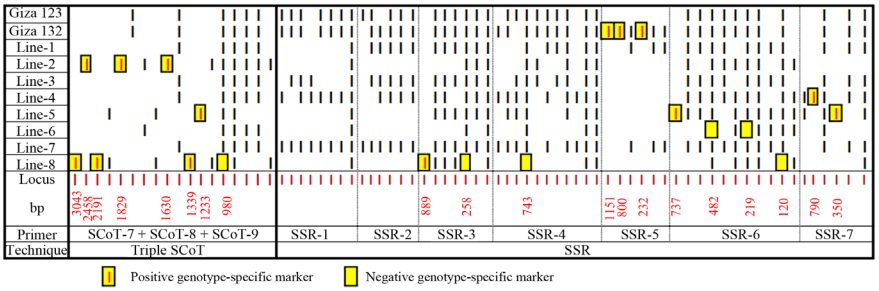

Figure 3. DNA-barcoding representation of Triple-SCoT and SSR fingerprints of the ten barley genotypes according to Adhikari *et al.* (2015).

Table 5. Distribution of amplified fragments in the ten barley genotypes using Triple-SCoT and SSR markers.

Parameters	Triple-SCoT	SSR
Total number of amplified fragments	18	55
Average No. of polymorphic fragments per primer	17	7.14
Percentage of total polymorphic loci	94.44	90.91
Size range of amplified fragments (bp)	431-3043	82-1322
Average No. of genotype-specific markers per primer	8	1.71

between Line-1 and each of lines-3, 4 and 7, Line-3 and each of lines 4 and 7 and between Line-4 and Line-7 (Table 6). While, the lowest genetic similarity value (0.143) was detected between Giza 132 and Line-8 genotypes.

Also, genetic similarity was calculated for the seven SSR primers as presented in Table 7. The maximum value of similarity (0.886) was observed between Giza 132 and Line-7 and the minimum similarity value (0.357) was recorded between Giza 132 and Line-6 genotypes.

The dendrogram generated by UPGMA cluster analysis of the data produced according to Triple-SCoT analysis (Figure 4) presented two main clusters divided into four groups, the first cluster contained the two genotypes Line-5 and Line-8 (group I), while, in the second cluster Giza 123 and Giza 132 were located at the same distance. Also, Line-1, Line-3, Line-4 and Line-7 were located at the same distance and they showed the highest genetic similarity (100%) as shown above in Table 6.

Table 6. Jaccard's similarity matrix of the ten barley genotypes according to Triple-SCoT analysis.

Genotypes	Giza 123	Giza 132	Line-1	Line-2	Line-3	Line-4	Line-5	Line-6	Line-7	Line-8
Giza 123	1									
Giza 132	1	1								
Line-1	0.909	0.909	1							
Line-2	0.5	0.5	0.533	1						
Line-3	0.909	0.909	1	0.533	1					
Line-4	0.909	0.909	1	0.533	1	1				
Line-5	0.364	0.364	0.4	0.267	0.4	0.4	1			
Line-6	0.727	0.727	0.8	0.667	0.8	0.8	0.4	1		
Line-7	0.909	0.909	1	0.533	1	1	0.4	0.8	1	
Line-8	0.143	0.143	0.154	0.333	0.154	0.154	0.462	0.154	0.154	1

Table 7. Jaccard's similarity matrix of the barley genotypes according to SSR analysis.

Genotypes	Giza 123	Giza 132	Line-1	Line-2	Line-3	Line-4	Line-5	Line-6	Line-7	Line-8
Giza 123	1									
Giza 132	0.878	1								
Line-1	0.771	0.816	1							
Line-2	0.582	0.525	0.571	1						
Line-3	0.845	0.779	0.862	0.600	1					
Line-4	0.831	0.795	0.704	0.571	0.750	1				
Line-5	0.677	0.592	0.678	0.682	0.700	0.697	1			
Line-6	0.400	0.357	0.455	0.690	0.444	0.431	0.615	1		
Line-7	0.878	0.886	0.816	0.492	0.805	0.867	0.648	0.393	1	
Line-8	0.462	0.414	0.478	0.581	0.468	0.453	0.634	0.538	0.414	1

On the other side, a dendrogram derived from UPGMA cluster analysis based on SSR data showed that cluster analysis separated the genotypes into two clusters at 0.497 similarities (Figure 5). The first group comprised three genotypes only (Line-2, Line-6 and Line-8) while the other seven genotypes formed the second cluster. As the results of Triple-SCoT analysis, Line-7, Line-4, Line-1, Line-3, Giza 132 and Giza 123 were the nearest similar genotypes and located together.

4. Discussion

Genotypes with low SSI values (less than 1) can be considered to be salinity tolerant [20], because they exhibited smaller yield reduction under stress compared with normal condition than the means of all genotypes. However, the low SSI values may not necessarily give a good indication of salinity tolerance of genotype. Low SSI estimates of a variety could be as a result of lack of yield production under normal conditions rather than an indication of its ability to tolerate salinity.

