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Genetic diversity and structure in a natural *Elymus caninus* population from Denmark based on microsatellite and isozyme analyses

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Abstract. Genetic diversity in a natural *Elymus caninus* population from Denmark was assessed using isozyme and microsatellite markers. A total of 119 individuals from 46 maternal plants were assayed. Microsatellite loci are shown to display higher levels of variation than isozyme loci. The mean number of alleles per locus was 1.04 for isozymes and 1.38 for microsatellites. The percentage of polymorphic loci for isozymes and microsatellites was 4.7% and 23.6% across the maternal plant, respectively. The genetic diversity at population level was 0.1 for isozymes, and 0.63 for microsatellites. The mean genetic diversity at maternal plant level was 0.027 for isozyme loci and 0.117 for microsatellite loci. The average of total allozyme diversity (H_T) was 0.22. The average of total microsatellite diversity was 0.56. Isozyme and microsatellite variation showed the same pattern of differentiation between maternal plants. More than 75% total genetic diversity was found among maternal plants. About 25% total genetic diversity was detected within maternal plants. Ten (22.7%) maternal plants produced heterozygous offspring at allozyme loci, and 30 (68.2%) maternal plants gave heterozygous offspring at microsatellite loci. Both types of markers revealed a relatively high genetic diversity in this population.

Key words: Population genetics, genetic diversity, microsatellite, isozyme, *Elymus caninus*.

Introduction

The knowledge of genetic diversity and population structure is a prerequisite for the successful management of conservation programs. A range of investigations on the genetic diversity in Eurasian *Elymus* species have being carried out at the Department of Plant breeding Research, Swedish University of Agriculture Sciences (Sun et al. 1998a, b, c, 1999; Díaz et al. 1998; Díaz 1999). Particularly, the program is concentrating on four native species from Scandinavia, namely *E. caninus* (L.) L., *E. alaskanus* (Scrib. ex Merr.) Löve, *E. fibrosus* (Schrenk) Tzvel., and *E. mutabilis* (Dobr.) Tzvel. Starch gel electrophoresis was employed to investigate isozyme variation in 128 accessions of 26 *Elymus* species originating in Europe, Asia and North and South America. Considerable isozyme variation was observed among accessions both within and between species (Díaz et al. 1998). Levels and distribution of allozyme, RAPD and microsatellite variation in populations of *Elymus fibrosus* were analyzed (Sun et al. 1998c; Díaz et al. 2000). Isozymes, RAPDs and microsatellites revealed different pictures of the amount of the genetic variation, but agreement was found

regarding how this genetic variation is distributed among populations. Generally, *E. fibrosus* contains low genetic variation in its populations (Sun et al. 1998c, Díaz et al. 2000). Genetic variability was assessed within and among 13 *E. alaskanus* populations collected from Iceland, Norway, Sweden and Russia. Low allozymic variability was detected in these populations (Díaz 1999). Allozyme analysis was used to examine the genetic structure of 10 Finnish and Swedish population of *E. mutabilis*. No variation was found either within or among populations at six enzyme systems (Díaz 1999).

Elymus caninus was chosen as a model species during the development and modification of appropriate tools for the screening of genetic diversity in the genus *Elymus*. This species has a wide distribution area ranging from Iceland and the British Isles in the west to southern Siberia in the east and from the subarctic in the north to the Mediterranean in the south. Genetic diversity has been examined on a macrogeographical scale. It shows considerable inter-population variation in morphology, isozyme, prolamine patterns, RAPD and microsatellites (Díaz et al. 1996, 1998; Kostina et al. 1998; Sun et al. 1999).

Previous studies on the genetic diversity were focused on larger geographical scale. For the comprehensive investigation of genetic diversity, it is necessary to include the genetic diversity at microgeographical scale. No information on genetic diversity of microgeographical scale is available in this regard.

