African Journal of Biotechnology Vol. 11(22), pp. 6011-6017, 15 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1685
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Genetic variation patterns of *Medicago ruthenica* populations from Northern China

Rong Yu-ping[1]*, Cao Zhe[1], Zhu Ling-ling[1], Zhao Min[1], Zhang Yong-juan[1] and Bai Ke-yu[2,3]

[1]Department of Grassland Science, Animal Science and Technology College, China Agricultural University, Beijing 100193, China.
[2]Institute of Agricultural Resources and Regional Planning, China Academy of Agricultural Sciences, Beijing, 100081, China.
[3]Bioversity International Sub-regional Office for East Asia, Beijing, 100081, China.

Accepted 28 February, 2012

As a promising legume for forage in arid and semi-arid areas, *Medicago ruthenica* (L.) Trautv. is widely distributed in northern China. For this study, twenty four accessions from northern China were analyzed by allozyme and microsatellites markers to determine its genetic variation patterns, two assays were found different in the amounts of polymorphism. Higher amounts of polymorphism were detected by microsatellites. For allozymes and microsatellite markers, mean expected heterozygosity (He) of 24 populations ranged from 0.160 to 0.485 and 0.274 to 0.5316 respectively. Mean observed heterozygosity per locus (Ho) of 24 populations was 0.422 for allozymes and 0.4149 for simple sequence repeats (SSR) markers. For allozymes, average interpopulation genetic diversity (Hs) was 0.2733 and average intrapopulation genetic diversity (D_{ST}) was 0.0473, mean coefficient of gene differentiation for loci (G_{ST}) at the population level was estimated 0.1813. According to G_{ST}, gene flow at population level was 1.4427 for allozymes and 1.2040 for SSR markers, which indicated there was definite gene flow between populations. Low differentiations among populations at allozymes (F_{ST} = 0.1719) and SSR markers (F_{ST} = 0.1816) means genetic variations is mainly lied in populations. The results showed there was high genetic diversity in *M. ruthenica* populations.

Key words: *Medicago ruthenica*, genetic diversity, allozyme, micro-satellite, North China.

INTRODUCTION

Medicago ruthenica (L.) Trautv. a diploid (n = 2x = 16) perennial legume species widely distributed in Siberia, Mongolia and northern China, commonly grows on open hillsides, mixed grass steppes, sandy land and meadows (Small and Jomphe, 1989). It has long been placed in genus of *Trigonella* and *Melilotoides* because of the major morphological differences with other species of the *Medicago sativa* complex (Lesins and Lesins, 1979), however, taxonomists put it into section Platycarpae of *Medicago* according to the genetic analysis recently (Small and Jomphe, 1989).

This species is known to possess useful genes for dry, stony site with extremely low snowfall and very cold winters, and is therefore regarded as a promising legume for forage in arid and semi-arid areas (Wu et al., 1996; Campbell et al., 1997, 1999). However, the habitats of *M. ruthenica* undergo fragmentation in recent years due to grassland degradation and reclamation in northern China which could result in the loss of genetic diversity (Wu and Ci, 2002; Li et al., 2005). Maintenance of genetic diversity within populations is currently regarded as a primary goal in conservation efforts, as it will enhance the threatened species to adapt to future environmental changes (Bemadette et al., 2003; Jump and Penuelas, 2005). A preservation of natural populations and utilization of its germplasm resources successfully require a good understanding of their genetic variation pattern (Pressoir

*Corresponding author. E-mail: rongyuping@cau.edu.cn.

Abbreviations: **D_{ST},** Genetic diversity; **Hs,** interpopulation genetic diversity; **He,** expected heterozygosity; **G_{ST},** coefficient of gene differentiation for loci; **SSR,** simple sequence repeats.

Table 1. *Medicago ruthenica* collections used in this study, together with a description of their origin.