DNA fingerprinting is an important tool for characterization of germplasm and establishment of the identity of varieties/hybrids/parental sources in breeding program. SCoT primers, despite having certain disadvantages (dominant nature and stringent optimization of assay), can produce multilocus profiles

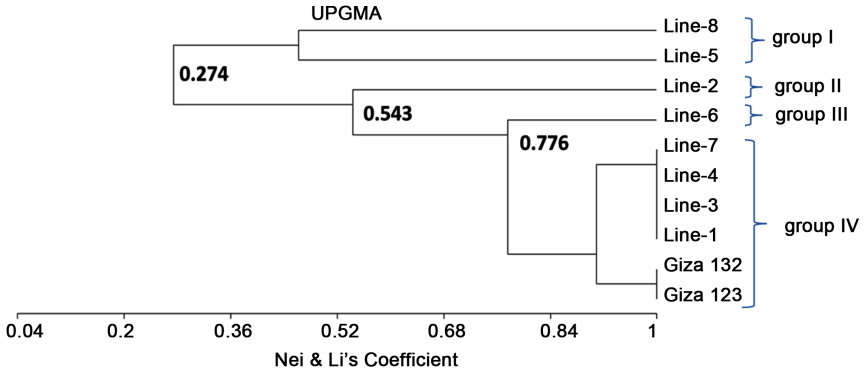

Figure 4. Hierarchical cluster analysis of the ten barley genortpes based on variations of Triple-SCoT patterns.

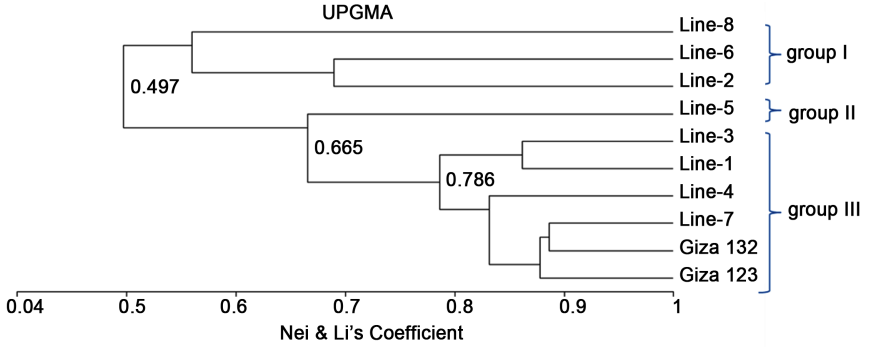

Figure 5. Hierarchical cluster analysis of the ten barley genortpes based on variations of SSR patterns.

widely scanning the genome even in the absence of any prior genetic/sequence information. Therefore, in the present investigation, we employed SCoT marker with different combinations beside SSR marker to evaluate ten barley genotypes, to determine the molecular relationships among them, and to develop a fingerprinting key (molecular barcode) for commercial varieties and produced lines.

4.1. Evaluation of SCoT and SSR Markers

The 14 primer and primer combinations evaluated in the ten barley genotypes revealed high levels of diversity, detecting a total of 94 and 55 amplified fragments, 79 and 50 of them were polymorphic, averaging 11.29 and 7.14 polymorphic amplified fragments per primer for SCoT and SSR analyses, respectively.

The percentage of polymorphic bands was higher for Triple-SCoT (94.44%) than for SSR (90.91%). The average number of polymorphic amplification products obtained with SSR (7.14) was fewer than that of Triple-SCoT (17) as shown in **Table 5**. Therefore, both Triple-SCoT and SSR markers were the most efficient marker systems depending on their capacity to reveal several informative bands in a single amplification.

In the present study, di and tri nucleotide SSR motifs CT, TC, GA and GAT were used. Out of these, CT and GAT motifs produced the maximum scorable loci (**Table 4**), thus revealing more coverage of genome indicating that the microsatellites content of repetitive motifs $(CT)_n$ and (GAT)n are more frequent than the repetitive motif targeted by the other SSR primers in barley. Also, SSR motifs CT and TC showed the maximum percentage of polymorphism (100%). The above observations were in conformity with previous studies on Swertia chirayita [21] and on Gerbera [22].

4.2. Relationships among Barley Genotypes

Although major emphasis of this work was to generate DNA profiles of the genotypes, the marker data were also used to study genetic relationships among barley genotypes. The ten genotypes were divided into two clusters based on each of Triple-SCoT and SSR markers. The dendrogram showed high similarity between the trees results from Triple-SCoT and SSR primers. Line-8 genotype proved to be the best one, while it scored the highest number (4) of genotype-specific markers in both Triple-SCoT and SSR primers. This genotype (Line-8) with Giza 123 genotype proved to be the best barley tolerant genotypes as indicated by field experiment. In order to establish genetic relationships between the salinity tolerant genotypes, genetic similarity was calculated using Jaccard's similarity coefficient, which ranged from 14.3% to 100% and 35.7% to 88.6% for Triple-SCoT and SSR, respectively. These values were lower than those previously reported on the basis of SSRs (60.5 to 93.9%) between Egyptian and ICARDA barley genotypes [18]. This is probably because of unequal distribution of SSR motifs throughout the genome in the form of clusters could not represent the entire genome well [23]. In the two commercial varieties (Giza 123 and Giza

132), higher genetic similarity was observed among the two cultivars, indicating that both barley genotypes are closely related to each other and sharing the common genetic pool. These results agree with Triple-SCoT similarity. These results confirmed the result obtained by SSR analysis published in previous study [24], indicating the wide genetic diversity among them.

The higher genotypic variation observed in the studied barley genotypes might be due to the considerable chemotypic (largely quantitative) diversity [25] [26]. Similar anomalies in cluster analysis were also observed [18] who examined genomic and expressed molecular diversity of barley genotypes through SCoT and SSR analysis. Thus, phylogenetic studies revealed tight groupings among the barley genotypes with some incongruities in position of genotypes. This inconsistency between the molecular and chemotypic variation observed among the cultivars in the present study suggests that genotype and environment interactions also led to the diversification of chemical constituents, rather than genotypic differences [27] [28].

