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GENETIC RELATIONSHIPS OF CHINESE GRAPE ACCESSIONS TO EUROPEAN AND AMERICAN CULTIVARS ASSESSED BY MICROSATELLITE MARKERS

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ABSTRACT

A total of 45 grape accessions were used to investigate the genetic polymorphism and relationships among Chinese and other European or America grape accessions by microsatellite markers. Eighty-six alleles were detected in 9 simple sequence repeat (SSR) loci with an average of 9.6 alleles per locus. Genetic similarity ranged from 0.38 to 0.83 with an average value of 0.58. This indicated sufficient diversity among the accessions. Based on cluster analysis and principal coordinate analysis, the results showed that all accessions could be divided into three major groups and the clustering pattern was related to the classical ecogeographical grouping: occidentalis, pontica and orientalis. Those from the same ecological group could cluster preferentially in most cases into one group, suggesting the positive correlation between the cluster results and the geographical origin of the grape accessions. Wine grapes were significantly differentiated from table grapes no matter whether the accessions were from China or other countries.

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Introduction

The grape (*Vitis vinifera* L.) is one of the most important fruit crops in the world. The genus *Vitis* consists of about 60 inter-fertile species (34). *Vitis vinifera* L. is the only species extensively used in the world (34). Viticulture spread along the Silk Road and it reached China in 2nd century and Japan in 3200 B.P. (27). Grape has been grown in China for more than 2000 years. China remains a rich source of *V. vinifera* genetic diversity; more than 40 species of *Vitis* are native to China.

Cultivars of *V. vinifera* are classified into three ecological groups (convar.): *pontica*, *orientalis*, and *occidentalis* (22). Most of indigenous cultivated accessions in China belong to Oriental cultivars such as 'Niunai', 'Lizixiang', 'Mulaga' and 'Hongjixin', etc. At present, the commercially cultivated grape accessions in China consist of old native varieties, more recently introduced widespread European cultivars and locally selected cultivars. Most grapes grown in China are European varieties (*Vitis vinifera* L.) or European-American hybrids. The principal table grape varieties cultivated in the country are 'Kyoho', 'Muscat Hamburg', 'Longyan' (Dragon Eyes), 'Jingxiu', 'Rizamat', 'Fenghuang 51', 'Red Globe', 'Jingzhaojin', 'Italia', etc. Popular wine-making varieties are 'Chardonnay', 'Cabernet Sauvignon', 'Merlot', etc. Most of the production of grapes in China annually were for table grapes (about 80 percent of the total grape production), and only about 10 percent of grape production was for wine making, while the other 10 percent was for processing into raisins. In China, as many as 1500 accessions are conserved in National Grape Germplasm Repository of Zhengzhou, Henan Province. However, only a limited number of Oriental cultivars have been characterized (15).

Molecular markers that reveal polymorphism at the DNA level have been shown to be a very powerful tool for characterization and estimation of genetic diversity. Compared with other DNA-based markers such as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), single nucleotide polymorphisms (SNPs) and amplified fragment length polymorphisms (AFLP), microsatellite or simple sequence repeat (SSR) markers have become valuable molecular tool for genetic fingerprinting due to their abundance, high degree of polymorphism, co-dominance and suitability for automation (2).

Many microsatellite markers have been developed and available in grape (6, 30, 32). Microsatellite markers have been extensively used in grape for different purposes: variety identification in collections, pedigree analysis, or genetic mapping (1, 31, 36). Several genetic diversity studies have been conducted in local, regional or national germplasm collection of grape using microsatellite markers, for example, Portugal (9), Turkey (19), Tunisia (35), Spain (18, 21), Hungary (12), Brasil (20), Italy (29) and France (25).

Until now, only few papers have been published on the DNA based molecular markers analysis of Chinese grape cultivars (15). Investigation of genetic relationship is very import for germplasm conservation, evaluation and utilization

