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Population genetic structure of two *Medicago* species shaped by distinct life form, mating system and seed dispersal

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• *Background and Aims* Life form, mating system and seed dispersal are important adaptive traits of plants. In the first effort to characterize in detail the population genetic structure and dynamics of wild *Medicago* species in China, a population genetic study of two closely related *Medicago* species, *M. lupulina* and *M. ruthenica*, that are distinct in these traits, are reported. These species are valuable germplasm resources for the improvement of *Medicago* forage crops but are under threat of habitat destruction.
• *Methods* Three hundred and twenty-eight individuals from 16 populations of the annual species, *M. lupulina*, and 447 individuals from 15 populations of the perennial species, *M. ruthenica*, were studied using 15 and 17 microsatellite loci, respectively. Conventional and Bayesian-clustering analyses were utilized to estimate population genetic structure, mating system and gene flow.
• *Key Results* Genetic diversity of *M. lupulina* (mean $H_E = 0{\cdot}246$) was lower than that of *M. ruthenica* (mean $H_E = 0{\cdot}677$). Populations of *M. lupulina* were more highly differentiated ($F_{ST} = 0{\cdot}535$) than those of *M. ruthenica* ($F_{ST} = 0{\cdot}130$). For *M. lupulina*, 55·5 % of the genetic variation was partitioned among populations, whereas 76·6 % of the variation existed within populations of *M. ruthenica*. Based on the genetic data, the selfing rates of *M. lupulina* and *M. ruthenica* were estimated at 95·8 % and 29·5 %, respectively. The genetic differentiation among populations of both species was positively correlated with geographical distance.
• *Conclusions* The mating system differentiation estimated from the genetic data is consistent with floral morphology and observed pollinator visitation. There was a much higher historical gene flow in *M. ruthenica* than in *M. lupulina*, despite more effective seed dispersal mechanisms in *M. lupulina*. The population genetic structure and geographical distribution of the two *Medicago* species have been shaped by life form, mating systems and seed dispersal mechanisms.

Key words: *Medicago lupulina*, *Medicago ruthenica*, microsatellite, genetic diversity, gene flow, forage legume.

INTRODUCTION

Life form, mating systems and seed dispersal are important adaptive traits shaping genetic structure and geographical distribution of plant populations (Levin, 1981; Loveless and Hamrick, 1984; Ennos, 1994; Hamrick and Godt, 1996; Bohonak, 1999; Clauss and Mitchell-Olds, 2006; Song *et al.*, 2006; Mable and Adam, 2007). Analyses of phenotypic variation of these traits together with population genetic variation should provide insights into the evolutionary history and processes of plant species (Barrett *et al.*, 1996; Juan *et al.*, 2004), which in turn will help determine evolutionary potentials and conservation strategies for natural populations.

Here, a population genetic study of two wild *Medicago* (Fabaceae) species, *Medicago lupulina* and *Medicago ruthenica*, which differ markedly in life form, mating systems, seed dispersal mechanisms and distribution ranges, is reported. The genus *Medicago* is distributed worldwide and consists of approx. 83 species, including two forage crops, *M. sativa* and *M. truncatula* (Small and Jomphe, 1989). Thirteen wild species of *Medicago* are found in China (Wei and Huang, 1998). They are adapted to a diverse range of habitats located in different geographical regions of China, from cold northern desert to warm and humid southern and central China, and from near the sea level in eastern China to high mountains in the Himalayas. These wild species hold a rich source of natural variation for the better understanding of plant population dynamics and for the improvement of *Medicago* cultivars. With rapid urbanization and overgrazing in China, however, these wild *Medicago* populations are threatened by severe reductions in number and size (J. Yan and H.-J. Chu, pers. obs.). Thus, there is an urgent need to investigate the population genetics and evolutionary dynamics of wild relatives of important forage crops.

Medicago truncatula has been chosen as one of the two representatives of the Fabaceae to have its entire genome sequenced. Taking advantage of the availability of genomic information for *M. truncatula* (http://medicago.org/), the population genetic structures of *M. lupulina* and *M. ruthenica* were studied using microsatellite markers. *Medicago lupulina* is annual, biennial or occasionally short-lived perennial, predominantly self-fertilizing, and widely distributed, whereas *M. ruthenica* is long-lived perennial, outcrossing, and much more narrowly distributed (Wei and Huang, 1998). *Medicago lupulina* has small indehiscent pods that facilitate

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long-distance seed dispersal by biotic and abiotic agents, whereas *M. ruthenica* has dehiscent pods and lacks effective mechanisms for seed dispersal.

Medicago lupulina, although never cultivated, has been grown as a green fodder or manure (Turkington and Cavers, 1979). The species is characterized as a water-saving and easily-maintained turf legume that contains a high level of protein and a low level of fibre suitable for grazing (Cao *et al.*, 2003). However, the biomass of the forage legume is relatively low. *Medicago ruthenica* is adapted to dry, stony habitats or desert with extremely low snowfall and very cold winters (Campbell *et al.*, 1999). It was considered to be superior to the cultivated species *M. sativa* in soil nutrient-use efficiency and thus might be more suitable for low-input systems (Campbell *et al.*, 1999). Both wild species are adapted to a much wider range of habitats than the cultivated species and are valuable genetic resources for developing better grazing legumes especially in drier and colder regions. Although the morphology, physiology, phenology, chromosomal variation and in-breeding of the two species had been studied previously (Lammerink, 1968; Sidhu, 1971; Hébert *et al.*, 1994; Mariani *et al.*, 1996; Qi, 1996; van Berkum *et al.*, 1998; Campbell *et al.*, 1999; Wilson, 2005; Li and Shi, 2006), their population genetic structures had not been characterized.

In the present paper, the genetic variation was investigated in a sample consisting of 16 *M. lupulina* populations and 15 *M. ruthenica* populations using 15 and 17 microsatellite markers, respectively. These included five populations of each species occurring sympatrically with those of the other species, and five microsatellite markers shared between the two species. This made it possible to evaluate to a certain extent the genetic differentiation between *M. lupulina* and *M. ruthenica* in the common environments and at the same loci. In characterizing the population genetic structures of the two species, the aim was to (*a*) quantify genetic variability; (*b*) estimate gene flow; (*c*) infer the correlation between the genetic relationships and geographical distributions; and (*d*) integrate the genetic information with phenotypic variation in mating system, life form and seed dispersal to understand the population dynamics and evolutionary processes of the two *Medicago* species.

