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Full Length Research Paper

Genetic variation of white clover (*Trifolium repens* L.) collections from China detected by morphological traits, RAPD and SSR

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White clover is an important legume naturalized in China. But genetic variation of Chinese local white clover germplasm has not been reported. There is no information for parents' selection and genetic resource conservation. The objective of this study was to investigate and characterize genetic variation of ten local white clover germplasms collected from different geographical regions of China. Morphological data analysis of white clover populations was coupled with molecular markers of random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) to investigate the genetic relationships among white clover germplasms of China, and four commercial cultivars were included for a comparison. The results revealed that the populations showed diverse morphological traits, RAPD and SSR patterns. Despite the certain similarities among the dendrograms of particular populations, the overall correlations among the distance matrices appeared to be rather low. In the present study, cluster analysis only slightly reflected the geographical origins of populations, and the dendrogram employing SSR data showed the closest agreement with collections' geographical origins. The analysis of molecular variance (AMOVA) analysis based on both RAPD and SSR markers revealed higher levels of intra-population variation than inter-population variation. Neither RAPD nor SSR based grouping showed obvious agreement with variation of leaf sizes and other morphological traits. Our results suggest that the combination of morphological traits and SSR analysis could be a useful method for assessing genetic variation in white clover, and could be helpful for breeders to plan crosses for positive traits.

Key words: White clover, *Trifolium repens*.L, morphological traits, RAPD, SSR.

INTRODUCTION

White clover (*Trifolium repens* L.) is an important legume in temperate climates (Treuren et al., 2005), and is a natural tetraploid with a chromosome number of 2n = 4x = 32 (Voisey et al., 1994). White clover is an allopolyploid species, which has a well-developed gametophytic self-incompatibility mechanism of disomic inheritance (Thomas, 1987). In essence, predominant out breeding and disomic inheritance indicates that white clover populations are composed of a heterogeneous mixture of highly heterozygous individuals. This caused high levels of genetic variation both within and between white clover populations (Voisey et al., 1994).

Information on genetic variation of germplasms is of great interest to population geneticists and plant breeders (Badr et al., 2002). The study of genetic relationships of plants may be used to identify suitable parents and to prevent progressive erosion in the genetic bases of breeding populations (Kolliker et al., 2001). In order to conserve genetic resources for plant improvement, it is necessary to preserve, maintain and document genetic diversity (Lane et al., 2000). It is particularly important for white clover, because of the high degree of self-incompatibility and high allelic diversity (Castric and

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Vekemans, 2004).

Initial white clover breeding efforts began in the 1930s (Woodfield and Caradus, 1994). During the last 60 - 70 years, substantial genetic improvement in white clover has been successfully achieved by classical breeding (George et al., 2006). It focused on selection for improved performance within existing ecotypes. Phenotypic recurrent selection within adapted germplasm pools has been a common way of population improvement in white clover (Woodfield and Caradus, 1994). Genetic variation of white clover is frequently detected by morphological and agronomic traits, such as root growth (Ennos, 1985), herbage yield and stolon density (Jahufer et al., 1995), and leaf size (Finne et al., 2000). But, these traits often show multigenic inheritance with strong environmental modification. Molecular markers that reveal polymorphism at the DNA level have proven to be a very useful tool for genotype characterization and estimation of genetic diversity (Treuren et al., 2005).

White clover originated from the Mediterranean region of Europe and was spread through Europe and Western Asia with migrating animals before recorded history (George et al., 2006). It is naturalized in China, especially in the southwest and middle regions. It also distributes in Changbai and Tianshan Mountain in the north of China. However, genetic variation of white clover in China has not been reported. Our objective was to evaluate the genetic variation of white clover germplasms collected in China, using morphological traits, random amplified polymorphic DNA (RAPD), and simple sequence repeat (SSR). Four white clover cultivars were included as contrast.

MATERIALS AND METHODS

Plant materials

Fourteen white clover populations were selected for this study, with 5 to 30 genotypes per population. These populations include ten local white clover collections from three different regions (middle, southwest, and northwest) China and four commercial white clover cultivars. White clover cultivars included were: Haifa (HF), Rivendel (RWD), Karina (KRN) and Alice (ALC). Haifa is a large leaved cultivar, derived from a line CPI 15648 in northeast Israel. Rivendel is a small leaf size cultivar bred from four families of small-leaved, older PAJBJERG material and was developed in Denmark. Karina is a medium leaf size cultivar. It was selected for absence of white V mark on leaves and developed in Germany. Alice is a medium-large leaved cultivar. It was bred from Kersey and Blanca in the United Kingdom (Caradus, 1986; Caradus and Woodfield, 1997).

Ten local white clover germplasms from different sites in China are Changji (CJ), Ziniquan (ZNQ), Nanshan pasture of Xingjiang province (XNS), Xiaodiwo (XDW), Tongshan (TS), Wuchang (WC), Puding (PD), Weining (WN), Guangnan (GN) and Zhenxiong (ZX). They were collected in Xinjiang province (northwest China), Hubei province (middle China), Guizhou province (southwest China), and Yunnan province (southwest China). The sites of Chinese local white clover collections are mapped in Figure 1.

Morphological traits

The field evaluation comprised three randomized complete blocks. All cultivars and local germplasms were grown with the same management in the experimental garden of China agricultural university. Measurements were begun at the full flowering stage in 2007. Nine morphological traits including height, leaf length, leaf width, leaf area, leafstalk length, footstalk length, stolon length, stolon density, and stolon thickness were recorded in triplicate and the data means were used for the analysis.