Detection of genetic variation among individuals within a population is critical for estimating population genetic parameters. It is also critical for evolutionary studies of mating systems and relatedness. *Elymus caninus* is supposedly autogamous, but to our knowledge, there is no previous report of the mating system for this species. Does genetic diversity exist among the individuals from the same maternity? And if so, what is the extent genetic diversity among the offspring plants from the same maternal plant? In the present report we have addressed these questions by isozyme and microsatellite markers, and provided genetic diversity information on microgeographical scale for *Elymus caninus*.

Materials and methods

Sampling. The *Elymus caninus* population for this study was collected from Slagelse, Falkenberg forest, Sjaelland province, Denmark (Collection No: Dk9604). It grows abundant in a mixed beech forest (*Fagus sylvatica*). The substrate is moraine. The individual spikes from separate plants were collected along two parallel transects. The collected individuals were generally separated by at least 1 m. Three seeds from each spike were sown separately in individual pots in a greenhouse. Totally 138 seeds from 46 spikes (maternal plants) were sown and 119 seeds germinated and grew into seedling.

Allozyme assays. Allozyme analysis was carried out for 119 individuals using horizontal starch gel electrophoresis. The following five enzyme systems were assayed: aconitase (ACO, EC 4.2.1.3), diaphorase (DIA, EC 1.8.1.4), malate dehydrogenase (MDH, EC 1.1.1.37), phosphoglucomutase (PGM, EC 5.4.2.2) and shikimate dehydrogenase (SKD, EC 1.1.1.25). Methods of enzyme extraction, electrophoresis and enzyme staining were as previously described (Díaz et al. 1998).

Microsatellite analysis. DNA was extracted from fresh young leaves of 119 individuals. Five microsatellite markers were isolated from *Elymus caninus* genomic libraries and described by Sun et al. (1998a). Forward and reverse primer sequences for each microsatellite studied are (5′ to 3′): ECGA1: AGCCCATATGTGAGGTGCA, TCG TTCACCATCCATTGTCTC; ECGA2: GTG-CCCGTATCACATCACT, AGTGGATCTTGA-GATTGGAGA; ECGA4: TGATCAAGAAGA-GGAACAT, GATAAGATCGTGACTCTCCT; ECGA22: GAAGGTGACTAGGTCCAAC, AT-AGTCTCGGTCAGGCTC; ECGA89: TTAGCT-CTTTACTTATTCAAAC, TCCTATGATCAAG-CACAAG. The PCR reaction mixture contained 0.2 mM of each deoxynucleotide, 2.0 mM $MgCl_2$, 1.5 U *Taq* polymerase, 15 pmol of each primer and 20 ng template DNA in a reaction volume of 10 µl. PCR was performed in an OmniGene Temperature Cycler (Hybaid) using one of the following two PCR conditions: (1) A PCR profile consisting of one cycle of 94 °C for 3 min, followed by 30 cycles

at 94 °C for 1 min, 54 °C for 45s and 72 °C for 1 min, and a final elongation step of 10 min at 72 °C for locus *ECGA22*. (2) A "touchdown" PCR consisting of 18 cycles of 94 °C for 1 min denaturation and 72 °C for 1 min extension. Annealing (30s) temperatures were progressively decreased by 2 °C every third cycle from 64 °C to 52 °C. The PCR reaction continued for 30 additional cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The reaction ended with a 10 min extension at 72 °C for other loci. The separation of PCR products followed Sun et al. (1998c).

Statistical analyses. We treated the individuals from the same maternity as one sub-population. The following parameters of genetic variation were assessed for the 44 sub-populations studied. Of the remaining two only one seed germinated, and were not included in this analysis. The mean number of alleles per locus (A), the percentage of polymorphic loci (P) and the gene diversity (H_e) were calculated. A locus was considered polymorphic if the frequency of its most common allele does not exceed 0.95 (the 95% criterion). "H_e" 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 (1978), which adjusts for sample sizes. The gene diversity index (H_e) is a more appropriate measure of variability for self-pollinating species where there are very few heterozygotes, but there may be several different homozygous types (Weir 1996, p. 150).