Origin	Collections	Altitude (m)	Longitude	Latitude	Habitat
Wuchuan county, Inner mongolia	HS	1737	111°28′55.4″	41°02′42.6″	Roadside
Abaga county, Inner mongolia	AQ1	1083	114°33′38.8″	43°56′09.9″	Desert steppe
	AQ2	1213	114°56′12.3″	44°15′03.5″	Dersert steppe
Xilinhot, Inner mongolia	XL	1149	115°48′30.2″	43°55′54.5″	Steppe
Dongwu county, Inner mongolia	DW	1010	116°45′49.1″	45°39′47.8″	Meadow steppe
	DB	967	117°59′28.9″	46°05′25.8″	Meadow steppe
	DM	859	118°37′58.7″	46°21′34.6″	Meadow
Yiershi county,Inner mongolia	YS	891	119°59′02.5″	47°19′54.9″	Meadow
Xinbaer county, Inner mongolia	XB	976	119°37′10.6″	47°24′26.2″	Forest meadow
Ewuke county,Inner mongolia	EG	703	118°59′52.4″	48°54′58.4″	Steppe
Chenbaer county, Inner mongolia	XS	602	119°40′54.4″	49°27′56.8″	Meadow
Eerguna county, Inner mongolia	EH	507	119°21′24.8″	50°12′26.3″	Meadow
Alukeer county, Inner mongolia	AL	364	120°22′25.9″	44°13′15.0″	Sandy land
Balinyou county, Inner mongolia	BL	708	119°01′26.1″ 4	43°40′02.8″	Steppe
Linxi county, Inner mongolia	LX	618	118°02′03.2″	43°14′15.0″	Steppe
Keshiketing county, Inner Mongolia	KJ	1304	117°03′31.7″	43°03′43.7″	Shrubland
	KH	1313	116°45′34.7″	42°56′51.7″	Sandy Shrubland
Lan county, Inner mongolia	ZS	1314	115°56′55.9″	42°40′00.3″	Steppe
	LQ	1362	116°13′16.5″	42°15′17.5″	Shrubland
	LH	1353	115°33′45.2″	42°11′05.9″	Shrubland
Guyuan county, Hebei province	HB1	1050	116°14′25.3″	41°37′32.1″	Swamp meadow
	HB2	1120	115°39′48.9″	41°45′56.2″	Steppe
	HB3	1126	115°40′13.6″	41°46′24.8″	Pen steppe
	HB4	1133	115°40′51.2″	41°46′11.3″	Steppe

and Berthaud, 2004; Badri et al., 2008).

A description of isozymes or allozymes for *M. ruthenica* has been done by many studies (Yang et al., 1994; Xia et al., 1997, 1998). Most of the allozymes studies were based on the banding patterns (Yang et al., 1994; Xia et al., 1998). Morphological polymorphism of 50 *M. ruthenica* accessions were evaluated in the field and represented considerable exploitable genetic variation (Campbell et al., 1997). Therefore polymorphism variation based on molecular markers of the population genetic characteristics may further promote its application in *M. ruthenica* characterization and germplasm conservation.

In this study, we analyzed the intra and inter-population genetic variation of *M. ruthenica* using six polymorphic allozyme loci and five polymorphic simple sequence repeats (SSR) markers. The study aims to: (1) investigate microsatellite and enzyme polymorphism and population structure of *M. ruthenica* and (2) examine the consistency of results from allozyme and microsatellite analysis.

MATERIALS AND METHODS

Plant materials and DNA extraction

Seeds of 24 natural populations of *M. ruthenica* (L.) Trautv., were collected from various locations at different elevation and habitat from Inner Mongolia plateaus and Great Xing'an Mountains, China. The information about the collections of *M. ruthenica* was shown in Table 1. Seed was sown with 10 plants of each collection in a pot experiment in the greenhouse at the Institute of Grassland Sciences, China Agricultural University, Beijing. Fresh leaf tissue of 10 individuals of each plant was bulked for DNA extraction. The procedure used for total genomic DNA extraction was followed by Rogers and Bendish (1988) modified protocol by Gherardi et al. (1998).

Allozyme analysis

Six enzymes systems were examined with 10 plants of each population, four of which, peroxidase (PRX, EC I.II.1.7), superoxide dismutase (SOD, EC1.15.1.1), esterase (EST EC3.1.1.1), and

Table 2. Gene diversity of 24 populations in *M. ruthenica* at 6 polymorphic locus.