The differences found among the dendrograms generated by Triple-SCoT and SSRs could be partially explained by the different number of PCR products analysed reinforcing again the importance of the number of loci and their coverage of the overall genome, in obtaining reliable estimates of genetic relationships among barley cultivars. Similar results were observed also in barley [4] [29].

4.3. Suitability of SCoT and SSR Markers in Barcoding

The most important features of a DNA barcode are its universality, specificity on variation and easiness on employment. This means that the gene segment used as barcode should have high variation between species, but should be conserved within the species so that the intraspecific variation will be insignificant [30] [31]. In this respect, SCoT marker was easy to apply, rapid, cost-not effective, reproducible and highly discriminating. In this report, we have successfully developed genotype-specific molecular markers, which might contain some important gene sequences that could be used for the development of molecular barcodes for exploitation of genetic resources.

With increasing ease and speed of DNA sequencing alongside decreasing costs, DNA barcoding will facilitate rapid and large-scale biodiversity surveys, both for inventory purposes and ecological studies. These could be performed without presorting of samples or the necessity for taxonomists to devote their time to highly repetitive identification rather than additional scientific research.

5. Conclusion

To our best knowledge, this is the first report of detecting molecular variations and relationships of barley genotypes using Triple-SCoT markers. This study demonstrates that these markers are powerful tool for generating fingerprinting keys and have the potential to identify genotype-specific markers for barley. The identification of genotype-specific markers and the generation of fingerprinting keys for salinity tolerance are important resources for the breeding and man-

agement of barley germplasm in comparison with the traditional breeding which needs long time and more cost.

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Identification and Characterization of Reverse Transcriptase Fragments of Long Interspersed Nuclear Elements (LINEs) in the *Morus notabilis* Genome

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Abstract

Reverse transcriptase (*rt*) fragments from LINE retrotransposons in the mulberry genome were analyzed in terms of heterogeneity, phylogeny, and chromosomal distribution. We amplified and characterized conserved domains of the *rt* using degenerate primer pairs. Sequence analyses indicated that the *rt* fragments were highly heterogeneous and rich in A/T bases. The sequence identity ranged from 31.8% to 99.4%. Based on sequence similarities, the *rt* fragments were categorized into eight groups. Furthermore, similar stop codon distribution patterns among a series of clones in the same group indicated that they underwent a similar evolutionary process. Interestingly, phylogenetic analyses of the *rt* fragments isolated from mulberry and 13 other plant species revealed that two distantly related taxa (mulberry and *Paeonia suffruticosa*) grouped together. It does not appear that this phenomenon resulted from horizontal transposable element transfer. Fluorescence *in situ* hybridization analysis revealed that most of the *rt* fragments were concentrated in the subtelomeric and pericentromeric regions of the mulberry chromosomes, but that these elements were not abundant in the mulberry genome. Future studies will focus on the potential roles of these elements in the subtelomeric and pericentromeric regions of the mulberry genome.

Keywords

Morus notabilis, LINE Retrotransposons, Reverse Transcriptase, Characterization, Fluorescence in Site Hybridization

1. Introduction

Transposable elements (TEs), which were first discovered in maize by Barbara

*These authors contribute equally to this work.

McClintock, are also known as "jumping genes" because of their ability to replicate and move to new genomic locations [1]. They are ubiquitous and abundant components of all eukaryotic genomes, and play important roles in the structural organization and evolution of genes and genomes [2]-[7]. Based on their mechanism of transposition, TEs are classified as retrotransposons (Class I) or DNA transposons (Class II) [8] [9] [10]. Class I retrotransposons move to new chromosomal locations *via* an RNA intermediate (*i.e.*, "copy and paste" mechanism). In contrast, Class II DNA transposons move *via* a DNA intermediate (*i.e.*, "cut and paste" mechanism) [6] [8]. Depending on whether or not they are flanked by long terminal repeats (LTRs), retrotransposons can be further classified as LTR or non-LTR retrotransposons [10]. Non-LTR retrotransposons are usually further divided into long or short interspersed nuclear elements (LINEs and SINEs, respectively) [10].

The LINE retrotransposons are ubiquitous, showing a great variation in structure and size [11] [12] [13]. A large body of knowledge on mammalian LINEs has been accumulated [14] [15]. In contrast, LINEs in plants have been poorly investigated. The first identified plant LINE retrotransposon was *Cin4* in *Zea mays*, which inactivates the *A1* gene following its insertion into the *A1* 3'-untranslated region [16]. Since that pioneer study, numerous other LINEs have been identified in taxa such as *Lilium speciosum* (*del2*) [17], *Arabidopsis thaliana* (*Tal 1-1*) [18], *Chlorella vulgaris* (*Zepp*) [19], *Hordeum vulgare* (*BLIN*) [20], *Oryza sativa* (*Karma*) [21], *Ipomoea batatas* (*LIb*) [22], *Beta vulgaris* (*BNR*) [13], and so on. Full-length LINEs have one or two open reading frames (ORFs) encoding proteins required for reverse transcription. The ORF1 sequence contains the *gag* gene, while genes for an endonuclease (en), reverse transcriptase (*rt*), and a cysteine-rich domain (Cys) encoding a putative RNA-binding motif are present in ORF2 [12] [23]. The *rt* is a key enzyme for retrotransposition and shares several conserved domains that are typical of retroviral RNA-directed DNA polymerases [24]. Previously studies have suggested that amplification of *rt* fragments using degenerate oligonucleotide primers complementary to the conserved domains of the *rt* is a feasible and efficient approach to evaluate the characterization of LINE retrotransposons in various plant species [18] [25] [26] [27] [28].