The distribution of the total genetic variation in the species was calculated using Nei's (1973) statistics. At each polymorphic locus, the total gene diversity is presented by H_T, which is partitioned into the mean allelic diversity within populations (H_S) and the allelic diversity among populations (D_{ST}). These quantities are related by the expression $H_T = H_S + D_{ST}$. The proportion of total genetic variation found among populations (G_{ST}) is calculated as the ratio, D_{ST}/H_T. The G_{ST} value will be used in this paper to provide a relative measure of population differentiation. Gene flow among populations was estimated using the indirect method of Slatkin and Barton (1989), that is based on the average number of migrants (Nm) between any pair of populations. Since G_{ST} is equivalent to Wright's between-population differentiation coefficient, Fst (Nei 1973), and Nm were calculated as follows: $Nm = (1 - G_{ST})/4G_{ST}$, where N is the effective population size, m the rate of migration, and G_{ST} the coefficient of gene differentiation. These analyses were performed using the BIOSYS-1 computer package (Swofford and Selander 1989).

Microsatellite data were also scored for each individual. Data matrices were entered into the NTSYS-PC program (Rohlf 1993). The data were analyzed using the Qualitative routine to generate Jaccard's similarity coefficients. Similarity coefficients were used to construct dendrograms using the UPGMA (unweighted pair group method with arithmetic average) and the SHAN (sequential, hierarchical, and nested clustering) routine in the NTSYS programs.

Results

Isozyme analysis. The five enzyme systems (i.e., ACO, DIA, MDH, PGM and SKD) assayed in this study were found to be encoded by 12 loci when 119 individuals were considered for one population. Of the 12 loci listed in Table 1, six (50%) were monomorphic. Of the six polymorphic loci five were diallelic and one showed three alleles (Table 1). For all of the polymorphic loci, the most common allele occurred at a frequency of $\geq$0.79 with the average of 0.88.

The allozyme variation was low in the 44 sub-populations analyzed (Table 2). Polymorphic loci were observed in 10 (22.7%) subpopulations. The mean gene diversity was 0.027, ranging from 0 to 0.2, with the DK44 having the highest value.

The distribution of allozyme variation is shown in Table 3. The average of total allozyme diversity (H_T) was 0.22. The diversity among subpopulations (0.17) was higher than the genetic diversity within subpopulations (0.05). The coefficient of gene differentiation ($G_{ST} = 0.76$) for all polymorphic loci indicates that 76% of the total allozyme variation resulted from differentiation among subpopulations. The overall gene flow (Nm) among maternal plants was estimated to be 0.08.

The dendrogram showed in Fig. 1 separated the sub-populations into two distinct

Table 1. Patterns of variability at individual isozyme and microsatellite loci in a population of *Elymus caninus*

Locus	Sample size	No. of alleles	Frequency of most common allele	Gene diversity
Isozymes				
ACO-1	119	3	0.79	0.35
ACO-2	119	2	0.84	0.27
ACO-3	119	1	1	0
ACO-4	119	1	1	0
DIA-1	119	2	0.92	0.15
MDH-1	119	1	1	0
MDH-2	119	1	1	0
MDH-3	119	2	0.90	0.18
PGM-1	119	2	0.87	0.23
PGM-2	119	2	0.96	0.07
SKD-1	119	1	1	0
SKD-2	119	1	1	0
Mean (all loci, n = 12)		1.58 (0.64)	0.94 (0.07)	0.10 (0.04)
Mean (polymorphic loci only, n = 6)		2.17 (0.37)	0.88 (0.06)	
Microsatellites				
ECGA1	119	6	0.69	0.5
ECGA2	119	7	0.45	0.8
ECGA22	119	4	0.6	0.58
ECGA4	119	5	0.52	0.63
ECGA89	119	9	0.59	0.65
Mean		6.2 (1.72)	0.57 (0.08)	0.63 (0.05)

groups. DK9, DK11, DK48, DK28, DK30, DK43, DK44 and DK46a formed one group, the remaining formed another group.