Loci	N	H_T	H_S	D_{ST}	G_{ST}	F_{ST}	N_M
POD-1	240	0.4485	0.3465	0.1020	0.2274	0.2276	0.8485
POD-2	240	0.3073	0.2481	0.0592	0.1926	0.1925	1.0486
EST	240	0.1316	0.0817	0.0498	0.3787	0.3796	0.4086
MDH	240	0.4250	0.3790	0.0460	0.1082	0.1082	2.0612
SOD-1	240	0.4980	0.4898	0.0082	0.0165	0.0166	14.833
SOD-2	240	0.1135	0.0948	0.0187	0.1648	0.1651	1.2645
Mean	240	0.3206	0.2733	0.0473	0.1813	0.1816	1.4427

N, The number of sample; H_S, gene diversity within populations; H_T, total gene diversity; D_{ST}, gene diversity among populations; G_{ST}, the proportion of the total genetic diversity partitioned among population; F_{ST}, the proportion of total genetic diversity partitioned among population; N_M, the gene flow estimate according to G_{ST}.

malate dehydrogenase (MD, EC1. 1. 1. 37), were used in this study due to their activity, high resolution and polymorphism. Methods of enzyme extraction, electrophoresis and enzyme staining were the same as those described in (Wang, 1996). In the current study, each locus was numbered sequentially, beginning with the most anodal migrating locus designated as 1, the next 2, and so on (for example, POD-1, POD-2, etc.).

Microsatellite analysis

Microsatellite variation were observed by 10 primer pairs specific for *M. sativa*, that is, afat15, afca1, Afcall, afca16, afct11, afct32, afct45, afct60, afctt1 and mtlec2a described by Diwan et al. (2000). Five microsatellite markers were isolated from these ten primer pairs because of stable and clear bands. Amplification products were separated by 6% denaturing polyacrylamide gel. After electrophoresis, the gel was stained with silver, following the method of Echt et al. (1996). Band and band size were detected with software Quantity-one (Bio-Rad. Hercules, CA, USA) on the Bio-Rad gel photographer system. The PCR system of each primer pairs were slightly modified accordingly from their origins (Diwan et al., 2000).

Statistical analysis and data treatment

Inter and intra-population genetic variation of *M. ruthenica* was calculated for allozyme locus and for SSR markers by three parameters: the mean number of alletes per locus (A), the proportion of polymorphic loci (P) and gene diversity index (He). 'He' is equivalent to the proportion of loci heterozygous per individual under Hardy-Weinberg expectations (= expected heterozygosity) and was calculated by the unbiased method of (Nei, 1973, 1978) which adjusts for sample sizes.

At each polymorphic locus, the total gene diversity is presented by H_T, which is partitioned into the mean allelic diversity within populations (Hs) and the allelic diversity among populations (D_{ST}). The proportion of total genetic variation found among populations (G_{ST}) is calculated as the ratio, D_{ST}/H_T. F_{ST} was used as measure of genetic differentiation among populations and was estimated according to Weir and Cockerham (1984) method. Overall F_{ST} among populations were computed, running the FSTAT program (Goudet, 1995). Gene flow (Nm) = (1- D_{ST})/ $4G_{ST}$ (Slatkin and Barton, 1989). All data analyses were performed against each sample using the software package Popgene, version 1.32 (Yeh et al., 1999).

RESULTS

Allozyme analysis

Six loci showed clear and consistent banding patterns: POD-1, POD-2, EST-1, MDH-1, SOD-1 and SOD-2, which have two locus of a and b. Banding patterns for the polymorphic loci were those expected plants, with POD-2a, EST-b and SOD-2b, which appeared to be the highest frequency among 24 populations, MDH-a, SOD-1a and SOD-1b were distributed evenly among populations. EST-a and SOD-2a were rare locus which presented only in four populations (HB1, DM, EH and LH) and seven populations (EH, EG, XB, DB, LH, AQ1 and YS) respectively. All 12 alleles were included in population LH and EH. The number of alleles at each polymorphic locus and gene diversity statistics at different loci were based on allozyme analysis of 240 individuals in each natural population of *M. ruthenica* (Table 2).

The SOD-1 locus indicated the highest level of diversity (H_T = 0.4980) followed by POD-1 (H_T = 0.4485) and MDH (H_T = 0.4250), while the rest of loci were below the average (H_T < 0.32). The mean intrapopulation gene diversity (Hs) was 0.2733, with a range from 0.087 (EST) to 0.4898 (SOD-1). The coefficient of gene differentiation (G_{ST}) for all polymorphic loci was 0.1813 ranging from 0.0165 (SOD-1) to 0.3787 (EST). This result reveals that, the mean total genetic diversity (H_T) observed at the polymorphic loci across 24 populations was 0.3206, proportionately 0.0473 resided among populations (D_{ST}) while 0.2733 was found within populations (Hs). Based on G_{ST} calculations, the number of migrants per generations among populations (Nm) was 1.4427, reflecting high gene flow among population. The proportion of the total genetic partitioned among population (F_{ST} = 0.1816) which had the similar meaning with G_{ST}, was consistent with G_{ST}.