Height was measured from the soil surface to the top of the canopy. Leaf length, leaf width, and leaf area were measured on the third leaf from the end of the stolon. Leaf length and leaf width were taken on the middle leaflet of the leaf. Leaf area (cm^2) was estimated by portable living leaf area meter (SHY-150, Harbin optical instrument factory). Petiole length and pedicel length were measured from the axil to the attachment point of leaf or flower. Stolon length was measured on a 5-internode piece. Stolon density was obtained from a 10 × 10 cm^3. Stolon thickness measurements were made at about 5 cm from the growing point of the stolon.

DNA extraction

Genomic DNA was extracted by the CTAB extraction procedure. Fresh leaves were ground to powder in liquid nitrogen and transferred to a 1.5 ml tube, to which 0.5 ml pre-heated (65℃) 2×CTAB (2%(w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl (2% β-mer-captoethanol)) extraction buffer was added. Samples were incubated for 60 min at 65℃. We then added 0.5 ml chloroform: iso-amyl alcohol (24:1) and mixed for about 5 min. Samples were centrifuged at 9500 g for 10 min, and supernatant was transferred to fresh tube. We added an equal volume of isopropanol, and high mw DNA spooled out upon mixing. Samples were centrifuge at 9500 g for 10 min. After we poured off the remaining supernatant, the spooled DNA was rinsed with 70% ethanol, air dried, and 0.1 ml TE, containing 20 ug/ ml RnaseA was added (Murray and Thompson, 1980). The extracted DNA was kept at 4℃ for RAPD and SSR analysis.

RAPD analysis

Final volume for PCR reaction was 25 µl, with 3 unit of Taq DNA polymerase, 20 ng of template DNA, 0.1 µM of random primer, 0.4 mM each of dATP, dTTP, dGTP, and dCTP, 1 mM $MgCl_2$, commercial Taq DNA polymerase buffer. Amplifications were performed as follows: 96℃ 4 min, 36℃ 50s , 72℃ 1 min 45 s, 1 cycle; 94℃ 50 s, 36℃ 50 s, 72℃ 1 min 45 s, 42 cycles; 94℃ 50 s, 36℃ 50 s, 72℃ 2 min 30 s, 1 cycle. PCR products were analyzed in 1.5 % (w/v) agarose gels in 1×TAE buffer. Gels were stained with 0.5 µg/ml ethidium bromide, and photographed in UV illumination.

Three primers (5'-GTGACGTAGG-3', 5'-ACGCGCATGT-3', 5'-GAGAGGCTCC-3') were chosen. The size of the markers was determined by 100 bp DNA ladder. RAPD analysis in this study was based on the result of Gustine and Huff (1999). Amplification products were separated by electrophoresis in 1.6% agarose gels, which contained ethidium bromide (0.5 µg/ml), with 1× TAE buffer (40 mM Tris-base, 20 mM sodium acetate, 2.0 mM EDTA, glacial acetic acid to pH 7.2) at 5 v/cm. A total of 1.5 µl 6× loading buffer (40% sucrose and 0.25% bromophenol blue) was added to each reaction prior to electrophoresis. After electrophoresis, the gels were photographed in ultraviolet light (Figure 6).

SSR analysis

The six SSR markers used were TRSSRA06E06, TRSSRA06B04, TRSSRB02E01, TRSSRB01E07, TRSSRA04B12, and TRSSRA0 3B05. Their characteristics and primer information were described previously (George et al., 2006) (Figure 6).

