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Genetic diversity and population structure analysis in wild strawberry (*Fragaria nubicola* L.) from Motuo in Tibet Plateau based on simple sequence repeats (SSRs)

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ABSTRACT

Germplasms resources of wild strawberry are rich in Motuo country of Tibet, China. In this study, we assessed genetic diversity of seventy wild strawberry germplasms from different geographical regions using simple sequence repeat (SSR) markers. The genetic diversity of wild strawberry was demonstrated by 189 polymorphic SSR-PCR bands obtained using ten selective primers. Additionally, the average polymorphic information content (*PIC*), total gene diversity and population diversity were 0.941, 0.331 and 0.214, respectively. At the population level, variation of strawberry accessions among population was higher than that within population. Based on arithmetic mean (UPGMA) dendrogram method, all samples were clustered into 6 groups and two subgroups. Combined with the results of UPGMA and Principle coordinate analysis, wild strawberry accessions tended to group by geographic origin. Thus, these findings will benefit for the protection and exploitation of wild strawberry, and provide theoretical basis for further study in the origin and phylogenetic systematics of wild strawberry.

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Strawberries (*Fragaria ananassa* Duch.) belong to the *Rosaceae* family and the *Fragaria* genus, which are important fruit throughout the world. Especially in China, 11 strawberry species are widely distributed in many provinces; therefore, it has rich germplasm resources. In China, the related work of breeding strawberry varieties had started from 1948 (Wang et al., 2008). However, cultivated strawberry species having narrow genetic base are lack for pest resistance and abiotic stress tolerance (Wang et al., 2008; Diamanti et al., 2012). In Motuo of Tibet (MT), heterogeneous environmental conditions always attracted more attention by researches (Liu et al., 2014). Accordingly, unique alpine environments have a strong influence on development and evolution of plant species. Thus, most alpine plants have better characteristics to survive from harsh climates at high altitude. In addition, this environment can contribute to form richness of plant species. In our previous study, we also found that there are rich strawberries resources in MT (Wang, 2014).

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Accordingly, we need make use of wild strawberry resources having novel traits to improve cultivated strawberry species. As extremely environmental conditions, there are few studies on wild strawberries from MT. Some previous study provided information on morphological properties of wild strawberries from MT, suggesting significant heterogeneity among resources (Wang, 2014). However, the information on genetic diversity and variation of morphological characteristics is limited and probably influenced by environmental factors. Therefore, we made use of SSR markers to assess genetic diversity of seventy wild strawberry germplasms from seven different locations in MT. And the results would be utilized for further germplasm conservation and breeding strategy in strawberry.

Nowadays, various molecular markers were used for analyze genetic diversity of plant species. Of these markers, simple sequence repeats (SSRs) has now become in fashion among these researches, and been used for measuring genetic diversity in many plant species (Da Cunha et al., 2014; Liu et al., 2014; Yook et al., 2014). For example, genetic diversity of 134 strawberry cultivars introduced since 1960s was analyzed (Sjulin and Dale, 1987). In addition, Amaya (2009) analyzed EST-SSRs diversity in 92 selected strawberry cultivars with widely diverse origins, and the results suggested breeding has produced a small but significant reduction on the genetic diversity of these cultivars. Furthermore, Tyrka et al. (2002) also reported a lower diversity and small genetic variation in 19 strawberry, with 15 originating from Europe and 4 from Japan or Canada.

Genetic diversity is the basis and most important components of biological diversity and it can be used to research population variation and maintain genetic variation (Zhang et al., 2011). Recently, much attentions have been paid to genetic diversity of wild species (Chen et al., 2014; Zhao et al., 2014). As we know, a wide range of variation could be found in strawberry species. Abundant genetic variation laid the foundation for varieties formation on one hand, and on the other hand, it also caused difficulties in species classification, phylogenetics and system evolution. Additionally, narrow genetic base could result in vulnerability to diseases, pests and environmental stresses in plants (Graham et al., 1996). Therefore, here, we analyzed genetic diversity of wild strawberry resources from MT using SSR markers. These findings will benefit for the protection and exploitation of wild strawberry, and provide theoretical basis for further study in the origin and phylogenetic systematics of strawberry.