Nei's (1973) gene diversity at populations level was calculated (Table 3). Among individual of each population, the proportion of polymorphic loci ranged from 33 to

Table 2. Gene diversity of 24 populations in *M. ruthenica* at 6 polymorphic locus.

Loci	N	H_T	H_S	D_{ST}	G_{ST}	F_{ST}	N_M
POD-1	240	0.4485	0.3465	0.1020	0.2274	0.2276	0.8485
POD-2	240	0.3073	0.2481	0.0592	0.1926	0.1925	1.0486
EST	240	0.1316	0.0817	0.0498	0.3787	0.3796	0.4086
MDH	240	0.4250	0.3790	0.0460	0.1082	0.1082	2.0612
SOD-1	240	0.4980	0.4898	0.0082	0.0165	0.0166	14.833
SOD-2	240	0.1135	0.0948	0.0187	0.1648	0.1651	1.2645
Mean	240	0.3206	0.2733	0.0473	0.1813	0.1816	1.4427

N, The number of sample; H_S, gene diversity within populations; H_T, total gene diversity; D_{ST}, gene diversity among populations; G_{ST}, the proportion of the total genetic diversity partitioned among population; F_{ST}, the proportion of total genetic diversity partitioned among population; N_M, the gene flow estimate according to G_{ST}.

Table 3. Observed number of alleles (A) at each polymorphic locus and genetic variation based on allozyme analysis of 24 populations of *M. ruthenica*.

Population	N	A	A_e	H_e	H_o	P (%)
HS	10	1.333	1.287	0.294	0.483	33
AQ1	10	1.667	1.423	0.251	0.367	67
AQ2	10	1.667	1.533	0.223	0.383	67
DW	10	1.667	1.608	0.333	0.466	67
DB	10	1.667	1.210	0.160	0.183	67
DM	10	1.833	1.377	0.247	0.316	83
YS	10	2.000	1.342	0.344	0.433	83
XB	10	1.833	1.656	0.359	0.466	83
EG	10	2.000	1.628	0.368	0.600	100
LH	10	1.667	1.553	0.307	0.500	67
EH	10	2.000	1.868	0.485	0.650	100
XL	10	1.833	1.484	0.288	0.450	83
AL	10	1.333	1.302	0.167	0.167	33
BL	10	2.000	1.576	0.345	0.517	100
LX	10	1.667	1.479	0.269	0.383	67
KJ	10	1.833	1.583	0.161	0.233	83
KH	10	1.333	1.326	0.174	0.300	33
ZS	10	1.667	1.463	0.273	0.383	67
LQ	10	2.000	1.808	0.451	0.716	100
XS	10	1.667	1.463	0.273	0.383	67
HB1	10	2.000	1.804	0.451	0.733	100
HB2	10	1.667	1.335	0.220	0.300	67
HB3	10	1.833	1.385	0.251	0.333	83
HB4	10	1.666	1.317	0.208	0.300	67
mean	10	1.743	1.492	0.321	0.422	76

N, The number of sample; A, mean number of alleles per locus; A_e, effective number of alleles per locus; H_o, mean observed heterozygosity; H_e, mean expected heterozygosity; P, percentage polymorphic of loci.

100%, and heterozygosity was from 0.160 to 0.485. In contrast, the significant differentiation (He = 0.485) appeared among individuals of EH population, and interpopulation gene flow was found. Population DB showed lower intrapopulation genetic variability. However, another two collections DM and DW from this region showed higher genetic diversity of 0.247 and 0.333 respectively, indicating that there were fewer gene flow

Table 4. Assessment of genetic diversity for accessions by micro-satellites. Observed number of alleles (Na) at each population, effective number of alleles (Ne), proportion of polymophic loci (P) and gene diversity statistics (He = Nei's gene divesity).