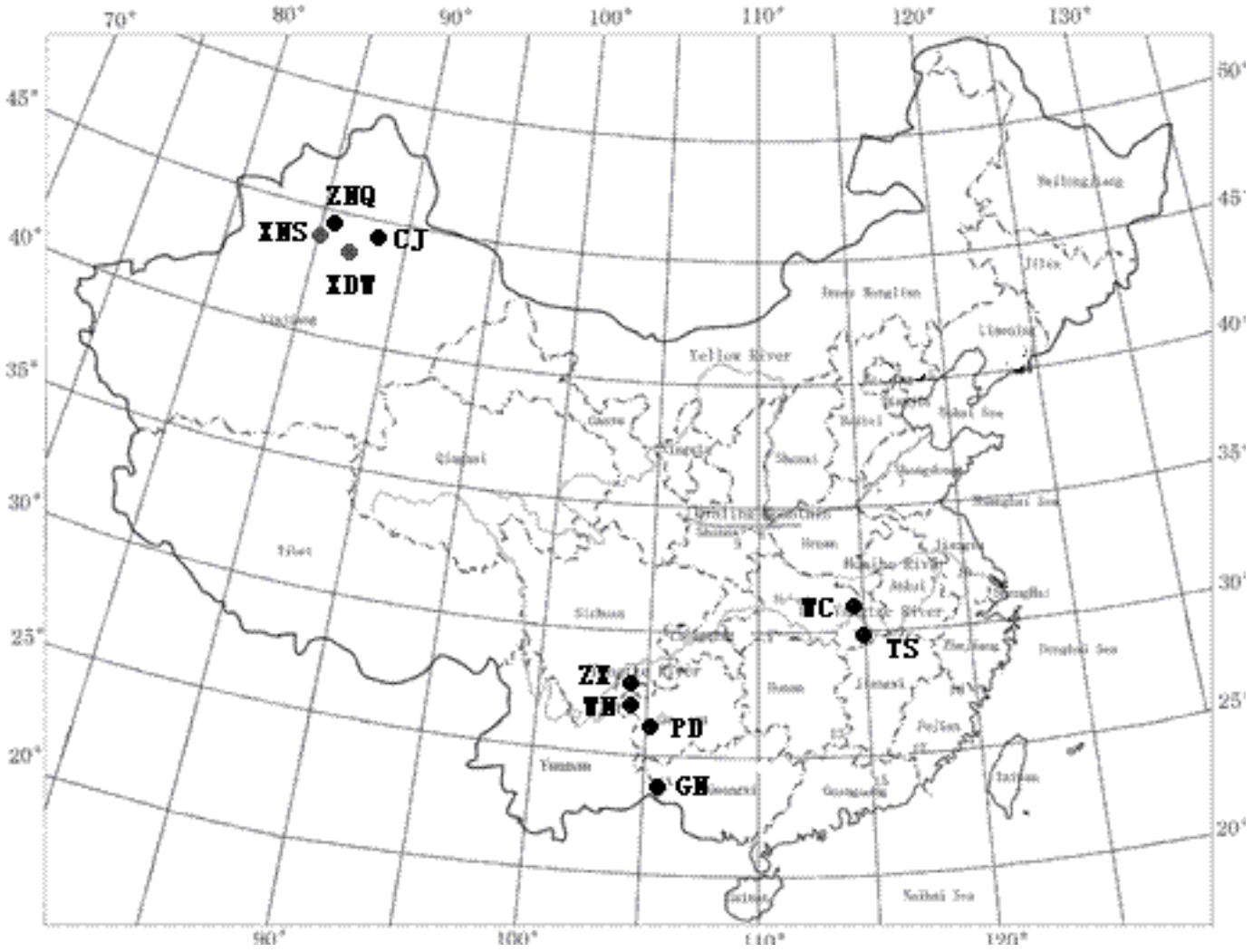

Figure 1. Locations of Chinese local white clover collections.

Amplification reactions of SSR loci were conducted as follows: 94℃ 10 min; followed by 94℃ 30 s, 65℃ 30 s, 72℃ 30 s, 35 cycles; and then 72℃ for 7 min. The reaction contained 20 ng DNA, 0.5 µM primer, 0.19 mM each of dATP, dTTP, dGTP, and dCTP, 1.25mM $MgCl_2$, commercial Taq DNA polymerase buffer, and one unit of Taq DNA polymerase in a 20 µl final volume. After amplification, 40 µl of loading dye (0.09% bromophenol blue, 0.09% xylene cyanol) were added to each reaction tube, then denaturing at 98℃ for 10 min. Amplification reaction products were separated on a 10% denaturing polyacrylamide gel, with 1× TBE buffer. Silver staining was used to visualize the fragments.

Analysis of data

Data from morphological traits were standardized by the formula:

X = (x-M) / sd

The mean value (M) of each trait was subtracted from the raw data (x) to rescale the difference of data, and the standard deviation (sd) was divided to eliminate the effect of different units of the trait. The standardized data were used to estimate Euclidean distance matrix by the SPSS version 11.5 (SPSS, 2002). The matrix was employed to form a dendrogram based on the unweighted paired group method of arithmetic averages (UPGMA) by mega 3.0 (Kumar et al., 2004). Because of the allotetraploid species background, it is difficult to distinguish allele over potentially more than 2 homoeoloci at SSR loci. Codominant SSR markers were coded as binary data. We scored SSR loci as dominant feature, and the alleles were classified simply as 'fragments'. Both RAPD and SSR fragments were scored as present (1) or absent (0) of homologous bands. For each individual plant only strong amplified loci were scored. In this way two matrices of phenotypes were assembled.

$$H = 1 - \sum_{k} x^2_{ik}$$

Genetic variation was measured as Nei's gene diversity H (Nei M, 1973) and Shannon's information index I (Shannon and Weaver, 1949), by using the software package Popgene, version 1.31 (Francis et al., 1999), where x_{ik} is the frequency of the kth allele in the ith subpopulation. Analysis of molecular variance (AMOVA) is a hierarchical analysis of variance. The significance with the different

Table 1. Genetic distance matrix for 14 white clover populations computed from morphological traits analysis.

	HF	RWD	KRN	ALC	ZNQ	XDW	XNS	CJ	PD	WN	ZX	GN	TS	WC
HF														
RWD	2.098													
KRN	3.968	3.141												
ALC	2.309	3.116	2.906											
ZNQ	4.729	3.285	4.205	1.733										
XDW	3.691	3.778	2.646	5.729	4.288									
XNS	1.365	2.364	2.27	2.412	2.692	3.357								
CJ	1.151	6.782	2.046	4.872	1.894	1.144	2.944							
PD	1.255	2.854	4.051	2.984	3.201	4.285	1.625	4.775						
WN	2.109	6.253	6.958	6.86	4.874	3.756	0.988	2.067	4.876					
ZX	2.918	3.265	3.795	2.906	2.676	2.598	2.585	2.206	3.817	5.238				
GN	4.563	3.413	3.785	4.137	4.465	2.773	1.515	1.202	3.477	1.893	2.505			
TS	3.14	3.448	2.557	2.758	2.902	5.384	2.57	1.957	7.346	5.205	2.671	2.423		
WC	2.634	1.672	1.478	1.977	4.298	6.494	3.482	3.143	4.152	2.44	1.087	1.493	2.679	

possible levels of genetic structure (within groups of populations, and among groups) is tested using non-parametric permutation procedures (Excoffier et al., 1992). The ten local Chinese populations were divided into three groups according to the regions (middle, southwest, and northwest) to calculate variance among groups, within and among populations. These were estimated by Arlequin version 3.0 (Excoffier et al., 2005).