Morus (mulberry) is a representative genus of the cosmopolitan family Moraceae (Rosales), and comprises of more than 13 species (over 1000 cultivars), which are widely distributed in Asia, Africa, Europe, and the United States [29] [30]. Meanwhile, mulberry attracts people for its delicious fruit and rich source of medicines against certain serious diseases [31] [32]. The relationship between mulberry and silkworm is part of the best example of "plant defense-insect adaptation" [33] [34]. The mulberry species, *Morus notabilis*, has a relatively small genome (estimated to be 357 Mb), and cytogenetic data suggest that *M. notabilis* is composed of 14 chromosomes ($2n = 14$) [35]. The previous studies published from our lab are the only papers describing the presence of LINE retrotransposons in the mulberry genome [35] [36]. Detailed characterization of

LINE retrotransposable elements in mulberry has not been carried out so far.

In the present work, our objective was to characterize the diversity of *rt* fragments of LINE retrotransposons from the *M. notabilis* genome, which were amplified and cloned using degenerate primers. Meanwhile, this present work also attempted to characterize their heterogeneity and phylogenetic relationships. In addition, fluorescence *in situ* hybridization (FISH) was used to clarify the distribution of these elements within the chromosomes. These results will lead us to better understand the LINE retrotransposons roles on the structural, functional, and evolutionary dynamics of mulberry genomes.

2. Methods

2.1. Plant Materials and DNA Isolation

Young leaves of *M. notabilis* C.K.Schn (Taxonomy ID: 981085) (2n = 14) were obtained from mulberry trees growing in Ya'an, Sichuan Province, China. The collected young leaves were stored in liquid nitrogen until used. Total genomic DNA used as a template for the cloning was extracted from the young leaves using a standard cetyltrimethylammonium bromide (CTAB) protocol [37].

2.2. Polymerase Chain Reaction (PCR) and Cloning of Amplicons

The *rt* sequences of LINEs were amplified from the genomic DNA of mulberry using degenerate primers (forward: 5'-GGGATCCNGGNCCNGAYGGNWT-3'; reverse: 5'-SWNARNGGRTCNCCYTG-3') [18]. The primers were synthesized by BGI (Shenzhen, China). PCR reaction mixture contained 20 ng DNA, 10 pmol of each primer, 0.25 mM of each dNTPs (Takara, Japan), 10× PCR buffer (including 3.5 mM $MgCl_2$, Takara, Japan), and 1 U rTaq polymerase (Takara, Japan). PCR amplification was carried out in 96-well thermal cycler (Applied Biosystems, USA). The PCR program was: 94˚C for 5 min; 35 cycles at 94 °C for 1 min, 50˚C for 1 min, and 72˚C for 1 min; 72˚C for 7 min. PCR products were analyzed on 1.5% agarose gels and purified using the Agarose Gel DNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's instructions. Purified products were cloned into the pMD19-T vector (TaKaRa, Japan) following the manufacturer's instructions. Two independent rounds of PCR amplification and cloning were carried out for the elements. The positive clones were verified by PCR and sequenced in both directions using M13 universal primers at Sangon Biotech (Shanghai, China). Clones were named according to the following rules: Mno stands for the *Morus notabilis*, L means the type of the element (L for LINE), and the serial stands for the clone number from *Morus notabilis.*

2.3. Sequence Data and Phylogenetic Analysis

Cloned sequences were compared with the previously characterized plant retroelement sequences in the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/) and Genetic Information Research Institute (GIRI) (http://www.girinst.org/) databases using BLAST [38]. The nucleotide and protein sequences were aligned using MUSCLE (version 3.8.31) with

default parameters [39]. Sequence identities were calculated by BioEdit (version 7.2.5) with BLOSUM62 matrix [40]. The locations of stop codons in *rt* sequences were demonstrated using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) [41]. Nucleotide sequences of the isolated *rt* fragments and 29 LINE *rt* sequences from 13 other plant species (**Supplementary material 1**) were aligned by MUSCLE (version 3.8.31) with default parameters [39]. In order to perform phylogenetic analysis, MEGA6 was used to find the best-fit substitution models for those datasets with default parameters [42]. The best substitution model (Tamura 3-parameter + G, T92 + G) was used to construct a phylogenetic tree according to the maximum-likelihood method with the pairwise deletion in MEGA 6 [42] [43] [44]. Tree topology was assessed by bootstrap analysis with 1000 resampling replicates.