MATERIALS AND METHODS

Plant materials

Medicago ruthenica Trautv. is distributed from central China to Mongolia and Siberia (Small and Jomphe, 1989). Our population sampling covers its entire distributional range in China (Fig. 1). *Medicago lupulina* L. has a broader distribution, occurring natively in temperate and subtropical Eurasia and North Africa (Dunbier, 1972). To compare its population genetic structure with *M. ruthenica*, populations of *M. lupulina* were sampled from the distributional range of *M. ruthenica*, including five sympatric populations of each species. In addition, populations of *M. lupulina* were sampled from north-western China. A total of 328 individuals were sampled from 16 populations of *Medicago lupulina* and 447 individuals were sampled from 15 populations of

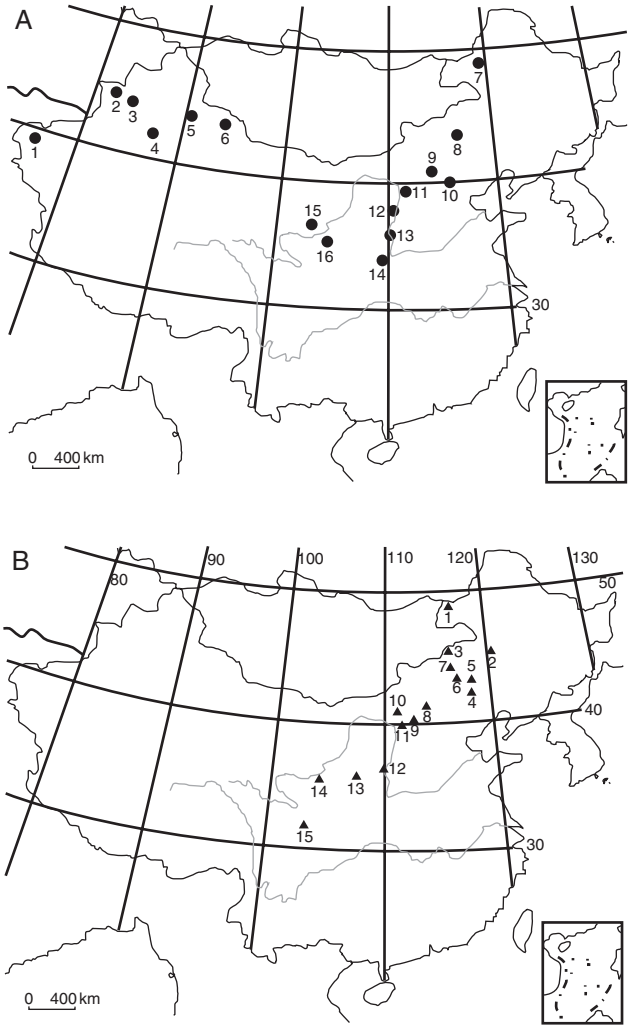

FIG. 1. Distribution of the populations sampled in this study: (A) *Medicago lupulina* (numbers correspond to abbreviations ML-1 to ML-16 in Table 1); (B) *Medicago ruthenica* (numbers correspond to MR-1 to MR-15 in Table 1).

Medicago ruthenica between August and October in 2004 and 2005 (Table 1). Populations were recorded by GPS co-ordinates. Leaf samples were collected from randomly selected individuals in each population and immediately dried using silica gel for DNA isolation.

Microsatellite analysis

DNA was isolated from approx. 0·5 g dried leaves using a modified cetyltrimethy lammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). DNA quality was tested on 0·8 % agarose gels. After measuring DNA concentration with an Eppendorf BioPhotometer, samples were diluted to 5 ng μL^{-1}.

For microsatellite analysis, 92 pairs of primers were selected from previous publications (Diwan *et al.*, 2000; Baquerizo-Audiot *et al.*, 2001; Julier *et al.*, 2003; Eujayl *et al.*, 2004) and the *Medicago* genome website (http://medicago.org/genome/downloads.php). These primers were tested using 32 individuals from each of *M. lupulina* and *M. ruthenica*. Fifteen and 17 primers that detected a suitable

TABLE 1. *Collection localities of* Medicago lupulina *(ML) and* Medicago ruthenica *(MR) populations*

Population	Latitude	Longitude	*N*	n_a	n_e	H_O	H_E	F_{IS}	*s*	Private allele
ML-1	39·44	75·988	23	2·6	1·7	0·014	0·334	0·959	0·979	0
ML-2	43·88	81·305	20	3·7	1·9	0·040	0·378	0·899	0·947	8
ML-3	43·45	83·283	20	2·9	2·1	0·030	0·397	0·928	0·963	1
ML-4	41·75	86·128	17	2·8	2·1	0·012	0·382	0·970	0·985	2
ML-5	43·70	89·604	23	2·7	1·7	0·012	0·292	0·961	0·980	1
ML-6	43·58	93·008	20	2·0	1·3	0·027	0·174	0·852	0·920	0
ML-7	48·90	119·837	18	2·4	1·3	0·015	0·175	0·920	0·958	4
ML-8	43·53	117·236	21	2·0	1·2	0·006	0·160	0·962	0·981	0
ML-9	40·89	113·885	21	1·9	1·3	0·006	0·183	0·965	0·982	0
ML-10	39·96	115·439	12	2·1	1·5	0·006	0·263	0·981	0·990	0
ML-11	39·44	111·461	22	2·0	1·2	0·024	0·130	0·820	0·901	1
ML-12	37·98	109·853	23	2·4	1·5	0·021	0·263	0·925	0·961	1
ML-13	36·32	109·651	20	1·9	1·3	0·000	0·168	1·000	1·000	3
ML-14	34·28	108·958	27	1·5	1·1	0·015	0·055	0·741	0·851	0
ML-15	36·94	102·589	21	3·3	1·5	0·044	0·265	0·840	0·913	9
ML-16	35·78	104·048	20	2·4	1·7	0·000	0·311	1·000	1·000	0
ML mean			20·5	2·4	1·5	0·017	0·246	0·920	0·958	1·875
MR-1	49·56	117·45	51	8·824	4·840	0·627	0·715	0·133	0·235	3
MR-2	45·13	112·49	46	9·000	5·449	0·636	0·729	0·139	0·244	4
MR-3	45·56	117·01	51	8·941	5·141	0·564	0·706	0·210	0·347	2
MR-4	42·29	119·02	20	6·941	3·927	0·619	0·684	0·121	0·216	3
MR-5	42·97	119·01	21	7·412	4·221	0·584	0·686	0·173	0·295	1
MR-6	43·26	117·53	35	8·000	4·359	0·599	0·692	0·149	0·259	6
MR-7	43·53	117·24	36	8·176	4·768	0·557	0·706	0·225	0·367	4
MR-8	40·89	113·87	20	6·941	4·003	0·581	0·660	0·145	0·253	5
MR-9	40·37	113·24	20	7·706	4·829	0·641	0·722	0·138	0·243	1
MR-10	40·91	111·69	35	7·941	4·728	0·603	0·722	0·179	0·304	2
MR-11	39·44	111·46	23	6·059	3·866	0·594	0·677	0·146	0·255	3
MR-12	36·31	109·65	20	6·000	3·772	0·551	0·617	0·135	0·238	1
MR-13	35·75	107·98	21	7·647	4·547	0·591	0·664	0·135	0·238	4
MR-14	35·80	104·06	20	5·647	3·664	0·457	0·608	0·273	0·429	3
MR-15	31·68	103·84	28	6·000	3·220	0·413	0·569	0·292	0·452	3
MR mean			30	7·416	4·356	0·574	0·677	0·173	0·295	3

N, number of individual plants; n_a, observed alleles number; n_e, effective allele number; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{IS}, inbreeding coefficient; *s*, selfing rate.

level of polymorphism in *M. lupulina* and *M. ruthenica*, respectively, were identified (Table 2). The two species shared five microsatellite loci, MTIC14, MTIC188, MTIC189, MTIC432 and AFct45.

