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Analysis of genetic diversity and population structure in accessions of the genus *Melilotus*

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ABSTRACT

Melilotus is one of the most important legume plants, in part due to its production of coumarin, but the genetic diversity among the 18 species remains unclear. In the present study, the analysis of genetic diversity of the *Melilotus* species was performed with simple sequence repeat (SSR) markers. From the PCR amplification, we selected 18 out of 70 primers that were used in previous studies. Three hundred twenty-six sampled individuals were assayed to study the genetic diversity and polymorphisms based on the SSR markers. All analyzed markers were polymorphic, and 287 alleles were identified, with 15.94 alleles per locus detected. The polymorphism information content (*PIC*) values ranged from 0.71 to 0.93, with an average of 0.87, which indicates that the markers were highly informative. Based on the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis, we found that the 18 species were divided into two clusters. *M. italicus* and *M. speciosus* from cluster A and *M. indicus* and *M. segetalis* from cluster B were closely related. Population structure analysis suggested that the optimum number of groups was three. From the analysis of molecular variance, 17.79% of the variance was due to species differences, 31.61% of the variance was due to differences among populations within species and the remaining 50.60% was due to differences within populations. The results of the present study showed that these SSR markers will benefit the *Melilotus* research community for genetics and breeding. Furthermore, this study also established the foundation for future breeding programs, genetic improvement and coumarin production in the *Melilotus* species.

1. Introduction

Melilotus is an important Leguminosae crop and is comprised of 19 annual or biennial species. All species are native to Eurasia or North Africa (Aboel-Atta, 2009). Members of the *Melilotus* genus have high seed yields and are more tolerant to extremes in environmental conditions, such as drought, cold and high salinity, relative to most other forages (Rogers et al., 2008; Sherif, 2009). As forage legumes, they can also perform symbiotic nitrogen fixation with a number of bacterial species (Bromfield et al., 2010). The nitrogen fixation rate of *Melilotus* is higher than that of other legumes, making it beneficial for crop rotations (Stickler and Johnson, 1959). It is important for agriculture and animal husbandry, as it is a green manure crop for grass and can be used as crop fertilizer. In addition to *Melilotus* being an important forage crop, there has been increased interest in its medicinal value, given its variety of biological activities from its coumarin, flavones and saponin constituents (Cong et al., 2012).

Coumarins are an important group of natural compounds that are found in different species of plants in nature, such as *Dipteryx odorata* Willd. (Ehlers et al., 1995), *Angelica archangelica* L. (Hawryl et al., 2000), *Melittis melissophyllum* L. (de Vincenzi et al., 1997) and *Mikania glomerata* Spreng. (Celeghini et al., 2001). It has been reported that coumarin content ranges from 0.05 to 1.30%, as deter-

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; h, Nei's (1973) gene diversity; H_E, expected heterozygosity; H_O, observed heterozygosity; I, Shannon's Information index; na, the observed number of alleles; ne, the effective number of alleles; NPGS, National Plant Germplasm System; NPL, the number of polymorphic loci; PCR, polymerase chain reaction; *PIC*, the polymorphism information content; PPL, the percentage of polymorphic loci; RFLP, restriction fragment length polymorphism; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; UPGMA, the unweighted pair-group method with arithmetic mean.

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mined from the coumarin content of 149 accessions in 15 *Melilotus* species. No coumarin was detected in an *M. segetalis* accession (Nair et al., 2010). In recent years, a preliminary study found that in addition to conventional carbohydrates, proteins, fats and oils, *Melilotus* contained a variety of chemicals, such as coumarin, flavones, phenolics, saponin and other varieties of chemical components with medicinal value. As one of the cheap abundant medicinal plant resources, *Melilotus* is worth further development for its vast market value (Cong et al., 2012).

At present, several molecular marker techniques are employed to assess genetic diversity in various crops, specially in legume plants. For example, single nucleotide polymorphism (SNP) in *Miscanthus* (de Cesare et al., 2010), restriction fragment length polymorphism (RFLP) in rice (Sun et al., 2001), amplified fragment length polymorphism (AFLP) in sesame (Uzun et al., 2003), random amplified polymorphic DNA (RAPD) in bean (Basheer-Salimia et al., 2013) and simple sequence repeats (SSR) (Wang et al., 2011) in switchgrass have all been used on a large scale, and there are different marker systems for studying genetic diversity in different contexts (Tam et al., 2005). In previous studies, genetic diversity of alfalfa (*Medicago sativa* L.) revealed by AFLP analysis and it suggested that 26 Iranian cultivated populations grouped into four main clusters with no correlation between genetic and geographical diversity (Keivani et al., 2010). The assessment of 58 faba bean (*Vicia faba* L.) genotypes using SRAP markers indicated the high genetic diversity and broad genetic basis due to the rich polymorphism rate (100%) and low genetic similarity (0.21) (Alghamdi et al., 2012). The analysis of genetic structure and diversity in 100 vegetable soybean accessions using simple sequence repeat (SSR) markers showed the vegetable soybean germplasms in China were relatively close and great consistency with the germplasm origins, seed coat colors or their pedigrees (Dong et al., 2014). SSR markers are the markers of choice because of locus specificity, hypervariability, co-dominance and high reproducibility (Powell et al., 1996; Varshney et al., 2009; Varshney et al., 2005) and have been proven to be promising for various genomic applications (Gupta and Varshney, 2000). However, current studies in the *Melilotus* genus mainly concentrate on morphology, cultivation techniques and chemical ingredients, and few studies on mutants, root nodules or genetic diversity have been reported.