Microsatellite analysis. All five microsatellite loci were polymorphic when the analysis was based on 119 individuals for one population. The number of alleles was detected from 4 for ECGA-22 to 9 for ECGA89. The average of alleles over the five loci was 6.2. The most common allele occurred at a frequency of ≥0.45 with the average of 0.63 (Table 1).

Microsatellite diversity was analyzed based on 44 sub-populations (Table 2). The mean of polymorphic loci over all sub-populations was 23.6%. Out of the 44 sub-populations, 30 were polymorphic. The unbiased gene diversity over all loci ranged from 0 to 0.447, with the average of 0.117. The sub-population DK11 had the highest level of gene diversity, followed by DK18.

The distribution of genetic diversity of the sub-populations is given in Table 4. The loci *ECGA4* (0.56) and *ECGA89* (0.51) showed the highest values of D_{ST} (measure of inter-subpopulation differentiation). These indicated the important role of the ECGA4 and ECGA89 loci in inter-subpopulation differentiation. The proportion of variation found among populations over all loci ($G_{ST} = 0.78$) indicates that 78% of the total genetic diversity was detected among the sub-populations. The overall gene flow (Nm) among maternal plants was estimated to be 0.07.

Genetic similarity among the 119 individuals was calculated for microsatellite data using the Jaccard algorithm. The similarities ranged from the 0 to 1.00. Dendrograms based

Table 2. Genetic variability at 12 isozyme (I) and 5 microsatellite (M) loci in all subpopulations

Population	Mean no. of alleles per locus		Percentage of polymorphic loci*		Gene diversity	
	I	M	I	M	I	M
DK1	1.0	1.8	.0	40.0	.000	.220
DK2	1.0	1.0	.0	0.0	.000	.000
DK3	1.0	1.0	.0	0.0	.000	.000
DK4	1.1	1.0	8.3	0.0	.050	.000
DK5	1.0	1.4	.0	40.0	.000	.093
DK6	1.1	1.2	8.3	20.0	.050	.067
DK7	1.1	1.0	8.3	0.0	.050	.000
DK8	1.0	1.0	.0	0.0	.000	.000
DK9	1.2	1.6	16.7	20.0	.100	.133
DK10	1.0	1.2	.0	20.0	.000	.067
DK11	1.2	2.4	16.7	80.0	.100	.447
DK12	1.0	1.6	.0	40.0	.000	.253
DK13	1.0	1.8	.0	40.0	.000	.173
DK14	1.0	1.4	.0	40.0	.000	.173
DK15	1.0	1.0	.0	0.0	.000	.000
DK16	1.0	1.2	.0	20.0	.000	.067
DK17	1.0	1.6	.0	60.0	.000	.240
DK18	1.0	2.4	.0	60.0	.000	.400
DK19	1.0	1.0	.0	0.0	.000	.000
DK20	1.0	1.0	.0	0.0	.000	.000
DK21	1.0	1.4	.0	20.0	.000	.127
DK22	1.0	1.6	.0	40.0	.000	.213
DK23	1.0	1.0	.0	0.0	.000	.000
DK24	1.0	1.0	.0	0.0	.000	.000
DK26	1.0	1.4	.0	20.0	.000	.120
DK27	1.0	1.2	.0	20.0	.000	.067
DK28	1.1	1.2	8.3	20.0	.056	.047
DK29	1.0	1.4	.0	20.0	.000	.147
DK30	1.1	1.8	8.3	40.0	.056	.180
DK31	1.0	2.6	.0	60.0	.000	.387
DK32	1.0	1.6	.0	40.0	.000	.173
DK33	1.0	1.8	.0	40.0	.000	.267
DK34	1.0	1.0	.0	0.0	.000	.000
DK35	1.0	1.4	.0	40.0	.000	.133
DK36	1.0	1.8	.0	60.0	.000	.360
DK38	1.0	1.4	.0	20.0	.000	.127
DK41	1.0	1.4	.0	40.0	.000	.120
DK42	1.0	1.0	.0	0.0	.000	.000
DK44	1.3	1.2	33.3	20.0	.200	.067
DK45	1.0	1.2	.0	20.0	.000	.067
DK46a	1.3	1.2	33.3	20.0	.200	.067
DK46b	1.0	1.0	.0	0.0	.000	.000
DK47	1.0	1.4	.0	20.0	.000	.147
DK48	1.3	1.0	25.0	0.0	.167	.000
Mean	1.04	1.38	4.7	23.6	.027	.117