Microsatellites were amplified by polymerase chain reaction (PCR). A 10-μL reaction contained 10–50 ng of template DNA, 1 × PCR reaction buffer, 1·5 mM of $MgCl_2$, 0·2 mM of dNTP mix, 0·5 μM of each primer, and 0·5 U of Taq polymerase (Invitrogen). Reactions were performed in a Gene Amp PCR system 9700 (PE Applied Biosystems) with the following programme: 5 min at 95 °C, then 30 cycles of 45 s at 95 °C, primer-specific annealing temperature for 30 s at 50–60 °C, and 72 °C for 1 s, followed by a final extension of 7 min at 72 °C. PCR products were denatured for 5 min at 95 °C and run on a 6 % polyacrylamide denaturing gels that were made 0·4 mm thick. A 25-bp DNA ladder (Promega, Madison, WI, USA) was used as the size standard. The gels were treated with silver stained to visualize DNA bands and score microsatellite alleles. The gel could reliably resolve alleles with 2-bp length difference.

Genetic diversity and mating system analyses

For each population, genetic diversity was estimated across all loci using the observed number of alleles (n_a), effective number of alleles (n_e), H_E, H_O, F_{IS}, and the number of private alleles. For each microsatellite locus, genetic polymorphism was assessed by calculating the total number of alleles (*A*, allelic diversity), the expected and observed heterozygosity (H_E and H_O). The inbreeding coefficient, F_{IS}, was calculated by FSTAT 2·9·3 (Goudet, 2001). GenAlEx 6 software was used to estimate n_e, n_a, A, H_O, H_E (Peakall and Smouse, 2006). Selfing rate was estimated as $s = 2F_{IS}/(1 + F_{IS})$ (Ritland, 1990). Deviation from the Hardy–Weinberg equilibrium and linkage disequilibrium were tested using the FSTAT program (Goudet, 2001). The significant values for the linkage disequilibrium were corrected for multiple comparisons by Bonferroni correction (Rice, 1989)

Genetic structure and genetic differentiation

STRUCTURE 2·2 (Pritchard *et al.*, 2000; Falush *et al.*, 2003) was used to test whether *M. lupulina* and *M. ruthenica* were genetically differentiated without *a priori* classification of individuals. The program STRUCTURE implements a model-based clustering method to demonstrate the presence of population structure, assign individuals to populations and identify migrants and admixed individuals (Pritchard *et al.*, 2000), by assuming that the markers are unlinked and at linkage equilibrium within populations.

TABLE 2. *Diversity statistics and summary of* F-*statistics of microsatellite loci for* Medicago lupulina *and* Medicago ruthenica

Locus	*A*	H_E	H_O	F_{ST}	*Nm*
M. lupulina					
Mtgsp_005g08.ag.21-1	10	0·395	0·006	0·491	0·259
Mtgsp_001b05.ag.17-1	7	0·384	0·031	0·476	0·276
MAA660870	5	0·162	0·003	0·604	0·164
MtSSRNFAL05	7	0·278	0·018	0·638	0·142
MTR58	16	0·524	0·012	0·411	0·358
MTIC210	9	0·275	0·003	0·564	0·193
MTIC251	3	0·063	0·002	0·762	0·078
MTIC339	3	0·037	0·000	0·062	3·774
MTIC345	8	0·323	0·090	0·421	0·344
MTIC451	8	0·222	0·020	0·670	0·123
MTIC14	6	0·187	0·000	0·604	0·164
MTIC188	11	0·258	0·015	0·585	0·177
MTIC189	9	0·267	0·016	0·551	0·203
MTIC432	5	0·184	0·037	0·612	0·158
AFct45	5	0·127	0·003	0·567	0·191
ML mean	7·5	0·246	0·017	0·535	0·218
M. ruthenica					
Mtgsp_005e04.taa.9-1	13	0·681	0·617	0·186	1·097
Mtgsp_002c04.ac.29-1	39	0·858	0·641	0·082	2·800
MtSSRNFAA02	7	0·445	0·484	0·082	2·788
MtSSRNFAL45	20	0·830	0·698	0·071	3·292
MAL369471	13	0·741	0·665	0·074	3·126
AI974357	16	0·668	0·572	0·154	1·377
MTIC48	24	0·735	0·573	0·139	1·547
MTIC134	9	0·574	0·450	0·141	1·527
MTIC249	9	0·534	0·299	0·155	1·367
MTIC343	17	0·808	0·671	0·068	3·422
MTIC354	11	0·453	0·489	0·057	4·175
MTIC471	13	0·674	0·493	0·174	1·189
MTIC14	6	0·475	0·302	0·244	0·776
MTIC188	22	0·859	0·835	0·072	3·214
MTIC189	22	0·885	0·834	0·053	4·425
MTIC432	28	0·710	0·603	0·211	0·936
AFct45	9	0·582	0·539	0·251	0·747
MR mean	16·4	0·677	0·577	0·130	1·672

A, Average number of alleles per locus; H_E, expected heterozygosity; H_O, observed heterozygosity; F_{ST}, coefficient of genetic differentiation; *Nm*, gene flow.

The five loci shared between the two species are indicated in bold.

The program calculates an estimate of the posterior probability of the data for a given *K*, Pr(*X*/*K*) (Pritchard *et al.*, 2000). The range of possible *K* was from 1 or 2 to the true number of populations plus 3 (Evanno *et al.*, 2005). Therefore, the number of populations (clusters), *K*, was set from 1 to 18. Under the assumption that admixture model and allele frequencies correlated, each *K* was replicated 3–5 times with different probability (ν) for 100 000 iterations after a burn-in period of 50 000 without prior information on the population of origin. Additionally, population divergence was quantified using θ, and an unbiased estimator of F_{ST} (Weir and Cockerham, 1984; Slatkin, 1995) was estimated using FSTAT version 2·9·3 (Goudet, 2001). Genetic differentiation within and among populations was further measured by analysis of molecular variance (AMOVA) using ARLEQUIN 3·1 (Excoffier *et al.*, 2005).

The POPULATION 1·2 software (Langella, 2000) was used to calculate the Nei's genetic distance (D_A) (Nei, 1983)among individuals within each species. Unrooted neighbor-joining trees based on Nei's genetic distance were constructed and visualized with the TREEVIEW software (Page, 1996). To investigate spatial genetic structure, the relationship between the matrix of pairwise genetic distance [$F_{ST}/(1 - F_{ST})$] and the matrix of the logarithm of geographical distances was analysed via a Mantel's test (Mantel, 1967) with 100 000 random permutations using the program IBD (Bohonak, 2002). Geographic distances between pairs of populations were calculated from linear distances between latitude and longitude positions (http://jan.ucc.nau.edu/~cvm/latlongdist.html).

Gene flow

Individual-based assignment tests have been used to estimate contemporary rates of gene flow and dispersal (Berry *et al.*, 2004; Mix *et al.*, 2006). A partial exclusion Bayesian-based individual assignment test (Rannala and Mountain, 1997), implemented in the GeneClass 2·0 (Piry *et al.*, 2004), was used to assess the recent gene flow between pairs of populations. Assignment probabilities are computed based on the resampling method of Paetkau *et al.* (2004). A total number of 1000 individuals was simulated and a threshold of 0·01 was used. The limitation of this method is that the migration rate observed during a short study might not accurately reflect long-term patterns of gene flow (Manel *et al.*, 2005). Therefore, F_{ST} was used to estimate historical rates of gene flow (*Nm*) according to Wright's island model of population genetic structure, where $F_{ST} \approx 1/(1 + 4Nm)$ (Wright, 1951; Slatkin and Barton, 1989; Gaggiotti *et al.*, 1999; Sork *et al.*, 1999).