Our previous results showed that the interspecific relationships within the *Melilotus* genus based on the phylogenetic tree are clearly monophyletic in the legume family (Di et al., 2015). A preliminary evaluation of agronomy and quality traits of 19 *Melilotus* accessions showed that coumarin content could vary from 0.16–1.02% (Luo et al., 2014). In this study, the SSR markers were used to study the genetic diversity and population structure among 50 accessions of 18 *Melilotus* species, totalling 326 plant samples that were collected. The SSR markers founded in this study will be a useful resource for genetic study and germplasm evaluation for coumarin production in the *Melilotus* genus.

2. Materials and methods

2.1. Plant materials and DNA extraction

Seeds from fifty accessions of eighteen *Melilotus* species were obtained from the National Plant Germplasm System (NPGS, America) as summarized in Table 1 and Fig. 1. The accessions were numbered from 1 to 50, a numbering that was maintained throughout the study. Seeds of all accessions were germinated, and the seedlings were cultivated for DNA extraction in a greenhouse at Lanzhou University in Yuzhong of Gansu Province, China.

Three to twelve individuals of each accession (Table 1), totalling 326 individuals, were sampled and used for polymorphism investigations of the selected SSR markers. Genomic DNA was extracted from the young leaves according to an SDS (sodium dodecyl sulfate) method (Shan et al., 2011). DNA samples were diluted to 50 ng/μL and stored at −20 °C prior to polymerase chain reaction (PCR) amplification.

2.2. PCR amplification

Seventy SSR primer pairs were selected (Supplemental Table 1); these primer sequences were published in previous studies (Winton et al., 2007; Zhou et al., 2014). Primers were excluded from the study if they failed to amplify consistently in PCR amplification. The 18 SSR primer pairs used in the final analysis are presented in Table 2.

The PCR was performed in a total reaction volume of 10 μL containing 1.0 μL DNA, 4.9 μL 2 × Reaction Mix (500 μM dNTP each, 20 mM Tris–HCl, 100 mM KCl, 3 mM $MgCl_2$), 0.1 μL 2.5 U/μL Golden DNA Polymerase, 1.0 μL each primer and 2.0 μL double distilled water. PCR cycling conditions were 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature (Supplemental Table 1), 30 s at 72 °C and a final extension step of 7 min at 72 °C.

2.3. Data analysis

The amplified bands were scored as present (1) or absent (0), and only reproducible bands were considered. The indexes of expected heterozygosity (H_E), observed heterozygosity (H_O) and polymorphic information content (*PIC*) were calculated as previously described (Chung et al., 2013). To evaluate the genetic diversity within species and populations, the following parameters were calculated: the number of polymorphic loci (NPL), the percentage of polymorphic loci (PPL), the observed number of alleles (na), the effective number of alleles (ne), Nei's (1973) gene diversity (h) and Shannon's Information index (I). The program POPGENE 32 (Yeh and Boyle, 1997) was used to calculate NPL, PPL, na, ne, h and I values. The analysis of molecular variance (AMOVA) was used to partition the total genetic variation among species, among populations within species and within populations by the method of AMOVA Version 1.55 (Excoffier, 1993). A cluster analysis was performed to generate a dendrogram using the unweighted pair-group method with arithmetic mean (UPGMA) and Nei's unbiased genetic distance with the help of the SAHN-Clustering by NTSYS-pc.V.2.1 (Rohlf, 2000) software package. A model-based approach implemented in the software program STRUCTURE 2.3 was used to subdivide the individuals into different subgroups (Falush et al., 2007; Pritchard et al., 2000). Due to the estimated 'log probability of data' [LnP(D)] of STRUCTURE overestimating the number of subgroups (Pritchard and Wen, 2003), we used the ad hoc measure ΔK (Evanno et al., 2005) to estimate the number of groups. The membership of each genotype was tested for the range of genetic clusters from $K = 1$ to $K = 8$ (each with 10 independent runs) with the admixture model.

3. Results

3.1. The polymorphism of SSR markers

In this study, 10 DNA samples from different *Melilotus* species were randomly chosen to conduct the primers screening. The primers that successfully amplified and produced clear and stable bands of the expected size by PCR amplification were selected. After the preliminary screening, 18 of 70 pairs of SSR primers with the obvious polymorphism were selected and used in the following analysis.

The eighteen SSR primers used in this study amplified 287 alleles with an average of 15.94 alleles per locus. The majority of the

Table 1
Details for 50 accessions of the 18 *Melilotus* species included for the study.