TABLE 1

List of grape accessions used in this study

Cultivar name	Pedigree	Species	Origin
Lizixiang	Ancient variety of China, Unknown	*V.vinifera* L.	China
Longyan	Ancient variety of China, Unknown	*V.vinifera* L.	China
Hetianhong	Ancient variety of China, Unknown	*V.vinifera* L.	China
Heijixin	Ancient variety of China, Unknown	*V.vinifera* L.	China
Hongjixin	Ancient variety of China, Unknown	*V.vinifera* L.	China
Manai	Ancient variety of China, Unknown	*V.vinifera* L.	China
Munage	Ancient variety of China, Unknown	*V.vinifera* L.	China
Niunai	Ancient variety of China, Unknown	*V.vinifera* L.	China
Pinger Putao	Ancient variety of China, Unknown	*V.vinifera* L.	West Asia
Niuxin	Ancient variety of China, Unknown	*V.vinifera* L.	China
Thompson Seedless	Ancient variety of West Asia, Unknown	*V.vinifera* L.	West Asia
Jingzaojin	Queen of vineyard×Thompson Seedless	*V.vinifera* L.	China, 1960
Zexiang	Muscat Hamburg×Longyan	*V.vinifera* L.	China, 1979
Cuiyu	Muscat Hamburg×Jingzaojin	*V.vinifera* L.	China, 1986
Guibao	Ispissar×Muscat BHPa	*V.vinifera* L.	China, 1988
Fenghuang 51	Muscat of Alexandria×Cardinal, uncertain	*V.vinifera* L.	China, 1988
Jingxiu	Pannoniariiiacse×(Muscat Hamburg× Monukka)	*V.vinifera* L.	China, 1994
Zaomana	Muscat Hamburg×Jingzaojin	*V.vinifera* L.	China, 1997
Xiangfei	(Muscat Hamburg×Pearl of Csaba)×Cardinal	*V.vinifera* L.	China, 1998
Jingya	Chance seedling of Black Olympia	*V.vinifera* L.×*V.larbrusca* L.	China, 1992
Luopuzaosheng	Sport of Jingya	*V.vinifera* L.×*V.larbrusca* L.	China, 2005
Muscat Hamburg	Black Hamburg×Muscat of Alexandria	*V.vinifera* L.	England, 1860
Pearl of Csaba	Mosknellier d'Hongrie×Nuscat Ottonel	*V.vinifera* L.	Hungary, 1904
Queen of vineyard	Elisabeth×Pearl of Csaba	*V.vinifera* L.	Hungary, 1916
Italia	Bicane×Muscat Hamburg	*V.vinifera* L.	Italy, 1911
Rizamat	Katta Kurgan×Parket	*V.vinifera* L.	Unknown
Centenial	Sport of Rosaki	*V.vinifera* L.	Turkey
Red Globe	C12~80×S45~48	*V.vinifera* L.	USA, 1982
Autumn Royal	Autumn Black×C741	*V.vinifera* L.	USA
Concord	Chance seedling, Uncertain	*V.larbrusca* L.	USA, 1852
Champbell Early	Moore Early×(Belvidere×Muscat Hamburg	*V.vinifera* L.×*V.labrusc*a L.	USA, 1852
Triumph	Concord×Chasselas Musque	*V.vinifera* L.×*V.labrusca* L.	USA, 1883
Kyoho	Campbell E× Centenial	*V.vinifera* L.×*V.labrusca* L.	Japan, 1945
Fujiminori	Honey Red× Pione	*V.vinifera* L.×*V.labrusc*a L.	Japan, 1985
Pinot blanc	Sport of Piont Gris	*V.vinifera* L.	France, 1896
Cabernet Sauvignon	Cabernet Franc x Sauvignon Blanc	*V.vinifera* L.	France
Merlot	Unknown	*V.vinifera* L.	France
Cabernet Franc	Unknown	*V.vinifera* L.	France
Chardonnay	Unknown	*V.vinifera* L.	France
Gongniang 1	Muscat Hamburg×*V.amurensis*	*V.vinifera* L. ×*V.amurensis*	China, 1951
Beichun	Muscat Hamburg×*V.amurensis*	*V.vinifera* L. ×*V.amurensis*	China, 1954
Beimei	Muscat Hamburg×*V.amurensis*	*V.vinifera* L. ×*V.amurensis*	China, 1965
Xiongyuebaiputao	(Muscat Hamburg× *V.amurensis*) × Longyan	*V.vinifera* L. ×*V.amurensis*	China, 1990
Zuoshan 1	Female of *V.amurensis*	*V.amurensis*	China, 1985
V.amurensis	Wild species	*V.amurensis*	China

Note: the year indicates the time when the cultivar was released

for future grape breeding programs considering the present need of cultivar improvement.

The objectives of the present study are to investigate the genetic polymorphism and relationships among the Chinese grape accessions which include main local grape varieties as well as some newly bred varieties in China and other European or America cultivars, and to determine the geographical difference on genetic diversity of the Oriental grape cultivars by microsatellite markers.