RESULTS

Genetic diversity and mating system

The level and pattern of estimated genetic variation differed substantially between populations of *M. lupulina* and *M. ruthenica* (Table 1 and Fig. 2). For *M. lupulina*, a total of 328 individuals from 16 populations was surveyed, in which 112 alleles were identified at 15 microsatellite loci.

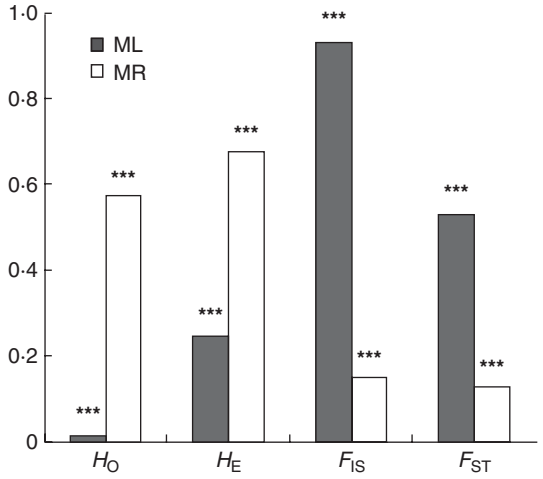

FIG. 2. Comparison of microsatellite diversity between *Medicago lupulina* (ML) and *Medicago ruthenica* (MR); ***$P < 0·001$.

The average of observed heterozygosity (H_O) within populations was 0·017, ranging from 0 to 0·044. The values are considerably lower than expected heterozygosity (H_E) assuming the Hardy–Weinberg equilibrium, which averaged 0·246.

In comparison to *M. lupulina*, *M. ruthenica* has a higher level of genetic variation. In 447 individuals from 15 sampled populations of *M. ruthenica*, 278 alleles were found at 17 loci. The average H_O of each populations is 0·574, ranging from 0·413 to 0·641. This is slightly lower than the average H_E of 0·677. *Medicago ruthenica* populations also possessed a larger average number of private alleles than *M. lupulina* populations (3 versus 1·9). However, the variation in the number of private alleles is greater among *M. lupulina* populations (range 0–9) than that of the *M. ruthenica* populations (range 1–5).

When the genetic variation was compared between the two species at the five shared microsatellite loci, *M. ruthenica* accessions also showed a higher allelic diversity than *M. lupulina* (Table 2). At these five loci, 17·4 alleles per locus were found for *M. ruthenica* and 7·2 alleles per locus for *M. lupulina*. This is similar to the averages of all sampled loci, which was 16·4 and 7·5 alleles per locus for *M. ruthenica* and *M. lupulina*, respectively.

The test for the Hardy–Weinberg equilibrium found that of 240 locus–population combinations in *M. lupulina*, 165, 128 and 109 or 68·8 %, 53·3 % and 45·4 % showed significant deviation at $P = 0{\cdot}05$, 0·01 and 0·001, respectively. For *M. ruthenica*, of 255 locus-population combinations, 112, 79 and 48 or 43·9 %, 30·9 % and 18·8 % showed significant deviation at $P = 0{\cdot}05$, 0·01 and 0·001, respectively. The test for the genotypic disequilibrium in all samples within each species found that 80 of 105 locus pairs in *M. lupulina* and 36 of 136 locus pairs in *M. ruthenica* showed significant deviation at the $P = 5$ %, but none of locus pairs was found to be in significant genotypic disequilibrium after the Bonferroni-type correction.

The average F_{IS} values were 0·920 (range 0·741–1·000) and 0·173 (range 0·121–0·292) for *M. lupulina* and *M. ruthenica*, respectively. From these values, the rates of self-fertilization of *M. lupulina* and *M. ruthenica* are calculated at 95·8 % and 29·5 %, respectively.

Population genetic structure and gene flow

The analysis of molecular variance (AMOVA, Table 3) revealed that the majority of genetic variation occurred among populations in *M. lupulina* (55·5 %) and within individuals in *M. ruthenica* (76·06 %). In contrast, the minimum partitions of genetic variation resided within individuals in *M. lupulina* (3·07 %) and among populations in *M. ruthenica* (10·81 %). Bayesian clustering without prior information about geographical origin of populations showed that the highest likelihood value (Ln PrX/K) occurred at $K = 16$ in *M. lupulina* and $K = 15$ in *M. ruthenica* (Fig. 3), where the number of clusters (K) was consistent with the natural populations sampled in this study. The result held for different values of v (the probability that an individual was an immigrant to a given population; $v = 0{\cdot}01$, 0·05 and 0·1).

Substantial genetic differentiation was found among populations for both *M. lupulina* ($F_{ST} = 0{\cdot}535$ $P < 0{\cdot}001$) and *M. ruthenica* ($F_{ST} = 0{\cdot}130$, $P < 0{\cdot}001$; Table 2). The Mantel's test showed that the genetic distance $[F_{ST}/(1 - F_{ST})]$ and the geographical distance of the populations of each species are positively correlated (*M. lupulina*: $r = 0{\cdot}27$, $P = 0{\cdot}0094$; *M. ruthenica*: $r = 0{\cdot}41$, $P = 0{\cdot}0018$; Fig. 4). The cluster analyses of population relationships based on genetic distance showed the populations from closely situated regions were grouped together (Fig. 5). Nevertheless, some exceptions are noteworthy. Populations MR-10 and MR-12 from central China were clustered with populations from north-eastern China. ML-7 in north-eastern China was clustered with populations from north-western China, ML1-5, rather than clustered with the nearest population ML-8. Similarly, populations ML-8 was clustered with ML-6 in western China.

Based on estimated F_{ST}, historical gene flow among the sampled populations (Nm) was calculated at 0·218 in *M. lupulina* and 1·672 in *M. ruthenica* (Table 2). With regard to the contemporary gene flow, the assignment tests

TABLE 3. *The analysis of molecular variance (AMOVA) for 16* Medicago lupulina *populations and 15* Medicago ruthenica *populations*

Source of variation	d. f.	Variance components	Percentage of variation	*P*
M. lupulina				
Among populations	15	2·39	55·50	<0·001
Among individuals within populations	312	1·83	41·43	<0·001
Within individuals	328	0·13	3·07	<0·001
Total	655	4·22		
M. ruthenica				
Among populations	14	0·68	10·81	<0·001
Among individuals within populations	432	0·83	13·13	<0·001
Within individuals	447	4·79	76·06	<0·001
Total	893	6·30		

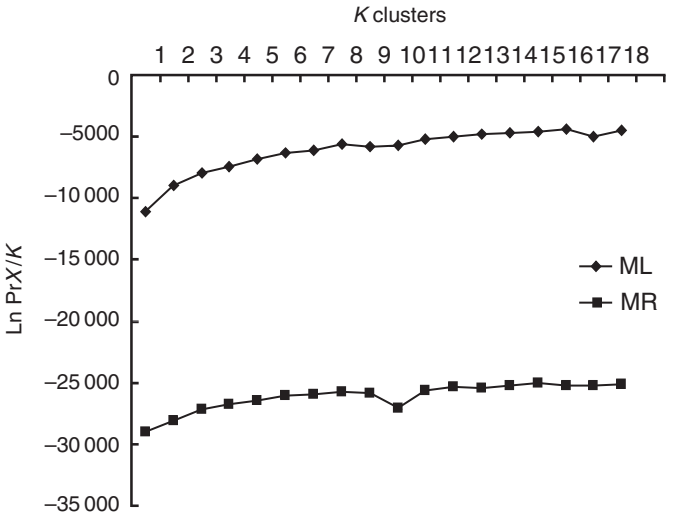

FIG. 3. Estimated posterior probability of K (1–18) for *Medicago lupulina* ($n = 328$) and *Medicago ruthenica* ($n = 447$) averaged over five runs, where K represents the number of clusters and n represent the number of samples.