1. Materials and methods

1.1. Plant materials and DNA extraction

A total of seventy strawberry (*Fragaria nubicola* L., $2n = 2x = 14$) samples exploited in this study were divided into seven groups on the basis of different geographical locations in Motuo County of the Tibet Plateau, China. All the detailed information of sampling sites has been listed in Table 1. Leaf samples of each individual were collected randomly and stored in −80 °C. Genomic DNA was extracted from preserved leaf tissues according to Doyle (1990). The concentration of extracted DNA was measured and purified. Finally, DNA samples were diluted to a uniform concentration of 50 ng/μl for the subsequent use.

1.2. SSR assays

Nineteen SSR primer pairs were used to characterize all samples based on good amplification, reproducible and high polymorphism bands. SSR-PCR conditions were performed in 25 μl volume containing 50 ng DNA templates, 10 pmol primer, 1 × PCR Buffer (including Mg^{2+}), 0.25 mM of *dNTPs* and 0.5 U *Taq* DNA polymerase (TakaRa, China). The reaction condition was programmed with 5 min at 94 °C for pre-denaturing, 40 cycles of 45 s at 94 °C, 45 s at a primer-appropriate temperature, 2 min at 72 °C, and a final cycle of 8 min at 72 °C. The amplification products were detected by 8.0% non-denaturing polyacrylamide gels, and visualized by silver staining protocol (Zhou et al., 2011).

1.3. Data analysis

Only reproducible, clear background and well-resolved fragments were scored as the presence (1) or absence (0). We estimated the genetic variations among all accessions, including Observed number of alleles (*Na*), Effective number of alleles (*Ne*), Nei's genetic diversity (*H*), Shannon's information index (*I*), Number of polymorphic loci and Percentage of polymorphic loci (*PPB*) according to Nei (1973). To explore the genetic variability among seven populations, total gene diversity (*Ht*), population diversity (*Hs*), inter-population differentiation (*Gst*) and gene flow (*Nm*) were calculated according to Nei (1973).

Table 1
Accession, sample size, code, locations, sampling altitudes of 70 wild strawberry accessions analyzed by simple sequence repeat markers (SSR).

Accession no.	Size	Code	Location	Altitude (m)
A	6	A1-A6	Motuo, Tibet Plateau	2130
B	9	B7-B15	Motuo, Tibet Plateau	2530
C	15	C16-C30	Motuo, Tibet Plateau	2630
D	12	D31-D42	Motuo, Tibet Plateau	3030
E	9	E43-D51	Motuo, Tibet Plateau	3230
F	5	F52-F56	Motuo, Tibet Plateau	3630
G	14	G57-G70	Motuo, Tibet Plateau	3730

The resolving power (*Rp*) was used to assess the power of the primers to discriminate all individuals (Prevost and Wilkinson, 1999). In addition, the degree of polymorphism for each SSR primer pairs was estimated based on the polymorphic information content (*PIC*), total number of fragments (*TNF*), number of polymorphic fragments (*NPF*), percentage of polymorphic fragments (*PPF*) (Botstein et al., 1980). The molecular genetic variation among and within populations were analyzed with analysis of molecular variance (AMOVA). To illustrate the genetic relationship of samples, principal coordinate analysis (*PCoA*) and unweighted pair group method with arithmetic mean (UPGMA) were obtained by Nei (1973).

2. Results

In this study, ten of 19 SSR primer pairs were finally used for SSR analysis (Table 1). Based on 10 SSR locis, a total of 189 bands were detected among the 70 wild strawberry samples. The number of bands per primers ranged from 12 (EFMvi136) to 24 (Fa1A-6 and SF-5-G02) with an average of 18.9 per primer, showing that these wild strawberry germplasms possessed a high level of genetic diversity. All SSR primer pairs were highly polymorphic (100%), and *PIC* values were from 0.904 to 0.993 with a mean of 0.941. The *Rp* value for the SSR locis was revealed with the highest in Fa1A-6 primer (21.03) and lowest in FxaAGA21F11 (1.54) (Table 2).