Code	Species	NPGS number	Origin	Latitude	Longitude	Sample size
1	*M. albus*	Ames 21597	Italy	N 41°52’	E 12°34’	6
2	*M. albus*	PI 90557	Manchuria, China	N 45°19’	E 124°29’	9
3	*M. altissimus*	Ames 18376	Nebraska, United States	N 41°29’	W 99°54’	5
4	*M. altissimus*	Ames 22881	Krasnodar, Russian Federation	N 44°8’	E 39°1’	3
5	*M. altissimus*	PI 275975	–	N 48°38’	W 4°18’	9
6	*M. altissimus*	PI 420163	France	N 46°13’	E 2°12’	7
7	*M. dentatus*	PI 108656	Armenia	N 40°4’	E 45°2’	12
8	*M. dentatus*	PI 595334	Xinjiang, China	N 41°49’	E 85°30’	3
9	*M. dentatus*	PI 90753	China	N 35°51’	E 104°11’	11
10	*M. elegans*	PI 250873	Iran	N 32°25’	E 53°41’	6
11	*M. elegans*	PI 260271	Shewa, Ethiopia	N 9°9’	E 37°48’	9
12	*M. hirsutus*	Ames 22882	Russian Federation	–	–	12
13	*M. hirsutus*	PI 129697	Sweden	N 60°7’	E 18°38’	11
14	*M. indicus*	Ames 21619	Nebraska, United States	N 41°29’	W 99°54’	8
15	*M. indicus*	Ames 24055	Egypt	N 26°49’	E 30°48’	3
16	*M. indicus*	PI 107562	Uzbekistan	N 41°23’	E 69°4’	8
17	*M. indicus*	PI 260756	Turkey	N 38°57’	E 35°14’	3
18	*M. indicus*	PI 308524	Peru	S 9°11’	W 75°0’	3
19	*M. indicus*	PI 43595	–	–	–	8
20	*M. indicus*	PI 532954	Nepal	N 28°0’	E 84°0’	3
21	*M. infestus*	PI 306326	Algeria	N 27°13’	E 2°29’	8
22	*M. infestus*	PI 306327	Italy	N 41°52’	E12°34’	6
23	*M. infestus*	PI 306328	Hungary	N 47°9’	E 19°30’	4
24	*M. italicus*	PI 317635	Czechoslovakia	N 14°28’	E 121°2’	3
25	*M. officinalis*	PI 304530	Turkey	N 38°57’	E 35°14’	5
26	*M. polonicus*	PI 108647	Former Soviet Union	N 54°30’	E 48°38’	10
27	*M. polonicus*	PI 314386	Former Soviet Union	N 45°5’	E 41°50’	5
28	*M. segetalis*	PI 317633	Algeria	N 27°13’	E 2°29’	5
29	*M. segetalis*	PI 317649	Czechoslovakia	N 48°2’	E 18°22’	5
30	*M. segetalis*	PI 43597	–	–	–	3
31	*M. siculus*	Ames 21249	Portugal	N 39°23’	W 8°13’	9
32	*M. siculus*	PI 129703	Malta	N 35°56’	E 14°22’	4
33	*M. siculus*	PI 318508	Greece	N 39°4’	E 21°49’	9
34	*M. siculus*	PI 33366	Former Soviet Union	–	–	6
35	*M. speciosus*	PI 317650	Manitoba, Canada	N 53°45’	W 98°48’	3
36	*M. spicatus*	Ames 18402	Nebraska, United States	N 41°29’	W 99°54’	4
37	*M. spicatus*	Ames 25647	Krym, Ukraine	N 44°24’	E 33°49’	8
38	*M. spicatus*	PI 317644	Algeria	N 27°13’	E 2°29’	4
39	*M. suaveolens*	Ames 18444	Nebraska, United States	N 41°29’	W 99°54’	5
40	*M. suaveolens*	Ames 23793	Mongolia	N 48°10’	E 91°45’	4
41	*M. suaveolens*	PI 593408	South Dakota, United States	N 43°58’	W 99°54’	7
42	*M. suaveolens*	PI 595395	Iowa, United States	N 41°52’	W 93°5’	8
43	*M. sulcatus*	PI 198090	Morocco	N 31°47’	W 7°5’	7
44	*M. sulcatus*	PI 227595	Tunisia	N 33°53’	E 9°32’	7
45	*M. tauricus*	Ames 18446	Nebraska, United States	N 41°29’	W 99°54’	7
46	*M. tauricus*	Ames 25789	Krym, Ukraine	N 44°24’	E 33°49’	4
47	*M. tauricus*	PI 67510	Krym, Ukraine	N 44°57’	E 34°6’	11
48	*M. wolgicus*	PI 317665	Denmark	N 56°15’	E 9°30’	8
49	*M. wolgicus*	PI 317666	Czechoslovakia	N 48°2’	E 18°22’	12
50	*M. wolgicus*	PI 502547	Russian Federation	N 50°45’	E 49°19’	6

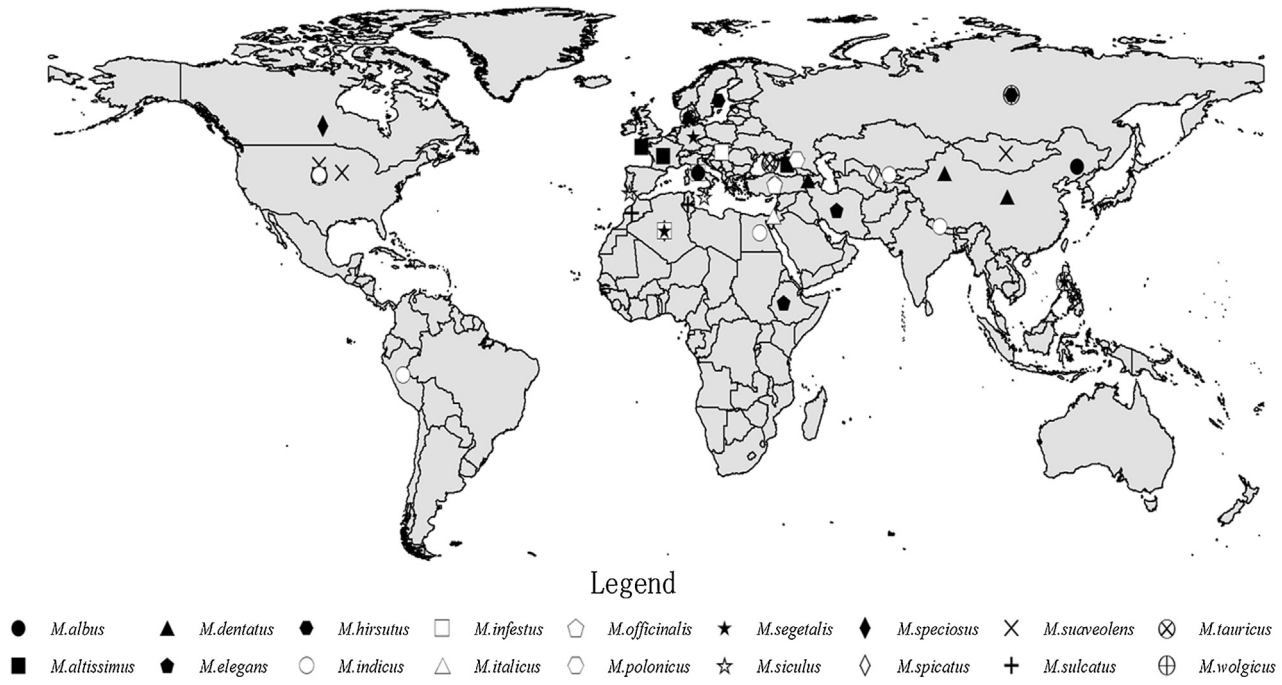

Fig 1. The distribution of 50 populations of 18 *Melilotus* species.