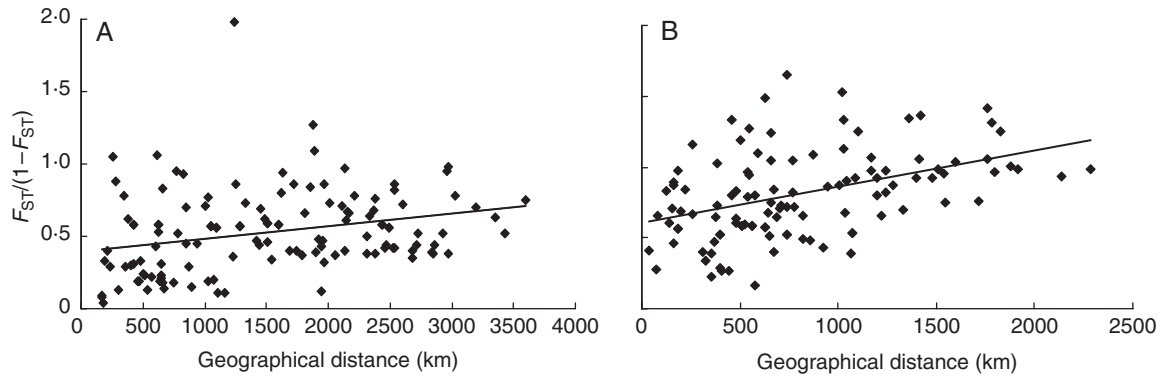

FIG. 4. Scatter plots of pairwise genetic distance [$F_{ST}/(1 - F_{ST})$] versus geographical distance (km) of all sampled populations of (A) *M. lupulina* and (B) *M. ruthenica*.

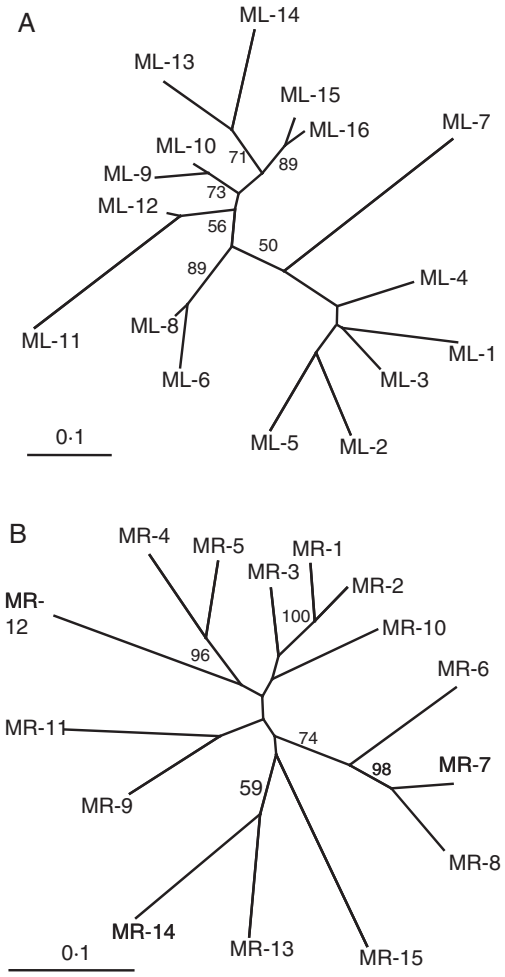

FIG. 5. Unrooted neighbor-joining tree showing relationships among the (A) 16 *M. lupulina* and (B) 15 *M. ruthenica* populations. Numbers associated with branches indicate bootstrap values higher than 50 %, based on 1000 replications. Refer to Table 1 for abbreviations of populations.

showed that an average of 80·8 % ($P < 0·01$) and 82·6 % ($P < 0·01$) of individuals were correctly assigned to their own source populations in *M. lupulina* and *M. ruthenica*, respectively (Fig. 6). For the remaining individuals, 3·4 % of *M. lupulina* and 1·4 % of *M. ruthenica* individuals did not belong to any of the populations sampled, and 15·7 % of *M. lupulina* and 16 % of *M. ruthenica* individuals were assigned to a population different from which they were collected.

DISCUSSION

Genetic variation

This study provides the genetic estimate of the mating systems of the two *Medicago* species. From F_{IS} values, selfing rates were estimated to be higher than 95 % for *M. lupulina* but lower than 30 % for *M. ruthenica*. Together with previous studies (Mulligan, 1972; Bena *et al.* 1998; Campbell *et al.*, 1999) and the observed heterozygosity (H_O), this suggests that *M. lupulina* is predominantly selfing whereas *M. ruthenica* is highly outcrossing.

The estimated mating system differentiation is consistent with differences in floral morphology, similar to the correlation between floral morphology and pollinator attraction found in other plant groups (e.g. Juan *et al.*, 2004; Gómez *et al.*, 2008). *Medicago lupulina* has relatively small flowers with yellow papilionaceous corollas of 2–4 mm long (Fig. 7A). *Medicago ruthenica* has larger and more showy flowers with yellow corollas of approx. 8 mm long tinged with dark purple on the outside of the petals and on the inside toward the base (Fig. 7C). It was observed that *M. ruthenica* was able to attract substantially more insect pollinators such as bees, bumblebees and butterflies.

Mating system and life form play important roles in shaping population genetic structure and distribution of plants (e.g. Loveless and Hamrick, 1984; Hamrick and Godt, 1989, 1996; Stenøien *et al.*, 2005; Clauss and Mitchell-Olds, 2006; Drummond and Hamilton, 2007; Mable and Adam, 2007; Michalski and Durka, 2007). The substantially higher selfing rate in *M. lupulina* could have contributed to a lower overall level of estimated heterozygosity ($H_E = 0·246$) than those from *M. ruthenica* ($H_E = 0·677$). A low level of heterozygosity ($H_E = 0·348–0·476$) was also found for the selfing species *M. truncatula* in the French Mediterranean region (Bonnin *et al.*, 2001; Ellwood *et al.*, 2006), whereas a higher level of heterozygosity ($H_E = 0·665–0·717$) was observed for an outcrossing species *Medicago sativa* (Flajoulot *et al.*, 2005).