2.4. Chromosome Preparation and FISH

Mulberry chromosome spreads were prepared using young leaves treated with 2 mM 8-hydroxyquinoline in darkness for 3 h at room temperature (24˚C). Samples were fixed in a methanol/glacial acetic acid solution (v/v = 3:1) for 2 h at 4˚C, incubated in 1/15 M KCl for 30 min, and digested by an enzyme mixture (5% cellulose and 2.5% pectinase) at 37˚C for 3 h. After the cell walls were completely degraded, samples were spread onto slides. According to the manufacturer's instructions for the PCR DIG Probe Synthesis Kit (Roche), probes were labeled with digoxigenin-11-dUTP using PCR with degenerate primers. Fluorescence *in situ* hybridization was completed according to a modified procedure [45]. Briefly, the prepared chromosomes (on slides) were treated with 100 μg/ml RNase for 15 min at 37˚C, and then digested with 1 μg/ml proteinase K for 10 min at 37˚C. Samples were denatured with 70% (v/v) formamide for 10 min at 72˚C, and then immediately treated for 5 min with each of 70%, 90%, and 100% (v/v) anhydrous ethanol solutions precooled to −20˚C. The hybridization mixture, which consisted of 2× SSC, 0.25 μg salmon sperm DNA, 10% (w/v) SDS, 50% (w/v) DS, 50% (v/v) formamide, and 400 ng labeled DNA probe, was denatured for 6 min at 96˚C. The slides and hybridization mixture were incubated at 80˚C for 10 min and then maintained at 37˚C for 16 h. The slides were washed with 10% (v/v) formamide for 10 s, 2× SSC at 37˚C for 5 min (five times), and 0.2% (v/v) Tween-20 at room temperature for 5 min. Digoxigenin was detected using FITC-conjugated anti-digoxigenin antibody (Roche), and chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Slides were viewed using a Leica DM2500 fluorescence microscope (Leica, Germany). Images were captured using the CV-M4+CL progressive scan charge-coupled device camera (DM2500, Leica) and analyzed using CytoVision software (version 7.3.1).

3. Results

3.1. Identification of *rt* Fragments

The sequences of the expected 580 bp amplicons [18] were compared with se-

quences available in the NCBI and GIRI databases using BLAST [38]. In total, two independent rounds of PCR and cloning yielded 43 clones with homology to known retroelements in the NCBI and GIRI (http://www.girinst.org/) database. All these elements were selected for further analysis. All the clones are deposited in GenBank under accession numbers: KT900650–KT900692 (**Supplementary material 2**).

3.2. Characterization of LINE *rt* Sequences

Nucleotide sequences derived from the isolated *rt* fragments were aligned and used to construct a phylogenetic tree using the maximum-likelihood method in MEGA6. The identified *rt* sequences were classified into eight groups (**Figure 1**). Group I contained the most *rt* clones (41.8%, 18/43), followed by Group VI (30.2%, 13/43). These two groups accounted for 72.1% of the 43 clones and were further classified into several subfamilies. Additionally, sequences of clones from the same group were of almost the same length, and were highly similar (> 97%, except for Group II) (**Table 1** and **Supplementary material 2**). The length of isolated LINE *rt* fragments ranged from 557 bp (MnoL_11, MnoL_13, and MnoL_15) to 595 bp (MnoL_31), with an average of 579 bp. The AT/GC ratio

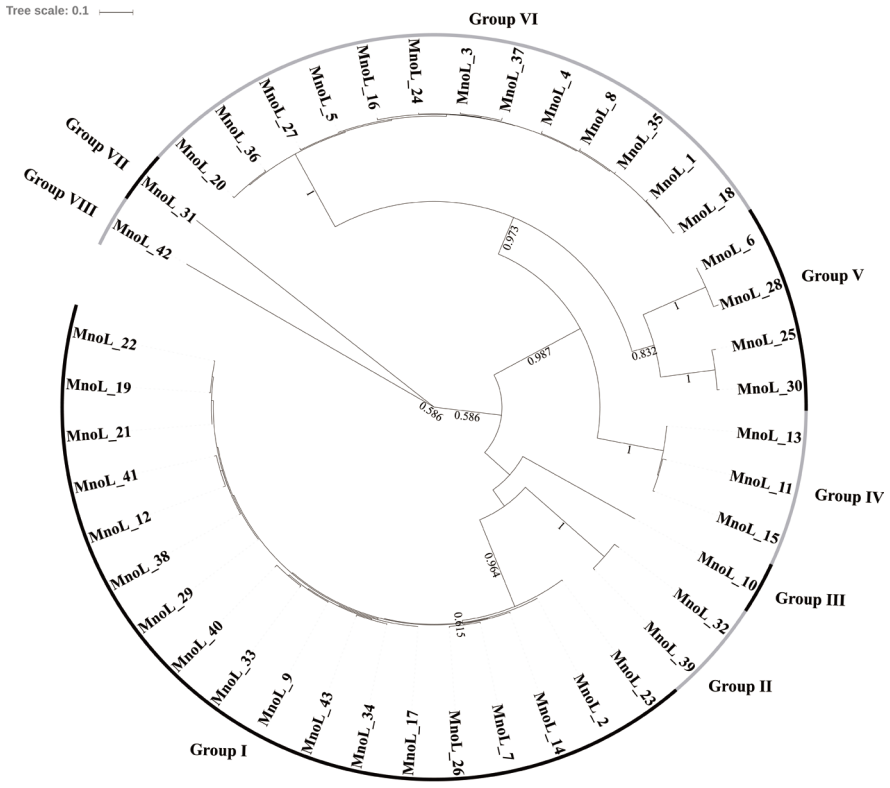

Figure 1. Phylogenetic analysis of reverse transcriptases from mulberry LINE retrotransposons. All cloned nucleotide sequences of reverse transcriptase fragments from mulberry were aligned by muscle (version 3.8.31) under default parameters. The best substitution model (Tamura 3-parameter + G), which was tested by MEGA6, was used to construct a phylogenetic tree based on a maximum-likelihood method with the pairwise deletion in MEGA6. Only more than 50% of the frequency of replicate (1000 replicates) trees were shown. The sequences were classified into eight groups: Group I to Group VIII.

ranged from 1.26 (MnoL_20 and MnoL_37) to 1.98 (MnoL_42), with an average of 1.37, which indicated the *rt* sequences are rich in AT (Table 1 and Supplementary material 2). Pairwise comparisons revealed that similarity among 43 *rt* nucleotide sequences ranged from 31.8% (MnoL_20 and MnoL_31, MnoL_31 and MnoL_36) to 99.4% (MnoL_17 and MnoL_29) (Supplementary material 3). These results suggest that the *rt* sequences isolated were highly heterogeneous.