Table 2
Polymorphism analysis of 50 *Melilotus* accessions with SSR primers. N_A, number of alleles, H_O, observed heterozygosity, H_E, expected heterozygosity, *PIC*, polymorphic information content.

Primer	N_A	H_O	H_E	*PIC*
Ms-018	10	0.08	0.81	0.79
Ms-020	10	0.08	0.84	0.82
Ms-034	15	0.06	0.88	0.87
Ms-051	13	0.02	0.85	0.84
Ms-058	11	0.00	0.85	0.84
Ms-068	21	0.96	0.91	0.91
Ms-202	13	0.03	0.90	0.89
Ms-218	13	0.00	0.89	0.88
Ms-281	18	0.08	0.90	0.90
Ms-349	14	0.03	0.88	0.87
Ms-357	14	0.03	0.88	0.86
MaMS04	21	0.17	0.92	0.91
MaMS06	19	0.07	0.90	0.90
MaMS08	20	0.17	0.93	0.92
MaMS09	9	0.12	0.75	0.71
MaMS10	22	0.06	0.94	0.93
MaMS13	18	0.04	0.89	0.88
MaMS15	26	0.18	0.94	0.93
Mean	15.94	0.12	0.88	0.87

primers had a high *PIC* and identified a high level of polymorphism. Across the 326 samplings, the polymorphism information content (*PIC*) values ranged from 0.71 to 0.93 with an average of 0.87, the expected heterozygosity (H_E) values ranged from 0.75 to 0.94 with an average of 0.88 and the observed heterozygosity (H_O) values ranged from 0 to 0.96 with an average of 0.12 (Table 2).

3.2. Genetic diversity analysis

The number of polymorphic loci, the percentage of polymorphic loci, the observed number of alleles, the effective number of alleles, Nei's (1973) gene diversity and Shannon's Information index were calculated for each accession and species (Table 3). The number of polymorphic loci values for accessions ranged from 0 (accession 36) to 54 (accession 7 and accession 12), and for the species ranged from 8 (*M. italicus*) to 95 (*M. indicus*). The highest percentage of polymorphic loci was 18.82% and 33.10%, respectively, which were recorded in two accessions (7 and 12) for species *M. indicus*, while the lowest was 0% for accession 36 and 1.05% for *M. italicus*. The observed number of alleles ranged from 1 to 1.1882 in accessions and 1.0105 to 1.3310 in species. The effective number of alleles varied from 1 to 1.0904 in accessions and 1.0074 to 1.0937 in species. The Nei's (1973) gene diversity values for accessions ranged from 0 (accession 36) to 0.0544 (accession 25), and for the species ranged from 0.0043 (*M. italicus*) to 0.0607 (*M. suaveolens*). Correspondingly, the highest Shannon's Information index was recorded for accession 25 (0.0821) and the lowest for accession 36 (0).

Analysis of molecular variance (AMOVA) was used to evaluate variance components among species, among populations within species and within populations (Table 4). According to AMOVA analysis, there were highly significant differences ($P < 0.001$) existed in genetic differentiation among species, among populations within species and within populations. Of the total genetic variance, 17.79% of the variance was due to species differences, 31.61% of the variance was due to differences among populations within species and the remaining 50.60% was due to differences within populations. Therefore, the results showed significant genetic differences among the 50 populations of 18 *Melilotus* species.

3.3. Cluster and population structure analysis

The relationship among the 18 *Melilotus* species based on genetic distance values was further determined by UPGMA cluster analysis. According to the UPGMA dendrogram (Fig. 2), the 18 species could be classified into two clusters. Cluster A contained 13 species, with the 5 remaining species in cluster B. Meanwhile, in cluster A and B, *M. italicus*/*M. speciosus* and *M. indicus*/*M. segetalis* were closely related, respectively. A dendrogram was constructed using data from the UPGMA cluster analysis based on the genetic similarity matrix from all individuals of the 50 accessions (Supplemental Fig. 1). The UPGMA analysis separated the 326 individuals into seven groups. Groups IV contained 26 accessions of 10 *Melilo-*

Table 3
Genetic variability within 50 accessions and 18 species detected by SSR markers.