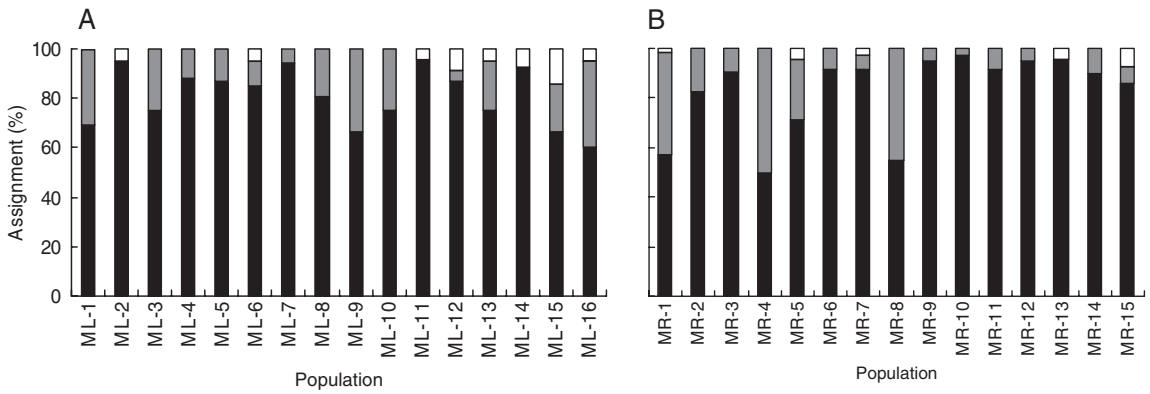

FIG. 6. Results of the assignment test for (A) *Medicago lupulina* and (B) *Medicago ruthenica*. Columns indicate the percentages of individuals per population correctly assigned to the source population (black), assigned to other sampled populations (grey) and not assigned to any sampled population (white).

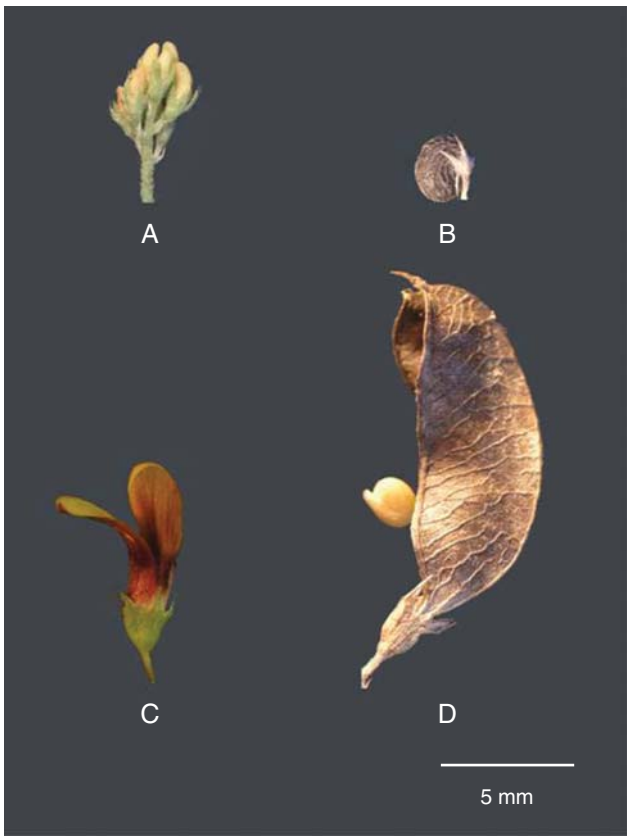

FIG. 7. Morphology of flowers and fruits of *M. lupulina* and *M. ruthenica*: (A, B) an inflorescence and an indehiscent fruit of *M. lupulina*; (C, D) a flower and a dehiscent fruit with an exposed seed of *M. ruthenica*.

These values are also comparable to the genetic diversity previously reported for other plant species with the similar mating system and life form. For instance, *Arabidopsis thaliana*, a selfing annual plant, had a much lower level of the genetic diversity ($H_E = 0{\cdot}01$, $0{\cdot}06$) than the self-incompatible perennial relatives *A. halleri* ($H_E = 0{\cdot}31$) and *A. petraea* ($H_E = 0{\cdot}41$) (Clauss *et al.*, 2002; Stenøien *et al.*, 2005). The present results are generally consistent with the trends of genetic variation observed in many flowering plant groups based on microsatellite data, with average H_E values being $0{\cdot}41$ for inbreeding populations versus $0{\cdot}65$ for outcrossing populations and $0{\cdot}46$ for annuals versus $0{\cdot}68$ for perennials (Nybom, 2004).

Population differentiation and gene flow

The estimate of population differentiation showed a much higher population genetic differentiation in *M. lupulina* ($F_{ST} = 0{\cdot}535$) than in *M. ruthenica* ($F_{ST} = 0{\cdot}130$). This fits well with the general estimates of approx. 5-fold higher F_{ST} in selfing, annual species than outcrossing, perennial species for flower plants (Hamrick and Godt, 1996).

The significantly positive correlation between genetic and geographical distances detected in *M. lupulina* ($r = 0{\cdot}2703$, $P = 0{\cdot}0094$) and *M. ruthenica* ($r = 0{\cdot}4113$, $P = 0{\cdot}0018$) indicates that spatial separation has played a role in shaping the population genetic structure of the species. There is a general tendency that closely situated populations are genetically more similar. However, a close relationship between two disjunct populations, ML-6 and ML-8, suggests that there exists a dispersal or gene flow corridor connecting these regions across the Mongolian grasslands. Although it was not possible to sample *M. lupulina* populations from Mongolia, finding genetically similar populations flanking the width of Mongolia implies that the gene pool from this broad region may be represented in the present samples.

While geographical isolation has played a role in population genetic differentiation, there is evidence for gene flow between populations of each species. Among individuals of *M. lupulina* and *M. ruthenica*, 3·4 % and 1·4 %, respectively could not be assigned to sampled populations, suggesting that they were immigrants from outside the areas sampled (Fig. 6). In addition, the assignment of 15·7 % and 16 % of individuals to populations different to their sampled populations may be a result of gene flow between these populations.

Mating system, seed dispersal and population structure and distribution

Plants and their genes migrate through seed and pollen dispersal. The study of the interplay between seed dispersal and

pollination is essential for understanding plant population structure and distribution (Govindaraju, 1988; Ennos, 1994; Bohonak, 1999; McCauley, 1997; Heuertz *et al.*, 2003; Juan *et al.*, 2004; Otero-Arnaiz, 2005). Seed dispersal of *M. lupulina* and *M. ruthenica* may be substantially affected by the distinct fruit morphologies (e.g. Janson, 1983; Gautier-Hion *et al.*, 1985; Willson and Traveset, 2000). Pods of *M. lupulina* are rough-ridged, indehiscent, up to 3 mm long and 1 mm wide and each contains one seed (Fig. 7B). Rough-surfaced, indehiscent pods are effectively dispersed by birds and animals through adhesion or ingestion (Lammerink, 1968; Dunbier, 1972; Sorensen 1986). In addition, indehiscent pods of *M. lupulina* can float in water for up to 12 d (Turkington and Cavers, 1979).

Medicago ruthenica, however, lacks an effective mechanism of seed dispersal. Its pod is up to 15 mm long and 5 mm wide with an oblong-falcate suture and contains three to five seeds (Fig. 7D). The dorsal suture is strongly convex and the ventral suture weakly convex to straight, facilitating dehiscence when pods mature. Pods of *M. ruthenica* dehisce and release seeds near the mother plant. Seeds have no wings and are dispersed mainly by gravity. This is similar to wild soybean which was reported to disperse seeds within 4·5 m of the mother plants (Oka, 1983; Kuroda *et al.*, 2006).