Genetic diversity levels of 70 samples were tested according to geographical area. And the results listed in Table 3, which showed clear differences in number of polymorphic loci and *PPB*. Interestingly, four indexes, *Na*, *Ne*, *H* and *I*, were highest in C group (1.788, 1.483, 0.280 and 0.418) and lowest for accessions from F population (1.344, 1.243, 0.137 and 0.201) (Table 3). The *Ht* and *Hs* among seven groups was 0.331 and 0.214, respectively, stating that a low level of heterozygosity in the all materials (Table 4). The mean value of the *Gst* was 0.354. At the population level, AMOVA was performed to analyze the genetic variation among 70 different samples according to their locations. And total variation from among population was 77%, 23% variation was from within population. Additionally, estimate of *Nm* was 0.914, manifesting clearly that gene exchanges among strawberry in MT was limited.

Cluster analyses were also performed using the UPGMA method according to Nei's genetic distance. All samples were clustered into 6 groups (Group I, Group II, Group III, Group IV, Group V and Group VI) (Fig. 1). Most of these samples clustered were strongly related to geographic origin. In addition, the *PCoA* analysis clearly divided the 70 strawberry individuals into six groups, which was in agreement with the UPGMA cluster analysis (Fig. 2).

3. Discussions

The wild strawberry from MT of Tibet is valuable resources of genetic determinants to resist disease and tolerance stress. However, despite intensive use of this wild strawberry, the progress of genetic research of this species has lagged behind that of many other crop species, because there are harsh alpine habitat, cold climate and lower temperatures across the Tibetan Plateau. Accordingly, it is difficult to collect these samples for researchers. Thus, it is necessary to determine genetic diversity

Table 2
Description of 10 simple sequence repeat primer combinations used in the analysis of 70 wild strawberry accessions and number of total bands, polymorphic bands, polymorphic rates, resolving power (*Rp*), polymorphic information content (*PIC*) estimated in all accessions.

No.	Primer pair	Sequence (5′–3′)	Tm (°C)	Number of total bands	Number of polymorphic bands	Polymorphic rates (%)	*Rp*	*PIC*
1	UFFa01E03	F: ACCCCATCTTCTTCAAATCTCA R: GACAAGGCCAGAGCTAGAGAAG	59	19	15	79	2.60	0.940
2	PBCESSRFXA9	F: TGACAAACATTCAACCACAC R: GTGCCCTCAGAAGACTACC	58	21	19	90	4.58	0.949
3	Fa1A-6	F: CAGTTTGCCCAACAACAAGG R: TTGATGGCAACAAATCACG	60	24	23	96	21.03	0.952
4	SF-5-G02	F: CCACCCTCCAATATAACCC R: AGGAGAACCAAGATTAAGCC	58	24	22	92	5.19	0.949
5	EMFn170	F: AAATCCTGTTCCTGCCAGTG R: TGGTGACGTATTGGGTGATG	60	19	18	95	4.74	0.939
6	FAC-001	F: CTTTTGCTGCTAGCTCTTTGTG R: TACGTACTCCACATCCCATTTG	64	20	17	85	7.35	0.993
7	Fa3C-2	F: TCTGCTTCTCTTGAACTGG R: GTATCTGGAGCCAAGAGG	56	20	20	100	3.68	0.946
8	Fa4A-1	F: AGGACAACTTCGAGAAGG R: CGAATTCGCTCTTCACAG	54	14	11	79	4.16	0.908
9	FxaAGA21F11	F: CAATTCACAATGGCTGATGACGAT R:GCACTCAGACATATTTTGGGAGGG	70	16	13	81	1.54	0.925
10	EFMvi136	F: GAGCCTGCTACGCTTTTCTATG R: CCTCTGATTCGATGATTTGCT	63	12	10	83	3.07	0.904
	Total	–	–	189	168	–	5.79	0.941
	Mean	–	–	18.9	16.8	88.9	57.9	9.41