Species	Accessions	NPL	PPL(%)	na	ne	h	I
M. albus	1	27	9.41	1.0941 ± 0.29	1.0582 ± 0.20	0.0344 ± 0.11	0.0513 ± 0.16
	2	37	12.89	1.1289 ± 0.34	1.0621 ± 0.20	0.0380 ± 0.11	0.0590 ± 0.17
	Malb[a]	65	22.65	1.2265 ± 0.42	1.0897 ± 0.20	0.0591 ± 0.13	0.0945 ± 0.19
M. altissimus	3	10	3.48	1.0348 ± 0.18	1.0154 ± 0.10	0.0098 ± 0.06	0.0156 ± 0.09
	4	6	2.09	1.0209 ± 0.14	1.0148 ± 0.10	0.0087 ± 0.06	0.0126 ± 0.09
	5	44	15.33	1.1533 ± 0.36	1.0572 ± 0.19	0.0360 ± 0.10	0.0582 ± 0.16
	6	13	4.53	1.0453 ± 0.21	1.0243 ± 0.13	0.0142 ± 0.07	0.0215 ± 0.11
	Malt[a]	91	31.71	1.3171 ± 0.47	1.0857 ± 0.20	0.0579 ± 0.12	0.0971 ± 0.18
M. dentatus	7	54	18.82	1.1882 ± 0.39	1.0767 ± 0.21	0.0477 ± 0.12	0.0755 ± 0.18
	8	3	1.05	1.0105 ± 0.10	1.0081 ± 0.08	0.0044 ± 0.04	0.0064 ± 0.06
	9	26	9.06	1.0906 ± 0.29	1.0407 ± 0.16	0.0249 ± 0.09	0.0389 ± 0.14
	Mden[a]	87	30.31	1.3031 ± 0.46	1.0890 ± 0.20	0.0601 ± 0.12	0.0996 ± 0.18
M. elegans	10	29	10.10	1.1010 ± 0.30	1.0454 ± 0.16	0.0292 ± 0.10	0.0460 ± 0.14
	11	20	6.97	1.0697 ± 0.26	1.0364 ± 0.16	0.0217 ± 0.09	0.0332 ± 0.13
	Mele[a]	68	23.69	1.2369 ± 0.43	1.0926 ± 0.22	0.0588 ± 0.13	0.0936 ± 0.19
M. hirsutus	12	54	18.82	1.1882 ± 0.39	1.0776 ± 0.21	0.0483 ± 0.12	0.0764 ± 0.18
	13	34	11.85	1.1185 ± 0.32	1.0562 ± 0.20	0.0334 ± 0.11	0.0516 ± 0.16
	Mhir[a]	73	25.44	1.2544 ± 0.44	1.0872 ± 0.22	0.0558 ± 0.13	0.0899 ± 0.19
M. indicus	14	28	9.76	1.0976 ± 0.30	1.0415 ± 0.17	0.0254 ± 0.09	0.0402 ± 0.14
	15	2	0.70	1.0070 ± 0.08	1.0048 ± 0.06	0.0027 ± 0.03	0.0040 ± 0.05
	16	10	3.48	1.0348 ± 0.18	1.0239 ± 0.14	0.0136 ± 0.07	0.0199 ± 0.11
	17	10	3.48	1.0348 ± 0.18	1.0259 ± 0.14	0.0144 ± 0.08	0.0209 ± 0.11
	18	4	1.39	1.0139 ± 0.12	1.0096 ± 0.09	0.0055 ± 0.05	0.0081 ± 0.07
	19	39	13.59	1.1359 ± 0.34	1.0792 ± 0.22	0.0473 ± 0.13	0.0712 ± 0.19
	20	3	1.05	1.0105 ± 0.10	1.0081 ± 0.08	0.0044 ± 0.04	0.0064 ± 0.06
	Mind[a]	95	33.10	1.3310 ± 0.47	1.0728 ± 0.14	0.0547 ± 0.10	0.0961 ± 0.16
M. infestus	21	29	10.10	1.1010 ± 0.30	1.0432 ± 0.16	0.0271 ± 0.09	0.0429 ± 0.14
	22	22	7.67	1.0767 ± 0.27	1.0415 ± 0.17	0.0249 ± 0.09	0.0380 ± 0.14
	23	6	2.09	1.0209 ± 0.14	1.0112 ± 0.09	0.0067 ± 0.05	0.0103 ± 0.07
	Minf[a]	58	20.21	1.2021 ± 0.40	1.0660 ± 0.18	0.0442 ± 0.11	0.0726 ± 0.17
M. italicus	24	3	1.05	1.0105 ± 0.10	1.0074 ± 0.07	0.0043 ± 0.04	0.0063 ± 0.06
	Mita[a]	3	1.05	1.0105 ± 0.10	1.0074 ± 0.07	0.0043 ± 0.04	0.0063 ± 0.06
M. officinalis	25	45	15.68	1.1568 ± 0.36	1.0904 ± 0.24	0.0544 ± 0.13	0.0821 ± 0.20
	Moff[a]	45	15.68	1.1568 ± 0.36	1.0904 ± 0.24	0.0544 ± 0.13	0.0821 ± 0.20
M. polonicus	26	27	9.41	1.0941 ± 0.29	1.0470 ± 0.18	0.0279 ± 0.10	0.0427 ± 0.15
	27	15	5.23	1.0523 ± 0.22	1.0334 ± 0.16	0.0192 ± 0.09	0.0286 ± 0.13
	Mpol[a]	48	16.72	1.1672 ± 0.37	1.0735 ± 0.21	0.0458 ± 0.12	0.0716 ± 0.18
M. segetalis	28	16	5.57	1.0557 ± 0.23	1.0392 ± 0.18	0.0218 ± 0.09	0.0319 ± 0.14
	29	22	7.67	1.0767 ± 0.27	1.0444 ± 0.17	0.0267 ± 0.10	0.0404 ± 0.14
	30	24	8.36	1.0836 ± 0.28	1.0559 ± 0.20	0.0323 ± 0.11	0.0477 ± 0.16
	Mseg[a]	73	25.44	1.2544 ± 0.44	1.0787 ± 0.17	0.0557 ± 0.11	0.0930 ± 0.18
M. siculus	31	20	6.97	1.0697 ± 0.26	1.0320 ± 0.14	0.0197 ± 0.08	0.0308 ± 0.12
	32	8	2.79	1.0279 ± 0.16	1.0181 ± 0.12	0.0102 ± 0.06	0.0152 ± 0.09
	33	30	10.45	1.1045 ± 0.31	1.0534 ± 0.18	0.0328 ± 0.11	0.0503 ± 0.16
	34	23	8.01	1.0801 ± 0.27	1.0468 ± 0.18	0.0276 ± 0.10	0.0416 ± 0.15
	Msic[a]	80	27.87	1.2787 ± 0.45	1.0768 ± 0.18	0.0529 ± 0.11	0.0895 ± 0.17
M. speciosus	35	4	1.39	1.0139 ± 0.12	1.0115 ± 0.10	0.0061 ± 0.05	0.0088 ± 0.07
	Mspe[a]	4	1.39	1.0139 ± 0.12	1.0115 ± 0.10	0.0061 ± 0.05	0.0088 ± 0.07
M. spicatus	36	0	0.00	1	1	0	0
	37	11	3.83	1.0383 ± 0.19	1.0238 ± 0.13	0.0137 ± 0.07	0.0204 ± 0.11
	38	16	5.57	1.0557 ± 0.23	1.0339 ± 0.16	0.0195 ± 0.09	0.0293 ± 0.13
	Mspi[a]	57	19.86	1.1986 ± 0.40	1.0782 ± 0.19	0.0517 ± 0.12	0.0829 ± 0.18
M. suaveolens	39	35	12.20	1.1220 ± 0.33	1.0695 ± 0.20	0.0429 ± 0.12	0.0649 ± 0.18
	40	7	2.44	1.0244 ± 0.15	1.0151 ± 0.11	0.0088 ± 0.06	0.0132 ± 0.09
	41	18	6.27	1.0627 ± 0.24	1.0395 ± 0.17	0.0229 ± 0.09	0.0341 ± 0.14
	42	14	4.88	1.0488 ± 0.22	1.0303 ± 0.15	0.0176 ± 0.08	0.0262 ± 0.12
	Msua[a]	80	27.87	1.2787 ± 0.45	1.0937 ± 0.22	0.0607 ± 0.13	0.0990 ± 0.19
M. sulcatus	43	19	6.62	1.0662 ± 0.25	1.0402 ± 0.17	0.0236 ± 0.09	0.0353 ± 0.14
	44	8	2.79	1.0279 ± 0.16	1.0141 ± 0.10	0.0084 ± 0.06	0.0129 ± 0.08
	Msul[a]	42	14.63	1.1463 ± 0.35	1.0763 ± 0.21	0.0470 ± 0.12	0.0718 ± 0.18
M. tauricus	45	25	8.71	1.0871 ± 0.28	1.0448 ± 0.18	0.0265 ± 0.10	0.0406 ± 0.14
	46	5	1.74	1.0174 ± 0.13	1.0119 ± 0.09	0.0069 ± 0.05	0.0101 ± 0.08
	47	18	6.27	1.0627 ± 0.24	1.0351 ± 0.16	0.0203 ± 0.09	0.0306 ± 0.13
	Mtau[a]	67	23.34	1.2334 ± 0.42	1.0762 ± 0.18	0.0519 ± 0.11	0.0855 ± 0.18