The difference in current distributional ranges of the two species could be attributed at least partly to seed dispersal abilities. *Medicago lupulina*, with more effective seed dispersal mechanisms, occurs in a much broader geographical range than *M. ruthenica*. The narrower distributional range of *M. ruthenica* does not seem to have been a result of population extinction. *Medicago ruthenica* populations that were studied in the field appeared healthy, and the level of genetic variation currently maintained within and among populations does not provide any indication that this outcrossing species has experienced a severe reduction in genetic variation.

Despite more effective seed dispersal of *M. lupulina*, the present genetic data showed that there has been a much higher level of historical gene flow between *M. ruthenica* populations. This is not surprising, given a greater pollen dispersal capacity of the outcrosser, *M. ruthenica* than the selfer, *M. lupulina*. In predominantly selfing species, individuals migrating into other populations may not effectively incorporate their private alleles into the local populations through cross-pollination. As a result, these alleles may easily get lost through drift if they are not favoured by selection. New alleles from other populations are likely to be less fit than the alleles of the native populations that have been selected by local ecological factors. Furthermore, migrating alleles are especially susceptible to loss through drift in annual species. For an out-crossing species, on the other hand, migration into new populations via seed dispersal allows alleles to be much more easily integrated into the gene pool of the recipient populations through cross-pollination. Less-fit alleles may be maintained in recipient populations at the heterozygous loci as long as they are at least partially recessive, and may persist for a relatively long period of time in a perennial species even though they are not postively selected into the local gene pool.

Taken together, this study between the two *Medicago* species with a combination of several distinct biological features has allowed us to gain a better understanding of population processes of plant evolution. As a perennial species, *M. ruthenica* benefits from an outcrossing mating system for the maintenance of genetic variation within populations. As a result, the populations can be more stable and less susceptible to pathogen and environmental changes. Consequently, there might have been relatively little pressure for the development of effective seed-dispersal mechanisms. In *M. lupulina*, on the other hand, effective seed-dispersal mechanisms are essential for a predominantly annual species. Indeed, effective seed-dispersal mechanisms could have broadened its distribution. For an annual species possessing effective seed dispersal, self-pollination provides reproductive assurance so that a single or a few seeds could potentially establish a new population in a new locality (Holsinger, 1996). Characterization of the population genetic structure of *M. lupulina* and *M. ruthenica* has provided an understanding of historical population dynamics of the two species and their current distribution.

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LITERATURE CITED

Baquerizo-Audiot E, Desplanque B, Prosperi JM, Santoni S. 2001. Characterization of microsatellite loci in the diploid legume *Medicago truncatula* (barrel medic). *Molecular Ecology Notes* **1**: 1–3.

Barrett SC, Harder LD, Worley AC. 1996. The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society of London, Series B* **351**: 1271–1280.

Bena G, Lejeune B, Prosperi JM, Olivieri I. 1998. Molecular phylogenetic approach for studying life-history evolution: the ambiguous example of the genus *Medicago* L. *Proceedings of the Royal Society of London, Series B* **265**: 1141–1151.

van Berkum P, Beyene D, Bao G, Campbell AT, Eardly BD. 1998. *Rhizobium mongolense* sp. nov., is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica*. *International Journal of Systematic Bacteriology* **48**: 13–22.

Berry O, Tocher MD, Sarre SD. 2004. Can assignment tests measure dispersal? *Molecular Ecology* **13**: 551–561.

Bohonak AJ. 1999. Dispersal, gene flow, and population structure. *The Quarterly Review of Biology* **74**: 21–45.

Bohonak AJ. 2002. IBD (Isolation by Distance): a program for analyses of isolation by distance. *Journal of Heredity* **93**: 153–154.

Bonnin I, Ronfort J, Wozniak F, Olivier I. 2001. Spatial effects and rare outcrossing events in *Medicago truncatula* (Fabaceae). *Molecular Ecology* **10**: 1371–1384.

Campbell TA, Bao G, Xia ZL. 1999. Completion of the agronomic evaluations of *Medicago ruthenica* [(L.) Ledebour] germplasm collected in Inner Mongolia. *Genetic Resources and Crop Evolution* **46**: 477–484.

Cao ZhZh, Feng YQ, Ma HL, Liu XN, Zhou YL, Xu ZM. 2003. *Medicago lupulina* – beautiful water-saving and easily-maintained turf legume. *Pratacultural Science* **4**: 58–60.

Clauss MJ, Mitchell-Olds T. 2006. Population genetic structure of *Arabidopsis lyrata* in Europe. *Molecular Ecology* **15**: 2753–2766.

Clauss MJ, Cobban H, Mitchell-Olds T. 2002. Cross-species microsatellite markers for elucidating population genetic structure in *Arabidopsis* and *Arabis* (Brassicaeae). *Molecular Ecology* **11**: 591–601.

Diwan N, Bouton JH, Kochert G, Cregan. 2000. Mapping of simple sequence repeat (SSR) DNA markers in diploid and tetraploid alfalfa. *Theoretical and Applied Genetics* **101**: 165–172.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Drummond CS, Hamilton MB. 2007. Hierarchical components of genetic variation at a species boundary: population structure in two sympatric varieties of *Lupinus microcarpus* (Leguminosae). *Molecular Ecology* **16**: 753–769.

Dunbier MW. 1972. Genetic variability in *Medicago lupulina* L. across a valley in the Weka Pass, New Zealand. *New Zealand Journal of Botany* **10**: 48–58.

Ellwood SR, Souza NKD, Kamphuis LG, Burgess TI, Nair RM, Oliver RP. 2006. SSR analysis of the *Medicago truncatula* SARDI core collection reveals substantial diversity and unusual genotype dispersal throughout the Mediterranean basin. *Theoretical and Applied Genetics* **112**: 977–983.

Ennos RA. 1994. Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* **72**: 250–259.

Eujayl I, Sledge MK, Wang L, *et al.* 2004. *Medicago truncatula* EST-SSRs reveal cross-species genetic markers for *Medicago* spp. *Theoretical and Applied Genetics* **108**: 414–422.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611–2620.

Excoffier L, Laval G, Schneider S. 2005. Arlequin version 3·0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**: 47–50.

Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**: 1567–1587.

Flajoulot S, Ronfort J, Baudouin P, *et al.* 2005. Genetic diversity among alfalfa (*Medicago sativa*) cultivars coming from a breeding program, using SSR markers. *Theoretical and Applied Genetics* **111**: 1420–1429.

Gaggiotti OE, Lange O, Rassmann K, Gliddon C. 1999. A comparison of two indirect methods for estimating average levels of gene flow using microsatellite data. *Molecular Ecology* **8**: 1513–1520.

Gautier-Hion A, Duplantier JM, Quris R, *et al.* 1985. Fruit characters as a basis of fruit choice and seed dispersal in a tropical forest vertebrate community. *Oecologia* **65**: 324–337.

Goudet J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices. version 2.9.3. http://www2.unil.ch/popgen/softwares/fstat.htm.

Govindaraju DR. 1988. Relationship between dispersal ability and levels of gene flow in plants. *Oikos* **52**: 31–35.

Gómez J, Bosch J, Perfectti F, Fernández JD, Abdelaziz M, Camacho JPM. 2008. Association between floral traits and rewards in *Erysimum mediohispanicum* (Brassicaceae). *Annals of Botany* **101**: 1413–1420.

Hamrick JL, Godt MJW. 1989. Allozyme diversity in plant species. In: Brown AHD, Clegg MT, Kahler AL, Weir BS, eds. *Plant population genetics, breeding and germplasm resources*. Sunderland, MA: Sinauer, 43–63.