Table 3
The information on observed number of alleles (*Na*), effective number of alleles (*Ne*), Nei's genetic diversity (*H*), Shannon's information index (*I*), number of polymorphic loci, percentage of polymorphic loci (*PPB*) for all wild strawberry populations by simple sequence repeat markers (SSR).

Population no.	Observed no. of alleles (*Na*) (mean ± SD)	Effective no. of alleles (*Ne*) (mean ± SD)	Nei's genetic diversity (*H*) (mean ± SD)	Shannon's information index (*I*) (mean ± SD)	No. of polymorphic loci	Percentage of polymorphic loci (*PPB*)
A	1.609 ± 0.490	1.382 ± 0.394	0.218 ± 0.107	0.323 ± 0.093	115	60.9%
B	1.524 ± 0.501	1.352 ± 0.417	0.195 ± 0.036	0.287 ± 0.104	99	52.4%
C	1.788 ± 0.410	1.483 ± 0.362	0.280 ± 0.187	0.418 ± 0.260	149	78.9%
D	1.646 ± 0.480	1.413 ± 0.384	0.238 ± 0.103	0.353 ± 0.188	122	64.6%
E	1.460 ± 0.500	1.256 ± 0.348	0.152 ± 0.090	0.230 ± 0.175	85	46.0%
F	1.344 ± 0.476	1.243 ± 0.366	0.137 ± 0.099	0.201 ± 0.185	65	34.4%
G	1.736 ± 0.454	1.455 ± 0.374	0.264 ± 0.195	0.393 ± 0.275	141	74.0%
Average	1.587	1.369	0.212	0.315	111	58.7%

Table 4
Total gene diversity, population diversity, inter-population differentiation (*Gst*), estimate of gene flow (*Nm*) seven populations of strawberry using simple sequence repeat primers.

Total gene diversity (*Ht*)	Population diversity (*Hs*)	Inter-population differentiation (*Gst*)	Estimate of gene flow (*Nm*)
0.331 ± 0.032	0.214 ± 0.017	0.354	0.914