Materials and Methods

Plant material

A total of 45 accessions including *Vitis vinifera*, *Vitis amurensis* and the hybrids of *Vitis vinifera* × *Vitis labrusca* and *Vitis vinifera* × *Vitis amurensis* collected from the national grape germplasm repository of Zhengzhou Fruit Research Institute at Chinese Academy of Agricultural Sciences, were analyzed. A number of most cultivated grape varieties in the world were chosen as a base for comparison. The information of the materials is listed in **Table 1**.

DNA preparation and SSR amplification

For each accession, total genomic DNA was extracted from newly expanded leaves using a mini-extraction kit according to the manufacturer's protocol (BioDev-Tech, Beijing, China) and stored at -20°C until needed. Six highly polymorphic SSR loci as suggested by This et al., (33): VVS2 (35), VVMD5 and VVMD7 (6), VVMD27 (7), VrZAG62 and VrZAG79 (32) and 3 additional markers: VrZAG112, VrZAG47 (32) and SCU06 (30) were used. For each SSR locus, annealing temperatures and number of alleles are shown in **Table 2**.

TABLE 2

Characterization of microsatellite markers used in this study

Locus	Annealing temperature (°C)	Numbers of allele (n)	H_e	H_o	PIC
VVS2	51	8	0.82	0.76	0.79
VVMD5	52	10	0.78	0.73	0.72
VVMD7	51	12	0.86	0.81	0.82
VVMD27	52	8	0.75	0.62	0.68
VrZAG62	52	9	0.82	0.72	0.75
VrZAG79	52	10	0.83	0.79	0.76
VrZAG112	52	10	0.76	0.69	0.71
VrZAG47	52	9	0.80	0.75	0.76
SCU06	50	10	0.78	0.62	0.68

Expected (H_e) and observed (H_o) heterozygosity and polymorphism information content (PIC) of the primers

PCR amplifications were carried out with a total volume of 20 μL, containing approximately 30 ng template genomic DNA, 0.4 μM of each primer, 0.2 mM of each dNTP, 2.0 mM $MgCl_2$ and 1.0U *Taq* polymerase. The PCR protocol consisted of one cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 50 sec, annealing at optimum Ta (**Table 2**) for 50 sec, and extension at 72°C for 1 min. A final extension cycle at 72°C for 8 min followed. DNA was amplified in an MJ Research Tetrad thermocycler (MJ Research Inc., Watertown, MA).

Amplification products from each primer pair were separated on 6.0% denaturing polyacrylamide gel and visualized by silver stain according to the protocol of the Promega kit (Madison, USA).

Data scoring and analysis

Microsatellite data were analyzed using Genealex ver.6.1 software (24) and the average of polymorphism information content (PIC) for each locus, expected (He) and observed (Ho) heterozygosity and allele number per locus were calculated.

For the statistical analysis, the patterns at all SSR loci were scored as 1 for band presence and 0 for band absence. Similarity coefficients based on SSR profiles were calculated according to Nei and Li (23).Genetic similarity data were used to construct dendrograms by unweighted pair group method with arithmetic average (UPGMA) method using the SAHN-clustering and TREE program of the NTSYS-PC software package, version 2.20 (28). Cophenetic correlation between clustering and similarity matrix was calculated to measure the goodness of fit of cluster analysis using COPH and MXCOMP options. Principal co-ordinate analysis (PCoA) was performed based on the similarity coefficients using DCENTER module to transform the symmetric similarity matrix to scalar product form and then EIGEN module was used to extract eigenvectors resulting into a PCOORDA. First three vectors were used to construct a three-dimensional coordinate plot.

Results and Discussion

Polymorphism of the SSR marker

Genetic variation patterns of 45 grape accessions were examined using 9 selected SSR primers. All primers produced clear and reproducible bands. The number of alleles ranged from 8 (in VVS 2 and VVM27) to 12 (in VVMD 7) with a total of 86 alleles and an average of 9.6 alleles per locus (**Table 2**), in agreement with previous analyses (6, 7, 31, 32). Allele numbers, expected and observed heterozygosities are shown in **Table 2**. Expected heterozygosity of the studied loci ranged from 0.75 (locus VVM27) to 0.86 (locus VVM7). The lowest observed heterozygosity was detected at SCU06 locus with 0.62 and the highest one at VVMD7 with 0.81. The mean of observed heterozygosity (Ho) over the ten loci (0.72) was comparable with prior studies (29).