* A locus is considered polymorphic if the frequency of the most common allele does not exceed .95

on the similarity values were constructed using UPGMA to determine the relationships among the individuals (Fig. 2). The dendrogram separated the 119 individuals into several groups. DK9-1, 2, 3, DK11-1, 2, 3 and DK43-1 form one group which was distant from other groups. DK44, DK46a and DK48, which clustered together also by isozyme data, grouped together by microsatellite data. DK4 and DK6 were clustered into one group, which was consistent to the isozyme result. Most individuals from the same maternity grouped together. Some individuals from the same maternity were allocated to different clusters. For example, DK41-3 was assigned into the cluster different from the DK41-1 and DK41-2 cluster. A plant is genetically no more similar to its immediate geographical neighbours than it is to more distant plants. Consequently, there was little relationship between spatial

Table 3. Genetic diversity analyses (Nei 1973) of 44 sub-populations (maternal plants) based on allozyme data

Locus	H_T	D_{ST}	H_S	G_{ST}
ACO-1	0.36	0.36	0	1.00
ACO-2	0.29	0.29	0	1.00
DIA-1	0.16	0.05	0.11	0.32
MDH-3	0.18	0.06	0.12	0.33
PGM-1	0.27	0.23	0.04	0.85
PGM-2	0.08	0.03	0.05	0.34
Mean	0.22	0.17	0.05	0.76

H_T = total gene diversity; H_S = gene diversity within sub-population; D_{ST} = gene diversity among sub-populations and G_{ST} = coefficient of gene differentiation