In order to describe genetic relationships among the Chinese white clover accessions, genetic distances were estimated using RAPD and SSR marker data. The Kimura 2-parameter model (Nei and Kumar, 2000) was used to calculate the inter-population distance matrix of fourteen populations.

$$D = a/2[(1-2P-Q)^{-1/a} + 1/2(1-2Q)^{-1/a}.$$

where P and Q are the frequencies of sites with transitional and transversional differences respectively, and *a* is nucleotide substitutions rate. Dendrograms were produced using the unweighted paired group method of arithmetic averages (UPGMA). These analyses were performed by mega 3.0 (Kumar et al., 2004).

RESULTS

Morphological traits analysis

Distance estimates based on nine morphological traits ranged from 0.988 to 6.958 with an average of 3.116 (Table 1). Cluster analysis based on the morphological data assigned the populations into two groups (Figure 2). The collection WC which had the highest means for all traits except stolon density separated from other genotypes firstly. The rest of the populations formed two clusters. The first cluster included TS, XNS, large leaved cultivar HF, medium leaf size cultivars ALC and KRN; ALC and KRN showed a high similarity. Collections CJ, ZNQ, XDW, WN, GN, PD, ZX and small leaf size cultivar RWD are in the second cluster. Populations in this cluster had relative low means for morphological traits. In this grouping collections CJ, ZNQ and XDW (originating from northwest of China) were closely clustered together; PD and ZX (both from southwest of China) also showed high similarities.

RAPD analysis

Random amplified polymorphic DNA analysis of the fourteen white clover populations on three RAPD primers produced a total of 30 bands, 29 of which were polymorphic, and the percentage of polymorphic loci was 96.67. These amplified fragments among the fourteen populations ranged in size from approximately 100 to 15000 bp (Fig. 5).

Estimates of Nei's genetic diversity, based on RAPD data, varied from 0.1412 to 0.2030 (Table 2) with an average of 0.1671, and the total gene diversity parameter was 2.3394. Shannon's total information index was 3.4876 (ranging from 0.2097 to 0.3110) and the average per accession was 0.2491. The diversity value was lowest for CJ and highest for WN.

The AMOVA analysis based on RAPD data did not show a significant partitioning of variance among groups, the variability was attributed to the individual plants within populations and populations within groups. The genetic variance among populations within groups was 48.00% (Table 3), and the within population variance was 50.57%.

In order to visualize the relationship among populations, an inter-population distance matrix (Table 4) was generated and submitted for cluster analysis by the UPGMA procedure. The resulting dendrogram was plotted (Figure 3). Cultivars HF and RWD separated from the other twelve white cover accessions first at 0.08 distance unit. The rest of the populations formed two clusters: collections CJ, WC and WN clustered together with cultivars KRN and ALC; the other seven collections occurred in the other cluster. According to the dendrogram, groupings