Table 3 (*Continued*)

Species	Accessions	NPL	PPL(%)	na	ne	h	I
M. wolgicus	48	12	4.18	1.0418 ± 0.20	1.0255 ± 0.14	0.0148 ± 0.08	0.0220 ± 0.11
	49	16	5.57	1.0557 ± 0.23	1.0343 ± 0.16	0.0197 ± 0.09	0.0294 ± 0.13
	50	5	1.74	1.0174 ± 0.13	1.0086 ± 0.08	0.0053 ± 0.04	0.0082 ± 0.07
	Mwol[a]	57	19.86	1.1986 ± 0.40	1.0771 ± 0.19	0.0509 ± 0.12	0.0815 ± 0.18

NPL—the number of polymorphic loci; PPL—the percentage of polymorphic loci; na—observed number of alleles; ne—effective number of alleles; h—Nei's (1973) gene diversity; I—Shannon's Information index.

[a] Diversity within species, species were abbreviated by its first letters of Latin name.

Table 4
Analysis of molecular variance (AMOVA) for 50 accessions of 18 *Melilotus* species.

Source of variance	Degrees of freedom	Sum of squares	Mean square	Variance components	Total variance (%)	*P*-value
Among species	17	1392.562	81.915	2.974	17.79	<0.001
Among populations within species	32	1174.132	36.692	5.285	31.61	<0.001
Within populations	221	1869.645	8.460	8.460	50.60	<0.001