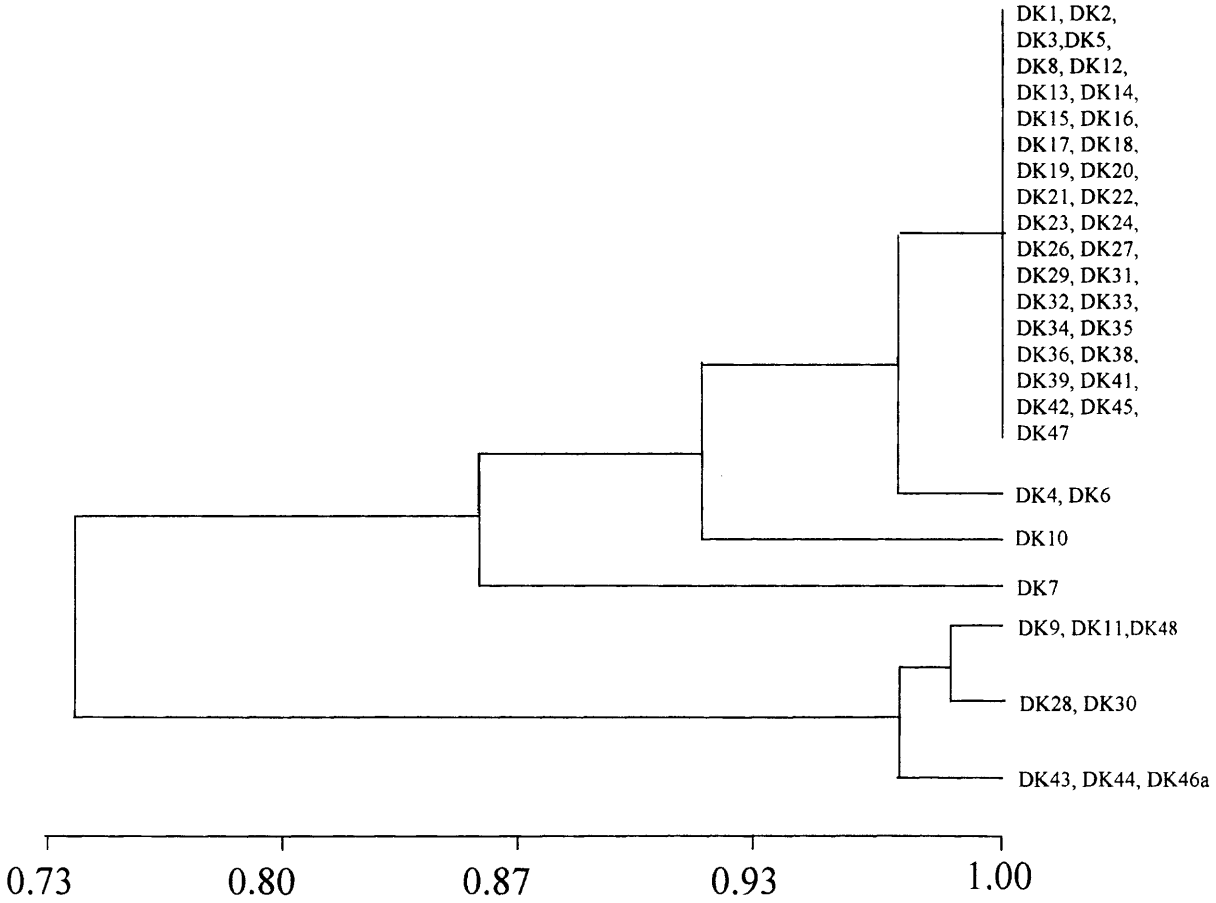


Fig. 1. UPGMA dendrogram of 44 maternal plants using Nei's genetic distances (Nei 1978) based on isozyme data

Table 4. Genetic diversity analyses (Nei 1973) of 44 sub-populations (maternal plants) based on microsatellite data

Locus	H_T	D_{ST}	H_S	G_{ST}
ECGA1	0.53	0.29	0.24	0.54
ECGA2	0.45	0.37	0.08	0.82
ECGA4	0.63	0.56	0.07	0.88
ECGA22	0.57	0.49	0.08	0.85
ECGA89	0.64	0.51	0.13	0.80
Mean	0.56	0.44	0.12	0.78

H_T = total gene diversity H_S = gene diversity within sub-population; D_{ST} = gene diversity among sub-populations and G_{ST} = coefficient of gene differentiation

proximity and genetic similarity within this population.