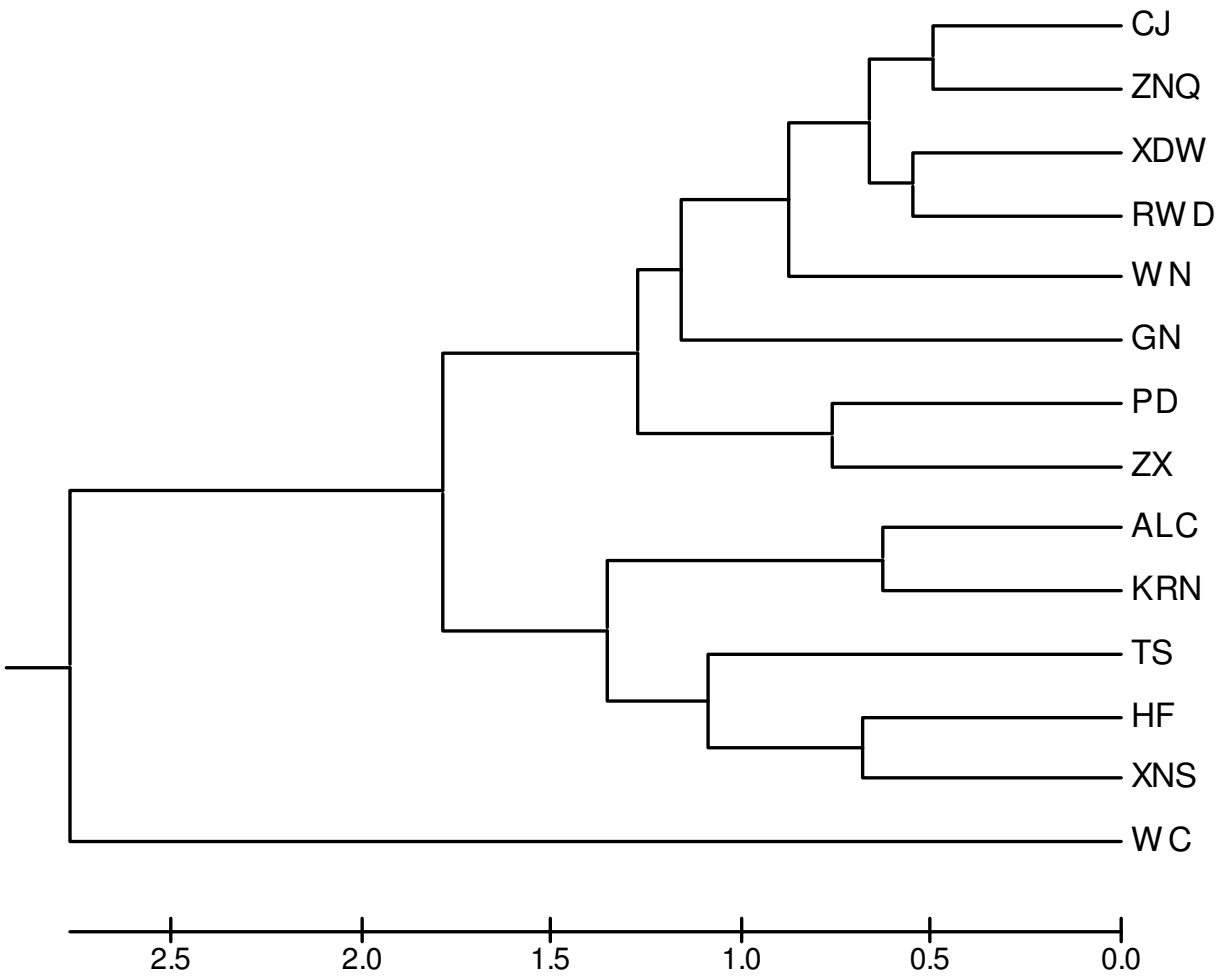

Figure 2. Dendrogram of fourteen white clover populations computed from morphological traits analysis.

Table 2. Nei's gene diversity and Shannon's Information index of 14 white clover populations based on RAPD and SSR data.

Populations	RAPD		SSR	
	Nei's gene diversity	Shannon's Information index	Nei's gene diversity	Shannon's Information index
RWD	0.1463	0.2177	0.1552	0.2310
KRN	0.1975	0.3010	0.1649	0.2576
ALC	0.1496	0.2238	0.1854	0.2822
HF	0.1979	0.2959	0.1511	0.2345
XDW	0.1475	0.2201	0.1500	0.2385
PD	0.2025	0.3052	0.1798	0.2685
TS	0.1882	0.2804	0.1384	0.2045
WN	0.2030	0.3110	0.1314	0.1987
CJ	0.1412	0.2097	0.1282	0.1945
XNS	0.1686	0.2502	0.1672	0.2619
ZX	0.1592	0.2375	0.2095	0.3108
ZNQ	0.1158	0.1659	0.1587	0.2335
WC	0.1775	0.2569	0.1072	0.1640
GN	0.1446	0.2123	0.1384	0.2045
Average	0.1671	0.2491	0.1547	0.2346

Table 3. Analysis of molecular variance (AMOVA) of 14 white clover populations based on RAPD and SSR data.

Source of variation	df	Sum of squares	Variance components	Percentage of variation††	P Value
RAPD					
Among groups†	3	160.706	0.07173	1.43	0.389
Among populations within groups	10	452.127	2.40919	48.00	< 0.0001
Within populations	241	611.614	2.53782	50.57	-
SSR					
Among groups†	3	25.351	-0.06726	-2.28	0.826
Among populations within groups	10	99.008	0.66926	22.69	< 0.0001
Within populations	153	359.216	2.34782	79.59	-

† The ten Chinese local populations were divided into three groups according to the geographical regions (middle, southwest, and northwest of China).
†† Components were significant at P < 0.001; the probability of obtaining a more extreme random value computed from non-parametric permutation approach (1,000 data permutations).

Table 4. Genetic distance matrix for 14 white clover populations computed from random amplified polymorphic DNA analysis and simple sequence repeats markers.

SSR† RAPD††	HF	RWD	KRN	ALC	ZNQ	XDW	XNS	CJ	PD	WN	ZX	GN	TS	WC
HF		0.415	0.416	0.394	0.446	0.47	0.414	0.445	0.463	0.381	0.409	0.451	0.462	0.443
RWD	0.918		0.46	0.433	0.409	0.394	0.455	0.419	0.408	0.424	0.368	0.401	0.409	0.429
KRN	0.705	0.767		0.375	0.367	0.378	0.428	0.365	0.381	0.406	0.395	0.371	0.353	0.38
ALC	0.624	0.428	0.491		0.375	0.39	0.438	0.368	0.4	0.407	0.387	0.376	0.41	0.42
ZNQ	0.719	0.432	0.625	0.342		0.216	0.374	0.287	0.297	0.371	0.328	0.349	0.316	0.31
XDW	0.707	0.677	0.756	0.516	0.27		0.283	0.154	0.306	0.295	0.342	0.324	0.305	0.272
XNS	0.604	0.914	0.625	0.64	0.449	0.327		0.31	0.411	0.42	0.392	0.387	0.423	0.384
CJ	0.53	0.622	0.464	0.4	0.397	0.663	0.55		0.375	0.31	0.311	0.355	0.323	0.279
PD	0.619	0.695	0.785	0.591	0.394	0.227	0.364	0.673		0.428	0.397	0.345	0.388	0.344
WN	0.708	0.661	0.608	0.407	0.409	0.478	0.393	0.481	0.573		0.377	0.385	0.401	0.39
ZX	0.846	0.597	0.62	0.517	0.271	0.384	0.451	0.394	0.472	0.477		0.365	0.398	0.393
GN	0.63	0.876	0.839	0.774	0.435	0.296	0.398	0.62	0.294	0.61	0.387		0.395	0.373
TS	0.631	0.787	0.783	0.625	0.35	0.212	0.304	0.604	0.247	0.494	0.363	0.268		0.374
WC	0.702	0.591	0.505	0.283	0.389	0.505	0.444	0.351	0.611	0.342	0.424	0.682	0.544	

† The upper right matrix is genetic distance matrix computed from simple sequence repeats markers.
†† The lower left matrix is genetic distance matrix computed from random amplified polymorphic DNA analysis.

were not distinctly associated with geographic origin, and clusters did not separate all cultivars from collections.