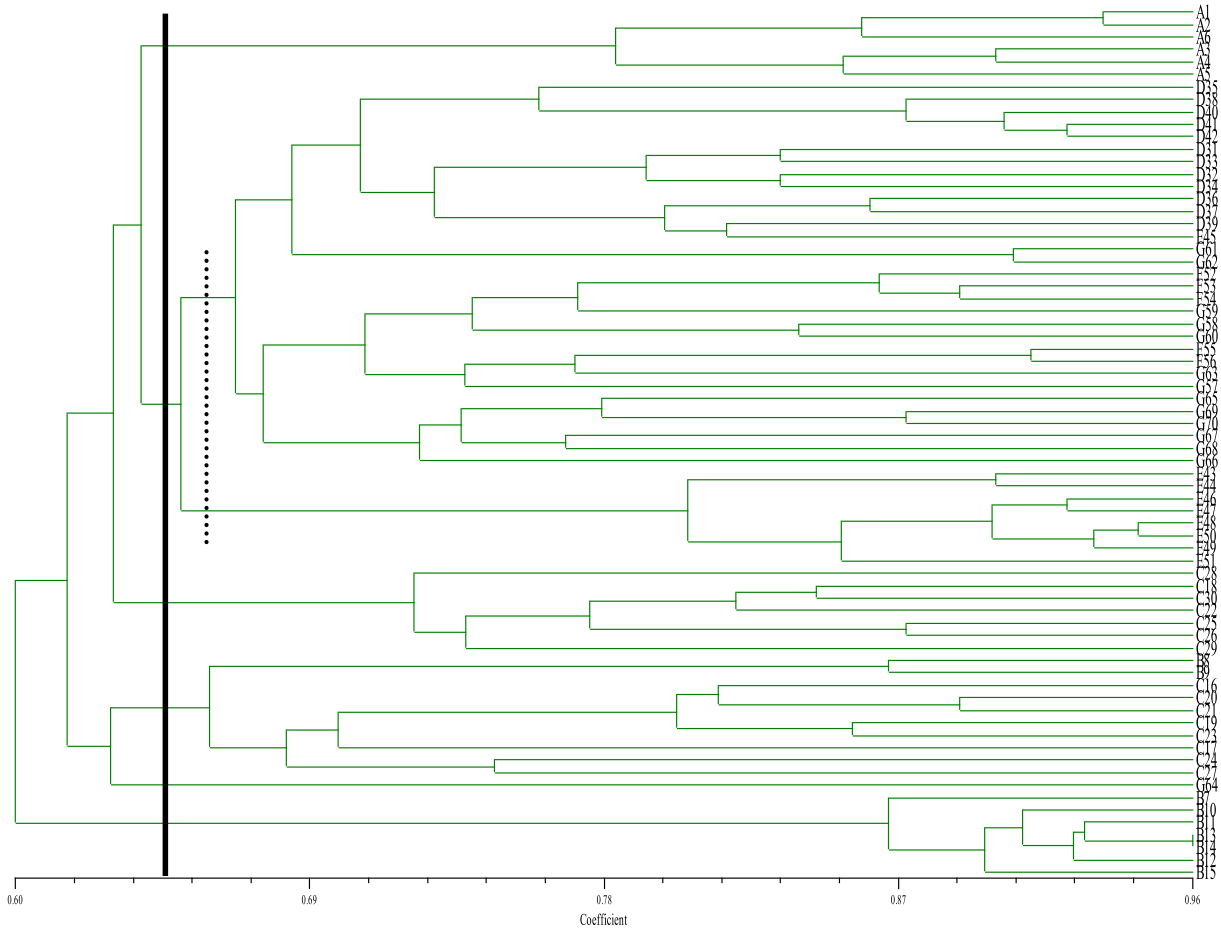

Fig. 1. Dendrogram of 70 wild strawberry accessions generated by nuweight pair group method with arithmetic mean cluster analysis based on 10 SSR primers. Simple similarity values are given at the bottom of the dendrogram. Numbers on the right of the dendrogram indicate the 70 strawberry accessions and correspond with Table 1. Black solid line indicate the groups, and imaginary line represent the subclusters.

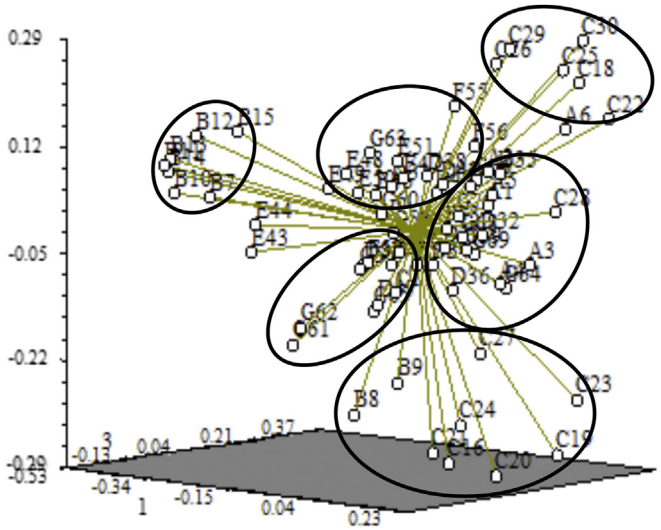

Fig. 2. Principle coordinate analysis of 70 wild strawberry accessions based on simple sequence repeat markers. The numbers at each dot indicates the 70 accessions and correspond with Table 1. The accessions within the six groups (six circles) are identical to those in the dendrogram constructed by unweight pair group method with arithmetic mean.

of wild strawberry from MT. In our study, simple sequence repeats are used for identification of wild strawberry accessions from MT.