Population	P	Sample size	Na	Ne	He
DM	0.393	50	2.0123	2.2	0.5316
LX	0.362	50	1.8759	2.4	0.4502
HS	0.3593	50	1.5220	1.6	0.4644
DB	0.3921	50	1.9661	2.4	0.4826
EG	0.1974	50	1.3638	2	0.3011
XS	0.1989	50	1.3643	1.8	0.274
EH	0.3508	50	1.8876	2.6	0.4256
AL	0.3250	50	1.8184	2.6	0.3756
LQ	0.3093	50	1.6736	2.2	0.3986
KH	0.3997	50	1.9835	2.4	0.4724
HB2	0.3844	50	2.0037	2.2	0.4988
ZS	0.3950	50	1.9749	2.6	0.4806
HB1	0.4252	50	1.8093	2.6	0.4256
LQ	0.2922	50	1.5440	1.8	0.301
AQ2	0.358	50	1.8696	2.4	0.4364
BL	0.384	50	1.8234	2.4	0.4408
HB4	0.3562	50	1.7796	2.6	0.4228
KJ	0.3538	50	1.9751	2.6	0.444
AQ1	0.3124	50	1.7159	2	0.3948
XL	0.3219	50	1.6464	2.2	0.357
DW	0.3146	50	1.7206	2	0.3962
HB3	0.3684	50	1.8651	2.2	0.378
YS	0.2996	50	1.6480	2.2	0.3724
XB	0.3636	50	1.8109	2.4	0.4344
Mean	0.3424	50	1.7264	2.3	0.4149

between these two populations and population DB. Clearly, population EH held more plentiful genetic diversity than other populations and should be in high priority for conservation.

Microsatellite analysis

To test the variation by microsatellite finger printing in the *M. ruthenica* populations, 10 microsatellites derived from *M. sativa* were used to fingerprint the 24 samples (Table 1). Among the 10 used SSR loci, five (50%) of them were polymorphic. The five SSR markers assayed in this study were found to be encoded across 24 samples by 15 alleles, with an average of three bands (alleles) per primer. Two to four alleles were found at each of the five loci. Among all populations, the proportion of polymorphic loci was 0.3423. Intrapopulation variation was detected by all five micro-satellites. Most amplified products were within 100 to 300 bp. A summary of the loci and alleles for each population is provided in Table 4. The polymorphic loci (P) ranged proportionately from 0.1974 to 0.4252 with a mean of 0.3424 for each population.

Among all the populations, observed heterozygosity, number of alleles and effective allele numbers were 0.4149, 2.3 and 1.73. Intrapopulation heterozygosity was higher than that of interpopulation, ranging from 0.2740 to 0.5316 (Table 4). Samples from different regions showed varied range of values. Observed number of alleles and effective allele number of intrapopulation varied from 1.3638 to 2.0123 and from 1.6 to 2.6 respectively (Table 4). Observed number of alleles and effective allele number at each polymorphic locus varied from two to four and from 1.5043 to 2.9655, respectively (Table 5).

Percentage of intrapopulation variance (82.8%) was much higher than that of interpopulation (17.2%), molecular interpopulation differentiation (F_{ST}) varies from 0.0728 to 0.2523 with a mean of 0.1719. Gene flow was from 0.7411 to 3.1859 with a mean of 1.2040 (Table 5).

DISCUSSION

In this study, the genetic diversity of cross *M. ruthenica* from Inner Mongolia plateau and Great Xing'an Mountains in China was analyzed by allozyme and microsatellite markers. Whilst it is possible that the sample size could influence the level of genetic variability, particularly for

Table 5. Observed number of alleles (Na) at each polymorphic locus, effective number of alleles (Ne) and F-statistics for 5 polymorphic loci in *M. ruthenica.*

Locus	Sample size	A	Ae	F_{is}	F_{it}	F_{st}	N_m
Afcall	452	3	1.7917	-0.5803	-0.4653	0.0728	3.1859
mtlec2a	324	2	1.5043	-0.5066	-0.2712	0.1563	1.3498
Afctt1	476	3	2.0332	-0.3830	-0.2217	0.1166	1.8944
Af245	420	3	1.7556	-0.2911	0.0028	0.2277	0.8482
Afca1	380	4	2.9655	-0.1449	0.1439	0.2523	0.7411
Mean	410	3	2.0100	-0.3664	-0.1314	0.1719	1.2040