SSR analysis

Six pairs of SSR primers were used to assess variation within and among the fourteen white clover germplasms. A total of 46 bands were produced, 44 of which were polymorphic (Fig 6), and the percentage of polymorphic loci was 95.65. Table 2 shows Nei's genetic diversity and Shannon's information index evaluation employing SSR data. Nei's genetic diversity ranged from 0.1072 to 0.2095 with an average of 0.1547, and the total diversity value was 2.1654. Shannon's total information index was 3.2847, with the lowest of 0.1640 (WC), the highest of 0.3108 (ZX), and an average of 0.2346. AMOVA performed on SSR and RAPD data showed no significant variance among groups (0%; P = 0.14370) (Table 3). Most of the variation resulted from genetic differences of single plants within populations (79.59%), rather than differences within groups (22.69%). The lowest distance parameter was for collections XDW and ZNQ(0.21600), which were from northwest China, while the highest value (0.46980) was calculated for large leaf cultivar HF and germplasm ZNQ (Table 4).

Grouping based on SSR data distinctly separated the white clover cultivars and collections (Figure 4). Cultivars

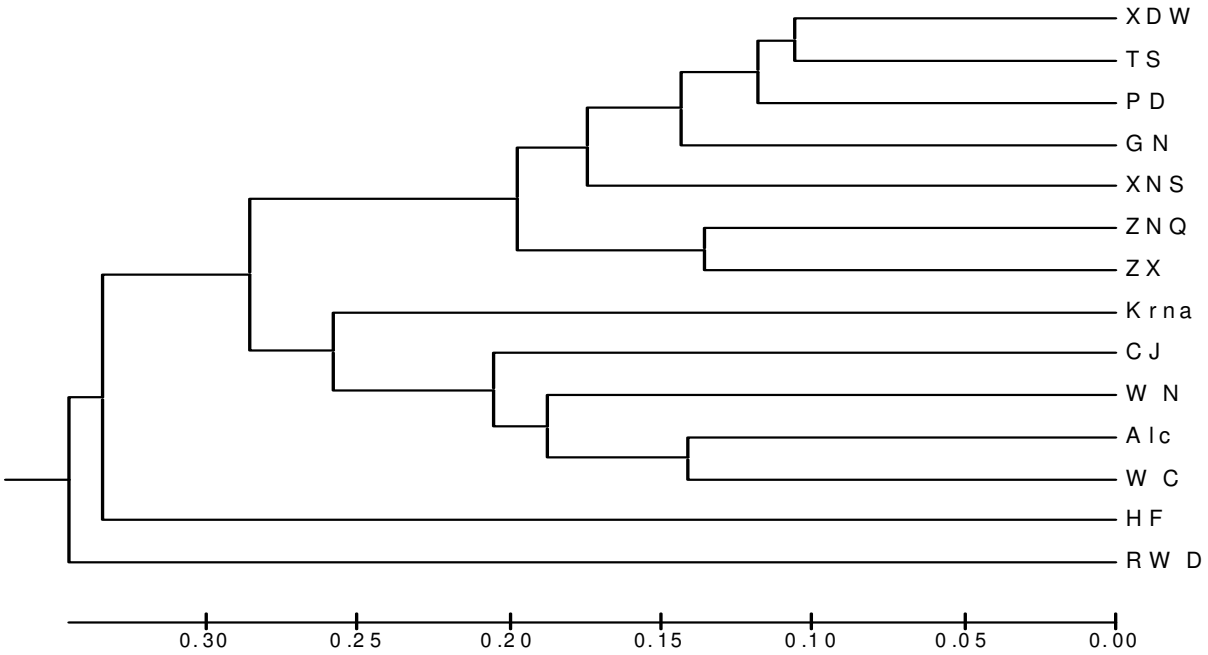

Figure 3. Dendrogram of fourteen white clover populations computed from random amplified polymorphic DNA analysis.