Pairwise comparison was conducted among all the accessions in this study. The pairwise genetic similarity values calculated using Nei and Li coefficients varied from 0.38 ('Queen of vineyard' and 'Cabernet Franc') to 0.83 ('Jingya' and 'Luopuzaosheng') with an overall mean of 0.58, which indicated sufficient diversity among them.

Cluster analysis and principal coordinate analysis

A dendrogram based on the similarity coefficient matrix of 45 accessions was generated using the UPGMA clustering method. All the accession were classified into three main clusters at the similarity level of 0.56 (**Fig. 1**). In the SSR based dendrogram (**Fig. 1**), broad clusterings related to the ecological groups and subspecies were evident, although these had low bootstrap supports (lower than 50% for main branches; data not shown). UPGMA cluster analysis separated the grape accessions into a large cluster of convar. *pontica* (Oriental cultivars), a small cluster of convar. *occidentalis* (Occidental cultivars) and another cluster of Euro-American hybrids (**Fig. 1**) as previously observed in other SSR-based analyses (14) in grape.

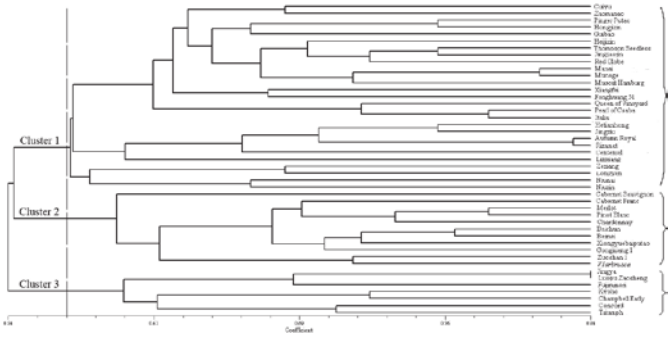

Fig. 1. Dendrograms generated using unweighted pair group method with arithmetic average (UPGMA) analysis, showing relationships between different grape accessions from China and other countries using microsatellite markers

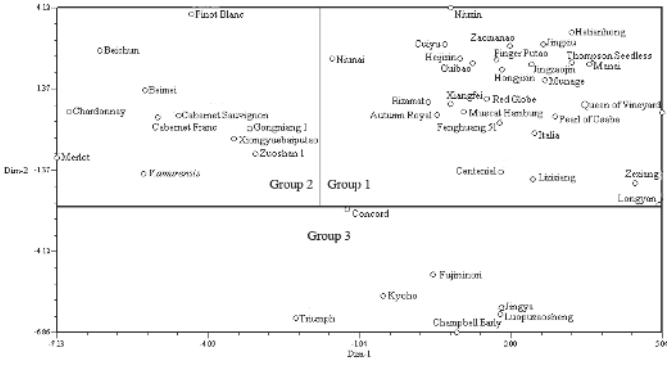

Fig. 2. Two-dimensional projection of the principal component analysis of 45 grape varieties based on 9 SSR markers along the first two principal axes

Cluster 1 is the largest and the most complex cluster, with 26 accessions included. Many important ancient Chinese accessions are in this cluster such as 'Heijixin', 'Lizixiang', 'Longyan', 'Munage' and 'Niunai'. The cluster consists mostly of accessions related to Oriental varieties of *V. vinifera*. Although Cluster 1 could be additionally divided into three subgroups, there were no distinction among the ancient and newly bred varieties in China and the accessions from other countries. They are mixed together and clustered into the subgroups randomly, suggested both their close relationships and a common origin. Some accessions showed some parentage relationships and were indeed grouped together, for example 'Cuiyu' and 'Zaomana' (both are offspring of 'Muscat Hamburg'× 'Jingzaojin'). 'Zexiang' and 'Jingzaojin' clustered closely with one of their parents 'Longyang' and 'Thompson Seedless', respectively. Most of the accessions grouped in agreement with the known pedigree, which indicated the validity of microsatellite markers to differentiate Oriental cultivars. Additionally, the bootstrap values in the branch were mostly below 20 (data not shown). All this resulted in the complex relationship and indicated the sufficient diversity among the accessions.