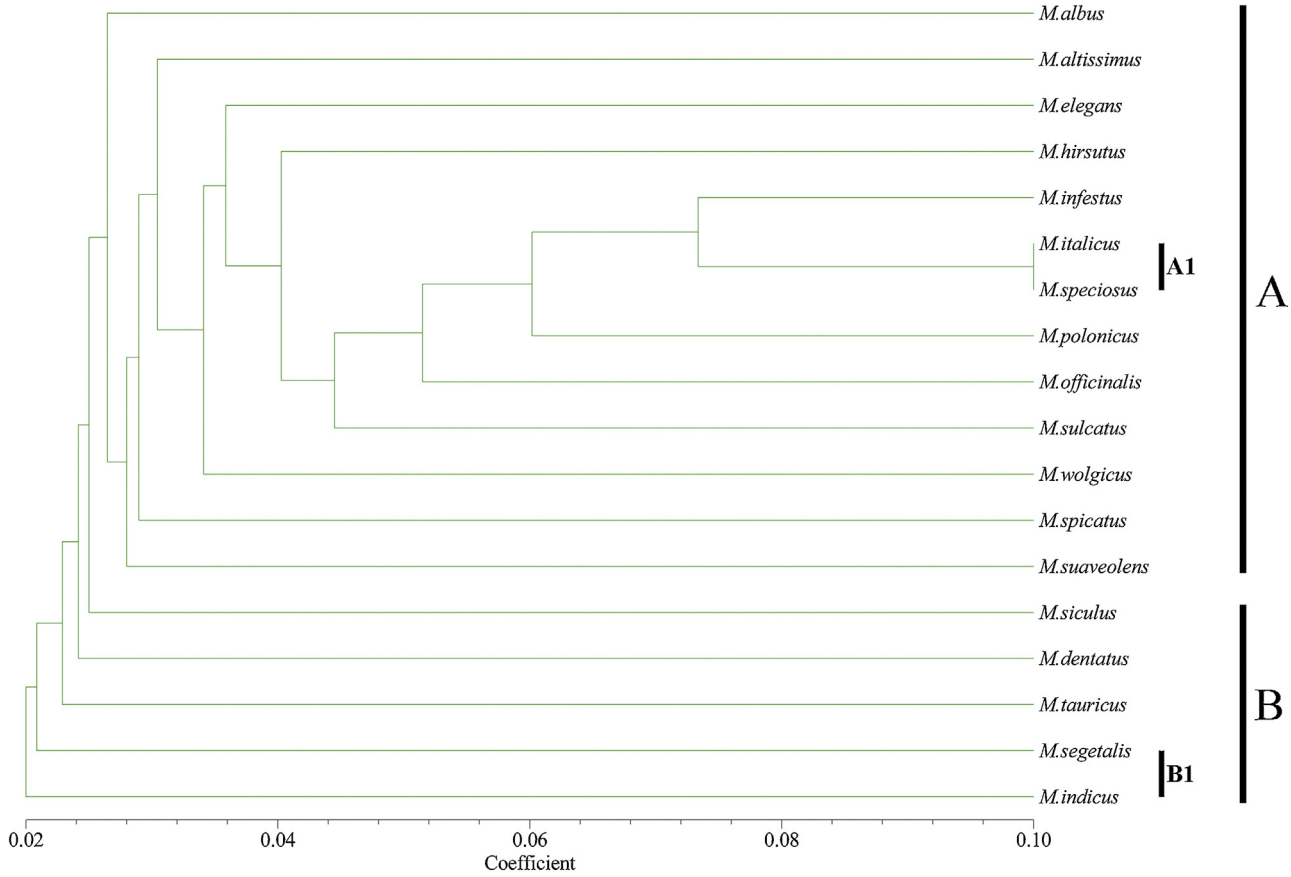

Fig. 2. UPGMA dendrogram based on Nei' genetic distance among 18 *Melilotus* using SSR marker analysis.

tus species. Group I, III, V, VI and VII consisted of 2, 5, 8, 5 and 3 accessions, respectively. Accession 10 of *M. elegans* was in group II.

Structure software was run for $K = 2–8$ based on the distribution of 18 SSR among the 326 individual plants. Based on maximum likelihood and delta K (ΔK) values, the number of optimum groups was three (Fig. 3). Among them, Group I contained 99 individuals for 18 accessions that consisted of *M. albus* (2 accessions), *M. elegans* (1 accession), *M. indicus* (5 accessions), *M. segetalis* (2 accessions), *M. siculus* (1accession), *M. suaveolens* (3 accessions), *M. sulcatus* (2 accessions) and *M. tauricus* (2 accessions). Group II contained 102 individuals for 15 accessions comprising four accessions of *M.altissimus*, three accessions of *M. infestus*, three accessions of *M. siculus*, one accession of *M. speciosus*, two accessions of *M. spicatus* and one accession each of *M. tauricus* and *M. wolgicus*. The remained 125 individuals for 17 accessions were assigned to Group III. The genetic structure revealed some species with admixture in each group, such as *M. siculus*, *M. spicatus*, *M. tauricus* and *M. wolgicus* in group 2 with admixture from group 1 and group 3, while species in group 1 and group 3 showed less admixture.

4. Discussion

In previous studies, biological characteristics were the main method of germplasm classification, but these results were often affected by the environmental conditions of crop growth or other factors, which reduced the accuracy of classification results. To avoid this, researchers began to use modern biotechnology tools at the molecular level to get a more exact germplasm classifi-

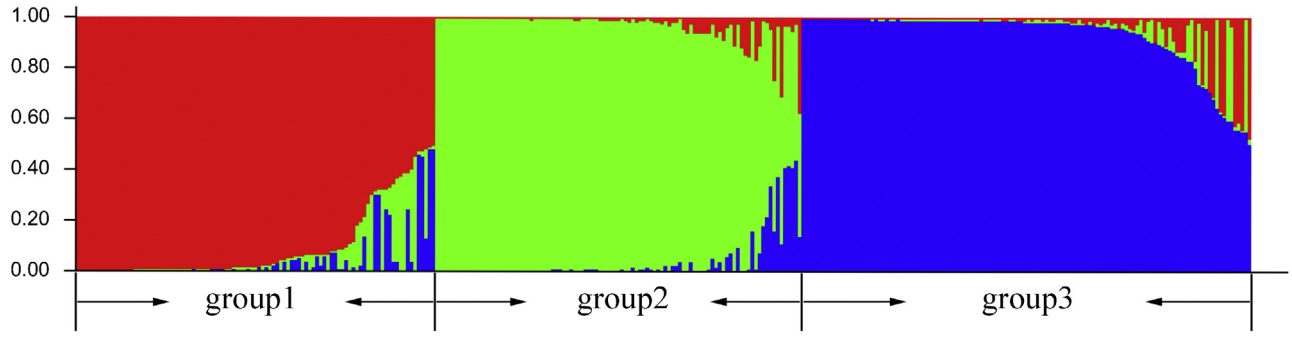

Fig. 3. Genetic structure of 326 individuals for 50 accessions as inferred by STRUCTURE with SSR markers data set.

Table 5
The three classifications of karyotype, molecular phylogeny and SSR makers in *Melilotus*.