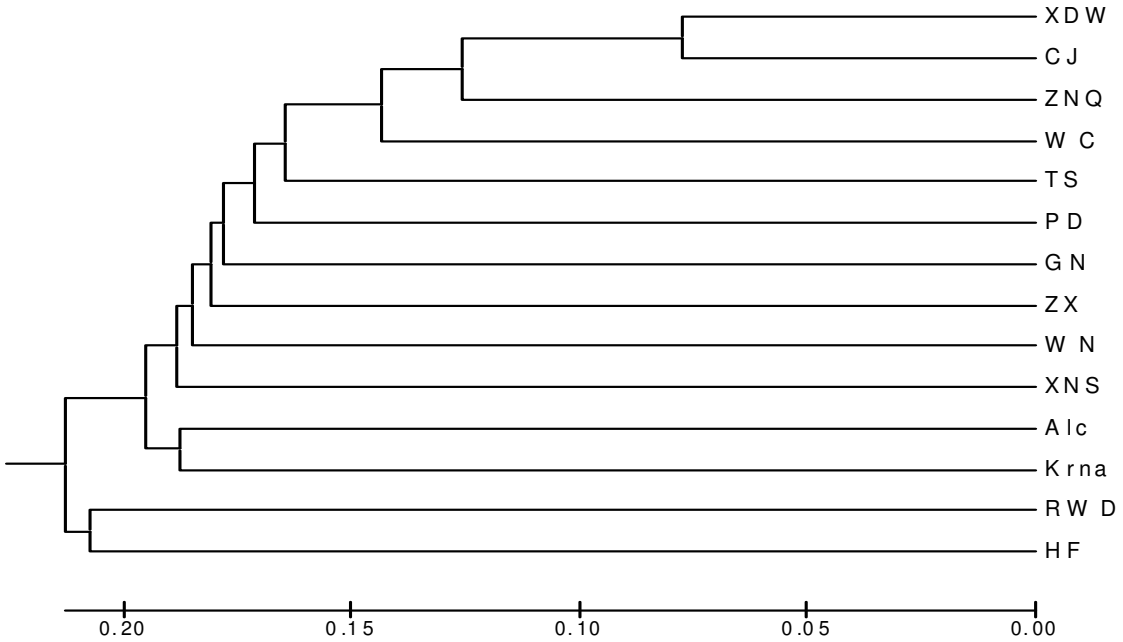

Figure 4. Dendrogram of fourteen white clover populations computed from simple sequence repeats analysis.

HF and RWD were in a single cluster, as well as ALC and KRN; the rest of the populations (ten white clover collections) were occurred in one cluster. The dendrogram showed relative agreement with white clover collections' region of origin. For the collections' cluster, no sub-cluster was formed. XDW, ZNQ and CJ (collections from northwest China) clustered together; germplasms from middle China (WC, TS) and southwest China (PD, GN, ZX and WN) gradually separated from the northwest ones.

DISCUSSION

Morphological data analysis of white clover populations

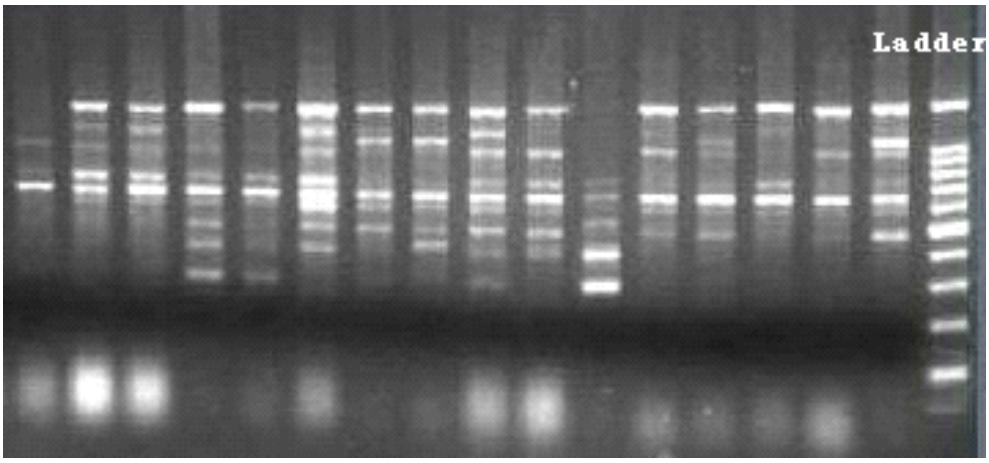

Figure 5. Profiles of white clover germplasm generate by RAPD primer (5'-GTGACGTAGG-3'). 100bp DNA Ladder 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1500 bp.

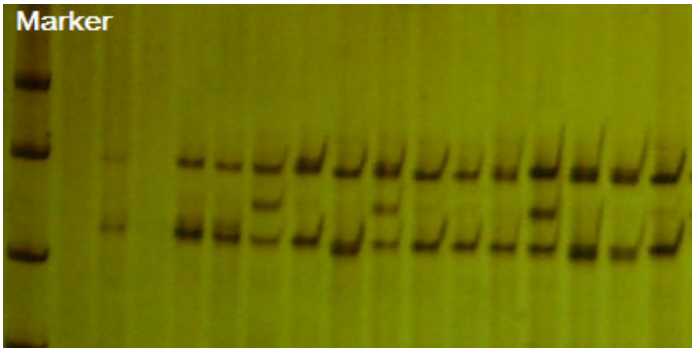

Figure 6. SSR profiles of white clover germplasm XDW generate by primer TRSSRA06B04.DNA marker 404, 527, 622bp

was coupled with molecular analysis (RAPD and SSR) to investigate the genetic relationships among ten white clover germplasms of China and four commercial cultivars. To our knowledge, this is the first report of an analysis of genetic diversity in local Chinese white clover germplasms. It is also the first work combining morphological, RAPD and SSR analysis to evaluate the genetic variability of white clover.

Relationships among populations were investigated by the analysis of molecular variance (AMOVA). AMOVA revealed high levels of intra- and inter-population variation detected by both RAPD and SSR markers, and a higher intra-population variation was observed based on SSR data. Differences among geographical regions based groups were not detected. Most of the variation was due to individual plants within white clover from a single sample site. A smaller part of variance was responsible for variation among populations within geographical regions. Similar results were reported for white clover employing RAPD analysis by Gustine and Huff (1999), where they found significant differences among populations collected from the USA, but no differences among the three sample states were observed. In studies based on analysis of SSR, George et al. (2006) showed consistent results. Similar observations were also found for other outcrossing pasture species, such as alfalfa (Mengoni et al., 2000). The result may be related to the allopolyploid and gametophytic self-incompatibility characters of white clover.As observed in the dendrogram employing SSR data (Figure 4), all of the ten local collections from China clustered into a single group. It was established that no distinct differences occurred among the geographical groups, and these groups of populations may have homologous germplasm sources. We observed slight correlations between geographical origins and population relationship patterns investigated by morphological traits, RAPD and SSR analyses. Kölliker et al. (2001) made a similar observation of a weak correlation between AFLP analysis and geographical origin for white clover cultivars and accessions. Because of the outcrossing and cross-breeding by pollinating insects, local populations may be significantly influenced by cultivars and germplasms introduced into an area. Variances among geographical regions were

decreased, and were smaller than those among sites within the same geographic regions.