Hamrick JL, Godt MJW. 1996. Effects of life-history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **351**: 1291–1298.

Hébert D, Fauré S, Olivieri I. 1994. Genetic, phenotypic, and environmental correlations in black medic, *Medicago lupulina* L., grown in three different environments. *Theoretical and Applied Genetics* **88**: 604–613.

Heuertz M, Vekemans X, Hausman JF, Palada M, Hardy OJ. 2003. Estimating seed vs. pollen dispersal from spatial genetic structure in the common ash. *Molecular Ecology* **12**: 2483–2495.

Holsinger KE. 1996. Pollination biology and the evolution of mating systems in flowering plants. *Evolutionary Biology* **29**: 107–149.

Janson CH. 1983. Adaptation of fruit morphology to dispersal agents in a Neotropical forest. *Science* **219**: 187–189.

Juan A, Crespo MB, Cowan RS, Lexer C, Fay MF. 2004. Patterns of variability and gene flow in *Medicago citrina*, an endangered endemic of islands in the western Mediterranean, as revealed by amplified fragment length polymorphism (AFLP). *Molecular Ecology* **13**: 2679–2690.

Julier B, Flajoulot S, Barre P, *et al.* 2003. Construction of two genetic linkage maps in cultivated tetraploid alfalfa (*Medicago sativa*) using microsatellite and AFLP markers. *BMC Plant Biology* **3**: 1471–1490.

Kuroda Y, Kaga A, Tomooka N, Vaughan DA. 2006. Population genetic structure of Japanese wild soybean (*Glycine soja*) based on microsatellite variation. *Molecular Ecology* **15**: 959–974.

Lammerink J. 1968. Genetic variability in commencement of flowering in *Medicago lupulina* L. in the south island of New Zealand. *New Zealand Journal of Botany* **6**: 33–42.

Langella O. 2000. POPULATIONS 1·2: population genetic software, individuals or population distance, phylogenetic trees. http://bioinformatics.org/~tryphon/populations/

Levin DA. 1981. Dispersal versus gene flow in plants. *Annals of the Missouri Botanical Garden* **68**: 233–253.

Li HX, Shi FL. 2006. Current situation of yield improvement and seed production of *Melilotoides ruthenica* in China. *Grassland and Turf* **3**: 14–16.

Loveless MD, Hamrick JL. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics* **15**: 65–95.

McCauley DE. 1997. The relative contributions of seed and pollen movement to the local genetic structure of *Silene alba*. *Journal of Heredity* **88**: 257–263.

Mable BK, Adam A. 2007. Patterns of genetic diversity in outcrossing and selfing populations of *Arabidopsis lyrata*. *Molecular Ecology* **16**: 3565–3580.

Manel S, Gaggiotti OE, Waples RS. 2005. Assignment methods: matching biological questions with appropriate techniques. *Trends in Ecology and Evolution* **20**: 136–142.

Mantel N. 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**: 209–220.

Mariani A, Pupilli F, Calderini O. 1996. Cytological and molecular analysis of annual species of the genus *Medicago*. *Canadian Journal of Botany* **74**: 299–307.

Michalski SG, Durka W. 2007. High selfing and high inbreeding depression in peripheral populations of *Juncus atratus*. *Molecular Ecology* **16**: 4715–4727.

Mix C, Arens PFP, Rengelink R, Smulders MJM, van Groenendael JM, Ouborg NJ. 2006. Regional gene flow and population structure of the wind-dispersal plant species *Hypochaeris radicata* (Asteraceae) in an agricultural landscape. *Molecular Ecology* **15**: 1749–1758.

Mulligan GA. 1972. Autogamy, allogamy, and pollination in some Canadian weeds. *Canadian Journal of Botany* **50**: 1767–1771.

Nei M, Tajima F, Tateno Y. 1983. Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution* **19**: 153–170.

Nybom H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology* **13**: 1143–1155.

Oka HI. 1983. Genetic control of regenerating success in semi-natural conditions observed among lines derived from a cultivated × wild soybean hybrid. *Journal of Applied Ecology* **20**: 937–947.

Otero-Arnaiz A, Casas A, Hamrick JL. 2005. Direct and indirect estimates of gene flow among wild and managed populations of *Polaskia chichipe*, an endemic columnar cactus in Centro Mexico. *Molecular Ecology* **14**: 4313–4322.

Paetkau D, Slade R, Burden M, Estoup A. 2004. Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology* **13**: 55–65.

Page RDM. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**: 357–358.

Peakall R, Smouse PE. 2006. GENALE6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288–295.

Piry S, Alapetite A, Cornuet JM, Paetkau D, Baudouin L, Estoup A. 2004. GeneCalss2: a software for genetic assignment and first generation migrants detection. *Journal of Heredity* **95**: 536–539.

Pritchard JK, Stephans M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Qi QH. 1996. Studies on properties of photosynthetic ecology in *Melilotides ruthenica*. *Acta Agrestia Sinica* **2**: 110–115.

Rannala B, Mountain JL. 1997. Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences of the USA* **94**: 9197–9221.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223–225.

Ritland K. 1990. Inferences about inbreeding depression based on changes of the inbreeding coefficient. *Evolution* **44**: 1230–1241.

Sidhu SS. 1971. *Some aspects of the ecology of black medick (*Medicago lupulina *L.)*. PhD Dissertation, University of Western Ontario, Canada.

Slatkin M. 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* **139**: 457–462.

Slatkin M, Barton NH. 1989. A comparison of three indirect methods for estimating average levels of gene flow. *Evolution* **43**: 1349–1368.

Small E, Jomphe M. 1989. A synopsis of the genus *Medicago* (Leguminosae). *Canadian Journal of Botany* **67**: 3260–3294.

Song BH, Clauss MJ, Pepper A, Mitchell-Olds T. 2006. Geographic patterns of microsatellite variation in *Boechera stricta*, a close relative of Arabidopsis. *Molecular Ecology* **15**: 357–369.

Sorensen AE. 1986. Seed dispersal by adhesion. *Annual Reviews in Ecology and Systematics* **17**: 443–463.

Sork VL, Nason J, Campbell DR, Fernández JF. 1999. Landscape approaches to historical and contemporary gene flow in plants. *Trends in Ecology and Evolution* **14**: 219–224.

Stenøien HK, Fenster CB, Tonteri A, Savolainen O. 2005. Genetic variability in natural populations of *Arabidopsis thaliana* in northern Europe. *Molecular Ecology* **14**: 137–148.

Turkington R, Cavers PB. 1979. The biology of Canadian weeds. 33. *Medicago lupulina* L. *Canadian Journal of Plant Science* **59**: 99–110.

Wei Z, Huang YL. 1998. *Flora Reipublicae Popularis Sinicae*, Tomus 42 (2). Beijing: Science Press, 314–316.

Weir BS, Cockerham CC. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**: 1358–1370.

Willson MF, Traveset A. 2000. The ecology of seed dispersal. In: Fenner M, ed. *Seeds: the ecology of regeneration in plant communities*, 2nd edn. Wallingford: CAB International, 85–110.

Wilson LC. 2005. *Characteristics of black medic (*Medicago lupulina *L.) seed dormancy loss in western Canada*. Master Thesis, University of Manitoba, Canada.

Wright S. 1951. The genetic structure of population. *Annals of Eugenics* **15**: 323–354.