F_{iT}, The excess of heterozygotes relative to panmictic expectation within entire samples; F_{iS}, the excess of heterozygotes relative to panmictic expectation with populations; Nm,Tte gene flow estimate according to $F_{st.}$

using microsatellite markers, and given the degree of polymorphism in the systems studied, the size of the study was still considerable. Considerable population differentiation as is expected for cross-pollinating taxa, population may accurately reflect most of the genetic diversity (Hamrick and Godt, 1991). Hamrick et al. (1989, 1997) generalized that in crossing species, the majority of the total genetic diversity resides within populations based on allozyme analyses. With random amplified polymorphic DNA (RAPD) markers, Nybom and Bartish (2000) compiled mean G_{ST} values of 0.59, 0.19 and 0.23 for selfing, mixed mating and outcrossing plant species, respectively. It is now well known that, for a wide range of plant species, the mating systems affects patterns of genetic variability both within and among populations. Outcrossing species commonly have higher levels of genetic diversity and lower differentiation between populations than selfing and clonal plants. Similar levels of intrapopulation genetic variation at allozymes and SSR markers were also found for studied populations of *M. ruthenica*.

Our results about SSR molecular marker of *M. ruthenica* showed the 82.8% intrapopulation variation and 17.2% interpopulations variation. This result is almost same as Nybom and Bartish (2000), which indicated that *M. ruthenica* is the typical outcross species.

Genetic diversity at the individuals level was high (H_T = 0.321), and percentage polymorphic of loci was high with values of 76% for allozymes which was higher than those of Hamrick's statistics (1989) of average plant level, which indicated that *M. ruthenica* maintained higher genetic diversity.

The Nei's G_{ST} statistic is usually used to estimate the proportion of total genetic diversity (H_T) residing among populations (D_{ST}); $G_{ST} = D_{ST}/H_T$. According to Hamrick and Godt (1989), outcross species are characterized by a high value of total allozyme diversity (H_T = 0.167), and a low value of the coefficient of gene differentiation (G_{ST} = 0.197), which means that while outcross species maintain lower allozyme diversity at their polymorphic loci than selfing species, most of this variation is found among individuals. In this study, the results indicate that *M. ruthenica* maintain high variation at polymorphic loci (H_T ranging from 0.1135 to 0.4980) and 0.2733 of this variation is found within population, rather than among populations (G_{ST} = 0.1813). A similar result was founded by Nybom and Bartish (2000) in a study of allozyme variation in populations of *M. sativa* (G_{ST} = 0.23). However, there were high levels of intrapopulation genetic diversity (82.8%) rather than that of intrepopulations (17.2%), which were mainly affected by mating system, seed dispersal ways and reproduction means.

In addition, the value of gene flow is over 1 means, the population divergent caused by genetic shift can be avoided. The gene flow (N_m) between populations according to SSR and allozyme analysis are 1.2040 and 1.44 respectively in *M. ruthenica* which are similar with the average value (N_m = 1.154) of outcross by entomophilous flower (Hamrick and Godt, 1989).

The estimates of genetic differentiation among populations based on molecular markers are generally higher than estimates based on allozymes (Ayre and Ryan, 1999). Similar results were derived from this study. Allozymes reflect the variation in gene-coding regions, and may thus be affected by strong natural selection causing allozyme loci differentiation. Indeed, based on allozymatic data, selection can be regarded as an important factor influencing the genetic structure and differentiation of *M. ruthenica* populations. Natural selection can operate with multi-loci allelic combinations as a unit (that is, co-adapted gene complexes) leading to stable superior multi-loci genotypes adapted to specific habitats (Nevo, 1998). Microsatellite loci reflect non-coding regions, and are selectively neutral which could be the reason that natural selection did not act on these loci and did not cause any differentiation among these populations. The high microsatellite allelic diversity and high frequency of private alleles do not contradict this explanation.

In conclusion, both SSR and allozyme analysis can be used for estimating genetic diversity pattern and its distribution in natural genetic *M. ruthenica* populations in China, which may benefit alfalfa breeding and germplasm conservation. SSR markers exhibited a high level of efficiency for detecting polymorphism in *M. ruthenica* populations compared with allozyme data. There was

high gene flow within populations of *M. ruthenica* caused by a cross-breeding system

ACKNOWLEDGEMENT

This work was supported by the project 2007CB106800 (973) and National forage industry system of China(CARS-35).

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