In general, precise identification of genetic diversity is essential for understanding plant species evolution and adaptation (Amos and Harwood, 1998; Jiang et al., 2012). High level in genetic variability was seen as healthy and adapt to the environmental conditions (Amos and Harwood, 1998; Peng et al., 2015). Here, a high genetic diversity (*PPB* = 88.9%) in wild strawberry from MT was obtained based on SSR technique. This result is not consistent with the study of Graham et al. (1996), who reported a lower genetic diversity in strawberry from Europe revealed using RAPD (*PPB* = 68%). In contrast with this result, a relatively higher genetic variability (*PPB* = 84.6%) was found in strawberry (*F. ananassa* Duch.) from United States and Canada using AFLP marker (Degani et al., 2001). Combined the results based on these different analytical methods, it is reasonable to use multiple tools to analyze genetic diversity of plant species. In addition, the high level of genetic diversity for wild strawberry from MT maybe attribute to the extreme and unique environments in MT, which contribute to form a stress inducing in genetic diversity of plant species. Furthermore, there is lack of interference by the human activities, resulting that potential gene flow is limited. Thus, wild strawberry in MT harbors high genetic diversity. From the present data, it is clear that wild strawberry from MT has rich genetic diversity. However, there is little information on comparison of genetic distances of relationship between wild strawberry and other strawberry species. In addition, there are few morphological and cytogenetical on wild strawberry from MT. On the face of it, therefore it is important and significant based on DNA molecular markers combined with multiple approaches.

To assess genetic variation in strawberry, morphological and physiological methods were used in the previously reports. For example, Dai et al. (2007) investigated 20 strawberry germplasm resources from Changbai Mountains and classified them into three species according to plant height, soluble solid content and chromosome number of root tip. However, it is important to notice that identification of germplasm resources only using these methods was difficult due to ecological plasticity of the morphological characters and varying environmental conditions. But various molecular marker techniques based on DNA can overcome these shortcomings. Accordingly, these markers provide accurate genetic information for breeding and collecting germplasm. To date, many molecular makers had been widely used for the identification of genetic relationship in strawberry including wild and cultivated. For example, Dong et al. (2011) analyzed the genetic relationship of 20 strawberry cultivars using EST-SSR markers. Moreover, Zhang et al. (2006) explored genetic diversity and relationship of 107 strawberry cultivars using AFLP, and the results showed cluster in line with genealogical relationship. As co-dominant marker, SSR markers offers more advantages, higher polymorphism and more discriminative than other techniques (Yao et al., 2007). Therefore, in our study, we combined with the methods of AMOVA, structure and UPGMA dendrogram to analyze the 70 accessions relationship from different geographic origin (Figs. 1 and 2).

Nybom (2004) found that effects of AMOVA-derived estimates for population diversity were related to life form categories, breeding system, seed dispersal and successional taxa. Generally, annual and selfing plant species spreading pollen by gravity had lower genetic variation than long-lived, outcrossed and animal-dispersed ones (Jiang et al., 2012). In MT, gene flow of wild strawberry only depends on seeds and pollen, which reduces the genetic differentiation among populations. However, this phenomenon was not verified by *Nm* value (0.914) (Table 4). This reason need to be further analyzed in later experiment. In contrast, wild strawberry from MT should have rich genetic base. The data presented in this paper showed that genetic variation among populations on wild strawberry from MT was 77%, which may be due to its widely geographical distribution and isolation at high level (Debnath et al., 2012). In addition, population size, spatial isolation and the lack of gene exchange

between populations can also contributed to form the high genetic variation (Diamanti et al., 2012). Accordingly, in this study, seventy wild strawberry resources form different geographic populations harbor rich genetic diversity, and this information may be invaluable for research in the origin and phylogenetic systematics of wild strawberry.

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