Categories	Classification	Subclassification	Species
Karyotype(Clevinger and Panero, 2000)	Type A		*M. albus, M. altissimus, M. dentatus, M. hirsutus, M. officinalis, M. polonicus, M. suaveolens, M. tauricus, M. wolgicus*
	Type B	B-1	*M. elegans, M. indicus, M. neapolitana*[a]
		B-2	*M. infestus, M. macrocarpus, M.* messanensis[a], *M. segetalis, M. speciosus, M. sulcatus*
	Type C		*M. italicus*
Molecular phylogeny(Di et al., 2015)	Clade I		*M. albus, M. altissimus, M. dentatus, M. hirsatus, M. officinalis, M. polonicus, M. suaveolens, M. tauricus, M. wolgicus, M. elegans*
	Clade II	Clade 1	*M. spicatus*[a]
		IIb	*M. indicus, M. segetalis*
		Clade 2	*M. infestus*
		IIa	*M. siculus*[a], *M. sulcatus*
			M. speciosus, M. italicus
SSR makers	A		*M. albus, M. altissimus, M. elegans, M. hirsutus, M. infestus, M. officinalis, M. polonicus, M. spicatus, M. suaveolens, M. sulcatus, M. wolgicus,*
		A1	*M. italicus, M. speciosus*
	B		*M. dentatus, M. siculus, M. tauricus,*
		B1	*M. indicus, M. segetalis*

[a]Species incongruence in karyotype and molecular phylogeny.

cation. SSR markers have been widely used for genetic diversity assessment, germplasm identification, genetic mapping, DNA fingerprinting and marker-assisted selection. Their wide use for the analysis of genetic diversity had been demonstrated in interspecific analysis (Erfani et al., 2012; Sa et al., 2013; Yook et al., 2014) and intraspecific analysis (Chen et al., 2015; Li and Geng, 2015; Spandana et al., 2012; Upadhyay et al., 2012). Using 110 SSR markers, the 261 Chinese adzuki bean accessions were classified into 10 subpopulations and 10 distinct clusters and showed low differentiation among populations because of the Fst index (0.046) (Chen et al., 2015). The genetic diversity of 69 accessions of miscanthus germplasm from Korea and other East Asian regions were revealed genetically diverse by combining analysis of 31 SSR markers and 14 morphological traits (Yook et al., 2014). From the diversity analyses in wild diploid alfalfa accessions with 89 polymorphic SSR loci, it revealed that the diploid genetic analyses and evaluations could contribute to alfalfa improvement and breeding exploration (Şakiroğlu et al., 2010). SSR markers can be the tool of choice for genetic diversity analysis and cultivar fingerprinting in crop species. The genetic diversity revealed by SSR loci was supported by the observed high values of mean allele numbers, expected heterozygosity and *PIC*. The average of 15.94 alleles per primer pair observed (Table 2) was higher than that seen in previous studies in *Melilotus* (Winton et al., 2007) and *M. sativa* L. (Zhou et al., 2014). Similarly, the mean expected heterozygosity (H_E) and polymorphism information content (*PIC*) of 0.88 and 0.87, respectively, were higher compared with other cultivated species previously studied using SSR markers, such as alfalfa (*M. sativa* L.) with an H_E value of 0.207 and a *PIC* value of 0.608 (Wang et al., 2013), and millet (*Setaria italica* L.) with an H_E value of 0.417 and a *PIC* value of 0.376 (Zhao et al., 2012). However, the observed heterozygosity (H_O) value was low, with an average of 0.12. High values for all of the measures of diversity indicated allelic richness in the analysed germplasm, which can be utilized in breeding programs to get the desired plant types for commercial cultivation. Thus, the present study suggested that it is necessary to improve the heterozygosity among these *Melilotus* accessions in order to study the genetic diversity and improve breeding programs. The SSR markers from this study with high polymorphism will be a useful resource for the identification of coumarin and for further genetic study.

For the karyotype analyses, 19 *Melilotus* species were grouped into three types: A, B and C. Type B was further divided into Type B-1 and Type B-2 (Table 5). The karyotype analyses also suggested that the species within each type were closely related (Fumiji and Kita, 1965). The grouping information based on molecular phylogeny analyses indicates that all *Melilotus* species were divided into two subclades, named clade I and clade II (Table 5). In clade II, *M. spicatus*, *M. indicus* and *M. segetalis* formed a subclade named clade 1; *M. infestus*, *M. siculus*, *M. sulcatus*, *M. speciosus* and *M. italicus* formed clade 2 (Di et al., 2015). In this study, according to the UPGMA cluster analysis by the SSR markers, the 18 species were classified into two clusters. There were 7 and 8 of the same species out of 13 species from cluster A that were consistent with type A and clade I, respectively. In cluster B, *M. indicus* and *M. segetalis* were closely

related and were similar to the subclade II b from clade II. In our study, the UPGMA dendrogram (Supplemental Fig. 1) showed that the 18 SSR markers were able to cluster 50 accessions of 18 *Melilotus* species to seven groups, suggesting that the SSR markers can be used for accession identification. Previous studies revealed that 8 *Melilotus* species were in one clade as determined by rbcL and 3-cpDNA analysis (Di et al., 2015), which was similar to this study, where group IV contained the same 8 species out of 10 species. These *Melilotus* species clustering within the same group may have closer genetic relationships. One accession of *M. elegans*, because of its origin region, was the sole member of one group. The dendrogram also showed that different accessions from the same species were clustered together or were divided into different groups.

In this study, the markers that we studied will be valuable resources for studying genetic diversity, population structure, germplasm collection and conservation of the *Melilotus* species. These markers have provided important information about the genetic structure of *Melilotus*, which can contribute significantly to future breeding and improvement programs for this species. Through the SSR analysis, the genetic diversity, genetic relationships and population structure among the *Melilotus* species will be useful knowledge for crop breeding and germplasm curation. Overall, these results demonstrated the value of the *Melilotus* species as an important resource for conducting genetic diversity studies and assisting in future conservation and research programs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2016.02.055.

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