The alignment of amino acid sequences of multiple isolated mulberry LINE *rt* rfragments evealed that all sequences contained several premature stop codons, with the exception of the MnoL_10 sequence (Figure 2). The MnoL_42 sequence had 16 premature stop codons, which is the most of any clone. Additionally, the premature stop codons were in similar locations in the sequences from the same group (Figure 2). Furthermore, all sequences carry frameshift mutation, except for the MnoL_10, MnoL_23, and MnoL_31 sequences (Supplementary material 4).

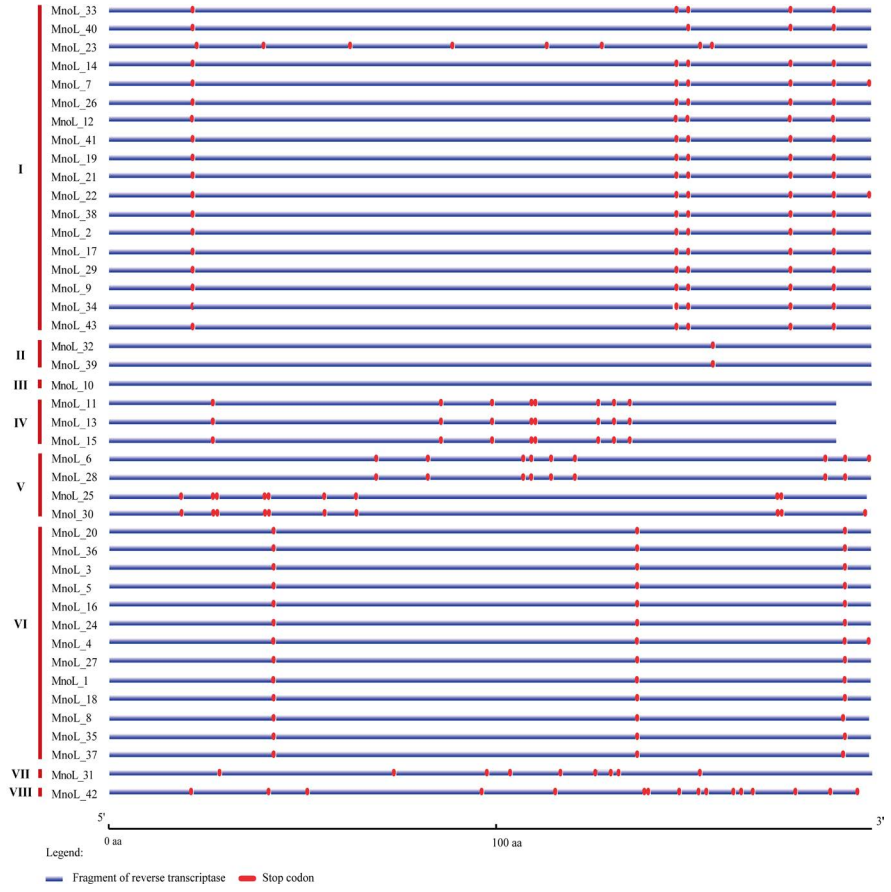

Figure 2. Premature stop codon positions in reverse transcriptase sequences from mulberry LINE retrotransposons. The locations of premature stop codons in *rt* sequences were demonstrated using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/). Each straight red line represents one of the eight groups of reverse transcriptase sequences (from top to bottom: Group I to Group VIII). The blue line means reverse transcriptase fragments. Red block appeared in the blue line means premature stop codons.

3.3. Phylogenetic Analysis of LINE *rt* Clones

Phylogenetic analyses indicated that the mulberry LINE *rt* sequences are homologous to *rt* sequences in other species (Figure 3). One interesting feature of the result is that PTLRT19 grouped with other mulberry *rt* sequences (Group V, VI, VII), instead of other PTLRT (PTLRT12, PTLRT4, and PTLRT14) from *Paeonia suffruticosa*. In fact, *P. suffruticosa* and *M. notabilis* are distantly related taxa. The phylogenetic between the LINE-*rt* sequences and the host species trees were incongruous (APG, The Angiosperm Phylogeny Group, http://www.theplantlist.org).