Except for morphological markers, the two molecular markers based population relationships were dissimilar with the leaf size differences. As revealed in the SSR dendrogram, the large leaf size cultivar HF (6.58 cm^2) and the small leaf cultivar RWD (1.75 cm^2) were assembled in a single cluster; TS (6.58 cm^2) and WC (9.22 cm^2) were closely related with ZNQ (2.35 cm^2), CJ (3.13 cm^2) and XDW (2.31 cm^2). It is obvious that there was no correlation between leaf size and the molecular (RAPD and SSR) markers' diversity patterns. In contrast, the RAPD based analysis of white clover (Gustine and Voigt, 2002) showed partial correlation between leaf characteristics and the relationships of populations. According to their study, all large leaf germplasms were in the same cluster, but a small leaf germplasm was also in this cluster too. They suggest that leaf area is not a good characteristic to distinguish the genetic relationship of populations. This was probably due to the function of primers. Primers used to gain the population relationships may originate from any part of the genome, while leaf size was controlled by certain genes. Some similarities were observed among dendrograms generated by different data sets. In both dendrograms obtained from the RAPD and SSR data, cultivars HF and RWD were completely separated from the other populations (Figures 3 and 4). This indicated that HF and RWD have distinct germplasm compared to the other collections. Cultivars ALC and KRN clustered closely in each of the three dendrograms based on morphological, RAPD and SSR data, respectively. This may be related to the similarity of morphological traits between ALC and KRN (11.17 cm, 10.90 cm for height; 11.67 cm, 11.20 cm for petiole length, and 1.50 cm, 1.93 cm for stolon thickness). In both morphological and SSR dendrograms, XDW, ZNQ and CJ (collections from northwest China) clustered together, while another northwest population XNS was distant from them. The dissimilarity of XNS to other northwest collections may be related to the relative high means of morphological traits of XNS. Despite the similarities mentioned above, no significant correlation was observed among three markers based population relationships. Neither RAPD nor SSR analysis was correlated with morphological trait analysis, and the correlation between RAPD and SSR based distance matrices appeared to be rather low ($r = 0.384$, $p < 0.01$). The populations showed diverse morphological traits, RAPD and SSR patterns. The range of genetic distance based on morphological traits was higher than RAPD and SSR markers. It may reflect the influence of the environment on the performances of the materials. The differences between morphological and molecular markers could be explained by the fact RAPD and SSR markers cover a larger proportion of the genome than the morphological markers and are less influenced by the environment than morphological markers (Kolliker et al., 2001). The molecular markers may originate from any part of the genome including coding and non-coding regions, and can cover either the full genome or large genomic segments, while morphological traits are controlled by a relatively small number of loci (Bruschi et al., 2003). Phenotypic plasticity can evolve independent from genetic variability, and it may bias the genetic variability based on morphological traits (Kölliker et al., 1999). Morphological traits may be modified by environmental conditions. The low correlation of grouping patterns obtained from RAPD and SSR data could be attributed to the different genomic constitution of two markers (Ravi1 et al., 2003). Therefore, the morphological traits, RAPD and SSR analysis will not necessarily produce closely matching results.

In conclusion, SSR analysis provided a better view of genetic relationships of white clover populations. The morphological traits analysis could not distinguish cultivars from collections, but showed a weak agreement with the collections' regions of origin. The grouping utilizing SSR data could distinctly separate cultivars from collections and showed a slight agreement with the geographic origins of the germplasms. It also showed a certain similarity with the morphological dendrogram. RAPD analysis could partially separate cultivars from collections. However, groups based on geographic origins could not be distinguished by RAPD. Neither RAPD nor SSR based grouping were consistent with leaf size. Our results suggest that the combination of morphological traits and SSR analysis could be a useful method for assessing genetic variation in white clover, and will be practical for breeders to use when planning crosses for positive traits.

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Abbreviations

ALC, Cultivar Alice; **AMOVA,** analysis of molecular variance; **CJ,** germplasms from Changji; **GN,** germplasms from Guangnan; **HF,** cultivar Haifa; **KRN,** cultivar Karina; **PCR,** polymerase chain reaction; **PD,** germplasms from Puding; **RAPD,** random amplified polymorphic DNA; **RWD,** cultivar Rivendel; **SSR,** simple sequence repeat; **TS,** germplasms from Tongshan; **UPGMA,** unweighted paired group method of arithmetic averages; **WC,** germplasms from Wuchang; **WN,** germplasms from Weining; **XDW,** germplasms from Ziniquan; **XNS,** germplasms from Nanshan pasture of Xingjiang province; **ZNQ,** germplasms from Ziniquan; **ZX,** germplasms from Zhenxiong; **CTAB,** cetyl trimethyl ammonium bromide; **EDTA,** ethylene diamine tetraacetic acid.

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