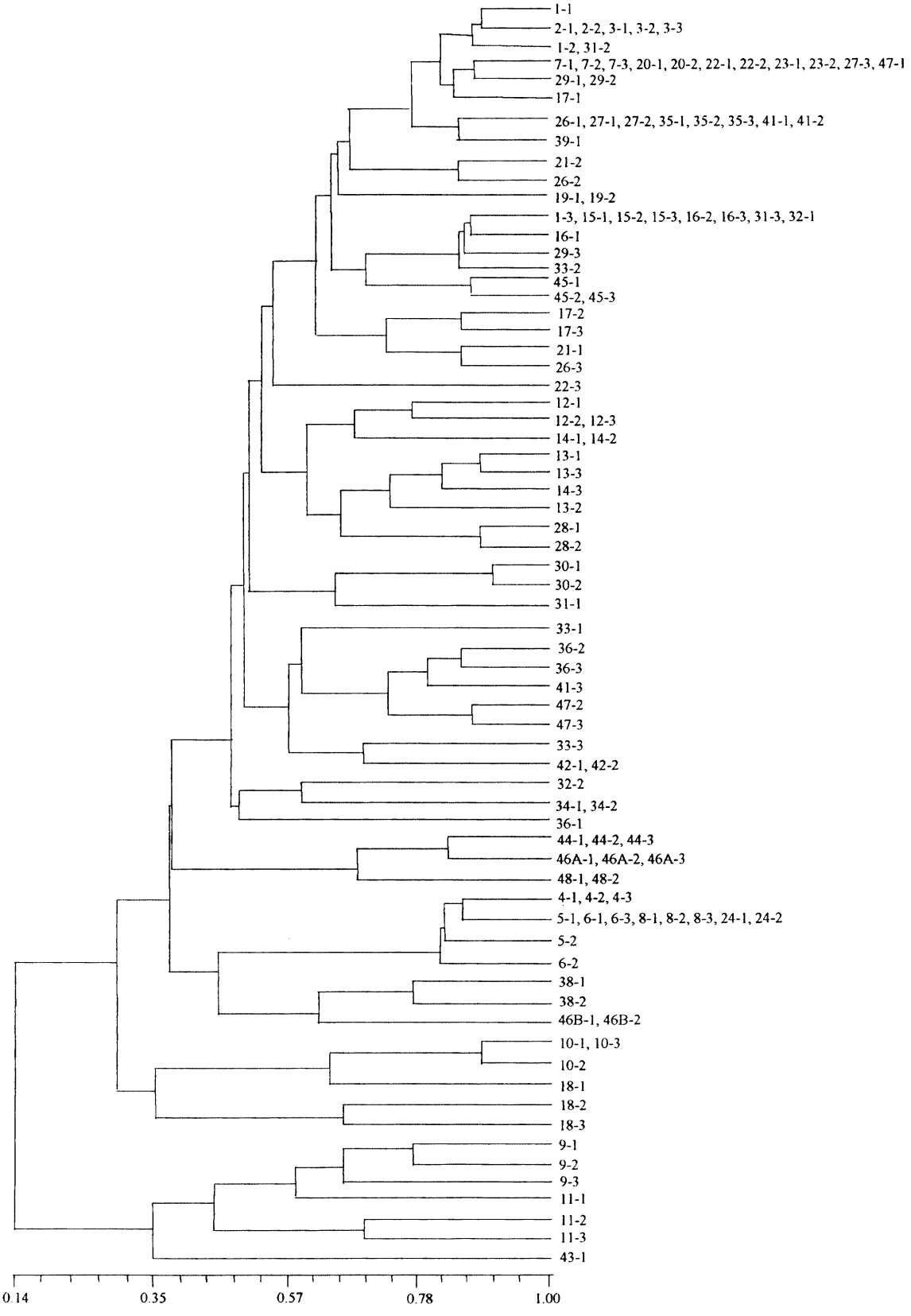
Discussion

The present investigation is a thorough study of a single population. The percentage of polymorphic loci for allozyme was 4.7% at the level of 44 subpopulations. The mean genetic diversity was 0.027 at the level of 44 subpopulations (maternal plants). The genetic diversity detected in this population was much higher than the diversity values detected in *E. fibrosus* (0.007) and *E. alaskanus* (0.005) at a larger geographical scale, thus indicating that in this *E. caninus* population exists higher allozyme diversity.

Sun et al. (1998c) estimated microsatellite diversity levels for 10 populations of *E. fibrosus*. They found an average percentage of polymorphic loci, P, of 53.3 and an average of gene diversity, *He*, of 0.252. The values of mean percentage of polymorphic microsatellite loci of the 23.6 and average gene diversity of 0.117 observed for this *E. caninus* population, were much lower than that observed for *E. fibrosus*. However, the highest gene diversity observed for DK11 subpopulation was 0.447, which was similar to the highest value ($He = 0.444$) (Sun et al. 1998c) observed for a *E. fibrosus* population. The genetic similarities calculated for all pairwise comparisons among the 119 individuals, which ranged from 0 to 1.00 for the microsatellite data also showed that the markers detected a good level of genetic diversity among these individuals of this population. The results indicate that this *E. caninus* population displayed a relative high microsatellite diversity.