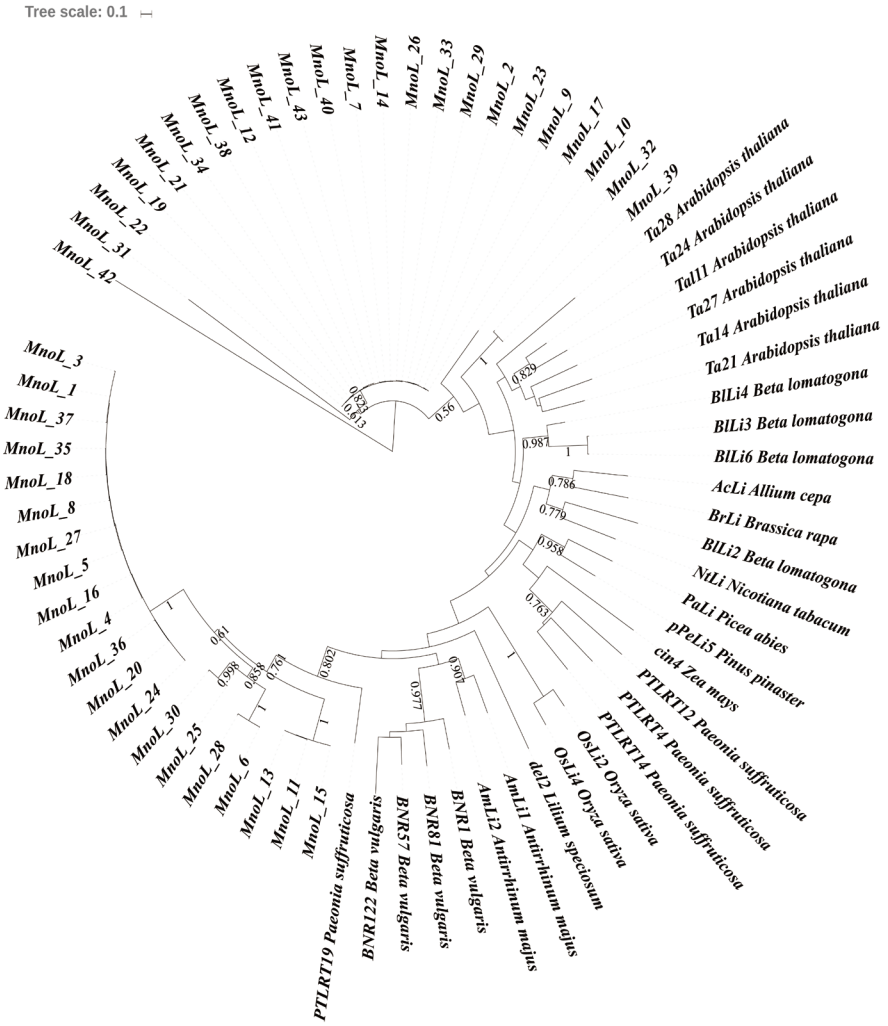

Figure 3. Phylogenetic analysis of reverse transcriptase fragments from mulberry and other thirteen plant species. All nucleotide sequences of reverse transcriptase fragments from mulberry and the representative members of other thirteen plant species were aligned by muscle (version 3.8.31) with default parameters. Firstly, MEGA6 was used to find the best-fit substitution models for those datasets with default parameters. The best substitution model (Tamura 3-parameter + G) was used to construct a phylogenetic tree, using the maximum-likelihood method with the pairwise deletion in MEGA6. Frequency (>50%) of replicate trees in which the associated taxa clustered together in the bootstraps test (1000 replicates) were shown. Detailed information of other thirteen plant species used in the present research was shown in supplementary material 1.

3.4. Distribution of LINEs in the Mulberry Genome

Fluorescence *in situ* hybridization (FISH) was performed to study the distribution of these sequences along mulberry chromosomes. Chromosomal localization of the LINEs elements was performed using a heterogeneous probe cocktail containing all isolated clones. FISH with such a cocktail revealed that hybridization signals were mainly concentrated in subtelomeric and pericentromeric regions (**Figure 4**).

4. Discussion

4.1. Characterization of LINEs

All cloned *rt* fragments could be classified into eight groups (**Figure 1**). Group I and VI consisted of the most *rt* clones (72.1%, 31/43). Only one clone (MnoL_42) contained in the Group VIII (**Figure 1**). The range of nucleotide sequence similarities between MnoL_42 and the other clones was only 32.8 to 37.2% (**Supplementary material 3**). Meanwhile, the number of premature stop codons in MnoL_42 was 16, which is much higher than in the other clones (**Supplementary material 2**). These results indicated that mutations accumulated progressively over evolutionary time. Combining these data with the phylogenetic analysis results (**Figure 1**) allows us to come to the conclusion that the MnoL_42 is an ancient LINE in mulberry.

The *rt* fragments amplified from the mulberry genome were highly heterogeneous. Almost all the 43 *rt* sequences described here contained frameshifts and premature stop codons (**Figure 2** and **Supplementary material 5**), which were the main causes of the observed heterogeneity. These results are consistent with those observed in other plants, including *Hordeum* species[20] and *Vicia* species [27]. Furthermore, the *rt* fragments from clones within the same group exhibited very few differences, suggesting that the heterogeneity among *rt* sequences is also the result of base substitutions, deletions, and insertions. As shown in **Figure 2**, similar stop codon distribution patterns among sequences of the *rt* fragments from the same group suggested that they went through a similar evolutionary process.

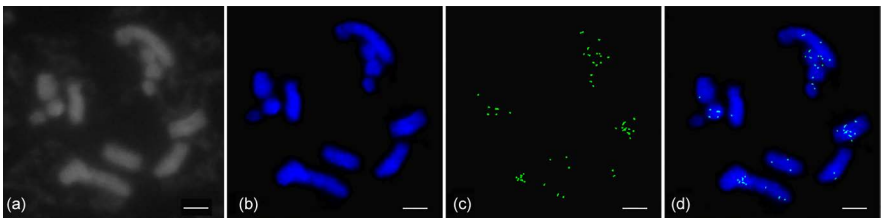

Figure 4. Chromosomal distribution of mulberry LINE retrotransposons by fluorescence *in situ* hybridization experiment. Images were captured using the CV-M4+CL progressive scan charge-coupled device camera (DM2500, Leica) and CytoVision software (version 7.3.1). (a) Cytological detection of mulberry chromosome; (b) blue fluorescence signals correspond to 4'-6-diamidino-2-phenylindole-stained DNA; (c) green fluorescence signals correspond to hybridization sites of reverse transcriptase probes; (d) the overlaid images of (b) and (c). Scale bar: 5 μm.