Cluster 2 consisted of 11 accessions and was characterized by the presence of wine grape from China and Europe. The well known international wine grape varieties such as 'Pinot blanc', 'Cabernet Sauvignon', 'Merlot', 'Cabernet Franc' and 'Chardonnay' grouped in this cluster. Some accessions with the parentage of *V. amurensis* from China were also grouped into this cluster. *V. amurensis* belongs to the East-Asia species group which is far from *V. vinifera* L. and mainly used as wine grape. Some valuable genetic resource of *V. amurensis* such as 'Zuoshan 1' which is a pistillate flower grape was explored in China. The other bisexual *V. amurensis* flowers accessions were bred and cultivated also widely in China. Wine grapes including Chinese and European accessions (Cluster 2) were significantly differentiated from table grapes (Cluster 1), as suggested by Aradhya et al. (4) who distinguished essentially French wine grapes from Eastern European table and dual-use grapes and Heuertz et al. (17), who found the use of table or wine grapes as a more important criterion than geographical origin for genetic differentiation.

Cluster 3 is characterized by the presence of one important accessions of *V. labrusca* L. ('Concord') together with some Euro-American hybrids ('Champbell Early', 'Triumph', 'Kyoho' and 'Fujiminori'). Sports are presumed to be the result of a mutation that results in a small difference in an otherwise identical cultivar. Microsatellite markers have at times been successfully and other times unsuccessfully used to discriminate clones (11, 26). 'Luopuzaosheng' is a sport of 'Jingya' selected in China and both of them are also in this cluster. They couldn't be distinguished by microsatellite markers in this study as shown in the dendrogram.

The correlation between the similarity coefficient matrix and the cophenetic matrix derived from the tree produced by UPGMA was almost 80% indicating a good fit of the cluster analysis. However, only a few nodes in the dendrogram were supported by large bootstrap values.

Previous researches (13, 14, 15, 16) reported that Oriental and Occidental cultivars formed different clusters based on microsatellite data from a limited number of cultivars. Oriental cultivars form a separate cluster from that of Occidental cultivars and these two clusters together form a higher cluster of *V. vinifera*. In this study, 26 Oriental accessions were employed and all of the accessions were closely grouped in cluster 1; Occidental cultivars grouped into cluster 2. This result was in agreement with the classification of species. This classification is supported by a cluster analysis of 222 cultivars of *V. vinifera*

based on simple sequence repeat (SSR) analysis (4). The UPGMA analysis (**Fig. 1**) confirmed the genetic divergence mentioned above. The results showed that Oriental cultivars have a certain degree of genetic difference from Occidental cultivars within the species *V. vinifera*.

The principal coordinate analysis (PCoA) further helped depicting the variability among these accessions in three-dimensional modes. Principal coordinate analysis was likewise performed based on the similarity matrix. The plot of first two coordinates for SSR analysis are given in **Fig. 2**. The classification of tested accessions derived from PCoA was similar to that of UPGMA analysis. Group 1-3 of the plot from PCoA exactly corresponded to Cluster 1-3 in the dendrogram from UPGMA analysis. The results showed a better correlation between genetic diversity and geographic origins of the accessions of *V. vinifera*, compared to those of UPGMA analysis. The first and second coordinates explained 6.8% and 6.0%, respectively, of the total variation. The first three eigen vectors accounted for 25.63% of the observed variation.

In this study, we fingerprinted a set of 45 grapes accessions by means of well-characterized microsatellite markers, in order to assess their genetic variability and relationships. We have thus demonstrated the usefulness and the reliability of microsatellite markers isolated from European cultivars in the genetic analysis of Oriental and East-Asia grape accessions. The present study demonstrates that SSRs are effective markers for assessment of genetic diversity in Oriental accessions of *V. vinifera*. The 9 microsatellite primers amplified high number of alleles in the studied here loci (**Table 2**). Other similar works involving the analysis of microsatellites in grape have also detected loci with highly variable numbers of alleles (4, 6, 31).

Conclusions

Based on cluster analysis and principal coordinate analysis, the results indicated that all accessions could be divided into three major groups and the clustering pattern was related to their ecological origin. Wine grapes were significantly differentiated from the table grapes no matter whether the accessions were from China or other countries. Chinese wine grapes which have the parentage of *V. amurensis* (East-Asia species group) grouped closely with wine grapes and indicated that there were very large genetic differences between table grape and wine grape.

The results of the present work elucidate the genetic relationships among the accessions of regional interest and further demonstrate that some genetic diversity of grape is still unexploited in China. Given the importance and the ancient origin of Chinese accessions, further investigation should be carried out to clarify their origin and evolvement.

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