As indicated in Table 1 and Supplementary material 2, the AT/GC ratio ranged from 1.26 to 1.98, with an average of 1.37. The results suggested that the *rt* sequences are rich in AT bases, which is important for the LINE copy and paste replication mechanism [12] [46]. An intact LINE element contains two open reading frames, ORF1 and ORF2. The ORF2 contains reverse transcriptase, which is a critical enzyme responsible for the replication process of LINE [12] [23]. One of the critical steps in the life cycle of LINE is that the ORF2 protein cleaves the first one DNA strand at the target. Due to the fact that the target sequence in this site is always rich, AT bases and the target site are usually similar to consensus TTAAAA [47] [48]; the AT bases content is high in the *rt* sequences to ensure that the target sites can be identified efficiently in the replication process of LINE.

Interestingly, there were no frameshifts or premature stop codons in MnoL_10 (Figure 2 and Supplementary material 5). All *rt* fragments from mulberry, with the exception of clone MonL_10, represented potential pseudogenes (possessed stop codons or frameshifts). It would be worthwhile carrying out further research on MonL_10, which may be a potential active transposable element.

4.2. Phylogenetic Analysis of LINEs

Interestingly, we found a phenomenon that PTLRT19 grouped with other mulberry *rt* sequences (Group V, VI, and VII), instead of other PTLRT (PTLRT12, PTLRT4, and PTLRT14) from *Paeonia suffruticosa* (Figure 3). While the two species (*P. suffruticosa* and *M. notabilis*) are distantly related taxa according to the APG (The Angiosperm Phylogeny Group, http://www.theplantlist.org). Similarity phenomenon had been found in other studies. For example, twenty-six genomes harbor at least one case of horizontal TE transfer (HTT), which may be important in TE-driven genome evolution, and these HTTs involve species as distantly related as palm and grapevine, tomato and bean, poplar and peach, and so on [49].

Table 1. Length, AT/GC ratio, and similarity [range (average)] of reverse transcriptase fragments from mulberry LINE retrotransposons.

	Number	Length (bp)	AT/GC	Identity (%)
LINE	43	557 - 595 (579)	1.26 - 1.98 (1.37)	31.8 - 99.4 (61.3)
Group I	18	583 - 584 (584)	1.35 - 1.40 (1.38)	97.4 - 99.4 (98.6)
Group II	2	587 - 587 (587)	1.28 - 1.28 (1.28)	97.9
Group III	1	586	1.46	-
Group IV	3	557 - 557 (557)	1.34 - 1.36 (1.35)	98.5 - 99.1 (98.7)
Group V	4	576 - 579 (578)	1.36 - 1.51 (1.43)	66.7 - 98.6 (77.5)
Group VI	13	575 - 575 (575)	1.26 - 1.32 (1.29)	97.7 - 99.1 (98.6)
Group VII	1	595	1.32	-
Group VIII	1	586	1.46	-

It is hypothesized that HTT may be the reason for this phenomenon observed in our research. Further analysis was performed in this work. Accordingly, three criteria have been defined for the detection of HTTs: (i) patchy distribution of the TEs in phylogenies, (ii) high sequence similarity of the TE between distantly related taxa, and (iii) phylogenetic incongruence between the TE and host species trees [50] [51] [52]. In the present work, although there is phylogenetic incongruence between the TE and trees of the two species, the range of nucleotide sequence similarities between PTLRT19 and mulberry *rt* sequences (Group V, VI, and VII) is only 0.520 to 0.562 (**Supplementary material 4**). These results suggest that the conclusion for PTLRT19 is that it is uncertain whether it represents a horizontal transfer event.

4.3. Chromosomal Localization of LINE Retrotransposons

The LINE distribution patterns are associated with LINE functions. For example, FISH experiments in *Cannabis sativa* suggested that differential accumulation of LINE retrotransposon elements onto the Y chromosome leads to sex chromosome heteromorphism [53]. Although the chromosomal distribution of LINEs has been analyzed in only a few plant species, the FISH results here revealed that the distribution of LINEs in mulberry chromosomes was similar to that in sugar beet and peanut chromosomes [28] [54]. Furthermore, the weak hybridization signals observed in this study indicated that LINEs were not abundant in the mulberry genome (**Figure 4**). Most of the hybridization signals were concentrated in subtelomeric and pericentromeric regions. The subtelomeric and pericentromeric regions are generally considered to correspond to the constitutively heterochromatic region. In fact, there is extensive DNA methylation in these regions [55]. Thus, we hypothesized that the tendency for LINEs to insert into these regions may be related to DNA methylation in mulberry.

Meanwhile, recent reports suggest that LINE insertion into promoters can influence promoter functionality and gene regulation, resulting in up or down regulation of reporter genes [56]. Insertion of a LINE into a gene can induce alternative splicing or change gene expression patterns, which can result in a change in the function of the gene [57]. Although we currently have no evidence that the mulberry LINEs described here are active and functional, previous studies have indicated that some LINEs are active and functional in other species [58]. So, our future studies will attempt to characterize the functions of mulberry LINEs more comprehensively, considering their localization in subtelomeric and pericentromeric regions.

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Conflict of Interest

All authors declare that there is no conflict of interest.

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