There are differences in the amount of genetic variation between allozymes and microsatellites (Tables 1 and 2), which is not an exceptional case. The results corresponded well with previous studies on comparisons of allozyme and microsatellite diversity in natural populations (Lanzaro et al. 1995; Lehmann et al. 1996; Sanchez et al. 1996; Meglecz et al. 1998; Sun et al. 1998c, 1999). Perhaps more comparable for allozyme and microsatellite data is G_{ST} as it is not determined solely on absolute values of heterozygosity or similarity, but on the relative distribution within and among subpopulations. The averages of G_{ST} values for both types of markers were very similar, with 0.76 for allozyme and 0.78 for microsatellite data. This result corresponds well with the report of Meglecz et al. (1998). They compared the differentiation pattern at allozyme and microsatellite loci in *Parnassius mnemosyne* (Lepidoptera) populations, and observed that the overall F_{ST} (similar to G_{ST}) values were similar for microsatellites and for allozymes, while the genetic distances obtained for microsatellites were larger than for allozymes. The G_{ST} values reported here also fell midway in the range of similar values calculated previously for *Elymus* species. For example, Sun et al. (1998c) and Díaz et al. (2000) studied genetic diversity in *E. fibrosus* populations and found $G_{ST} = 0.54$ for microsatellites, 0.65 for RAPDs and 0.63 for allozymes. Diaz et al. (1999) observed $G_{ST} = 0.62$ for allozymes when 54 *Elymus caninus* populations were studied. The $G_{ST} = 0.95$ was found for *E. alaskanus* populations (Díaz 1999).

The overall gene flow (Nm) among maternal plants was estimated to be 0.08 based on allozymes and 0.07 based on microsatellites. Gene flow within populations is caused either by seed or by pollen dispersal or both. In our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5-2
6-2
38-1
38-2
46B-1, 46B-2
10-1, 10-3
10-2
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11-1
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43-1
0.14
0.35
0.57
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1.00

case, only pollen dispersal was attributed to gene flow among maternal plants. Although formal analyses of the mating system of *E. caninus* are not available, the floral architecture of this species indicates that it is mainly self-pollinating. The *Nm* values based on both allozyme and microsatellite data presented here showed low gene flow among maternal plants, indicating a predominantly inbreeding mating system of this species. However, 10 (22.7%) maternal plants produced heterozygous offspring for allozyme loci, and 30 (68.2%) maternal plants gave heterozygous offspring for microsatellite loci. For a completely inbreeding species, all individuals from the same maternal plant are supposed to be homozygous. The mutation rate per locus per gamete per generation was 5×10^{-4}–10^{-5} for microsatellite loci (Edwards et al. 1992; Hearne et al. 1992), and 10^{-6}–10^{-9} for allozyme loci (Ayala 1976). These mutation rates could not explain the relatively high percentage of heterozygous offspring from the same parent. The reasonable explanation is that outcrossing may occur. Díaz et al. (2000) compared the genetic variation in populations where *E. caninus* grows alone or together with other *Elymus* species. They found that higher levels of variation were found when *E. caninus* grows together with *E. mutablis* than alone, thus indicating that gene flow may occur between *E. caninus* and *E. mutablis* when growing in the same area. Taken together with previous studies, the present data shows that *E. caninus* is a self-pollinating species with a relatively high outcrossing rate.

The cluster analyses based on the allozyme and microsatellite data separated the 46 maternal plants into several groups. Although the grouping of maternal plants based on isozyme and microsatellite data was not completely consistent with each other, both dendrograms consistently separated DK9, DK11 and DK43 into one group (Figs. 1 and 2). DK4 and DK6 were grouped together by isozymes and were also allocated into one group by microsatellites. The same observation was made for DK44, DK46a and DK48. There was no clear cut relationship between spatial proximity and genetic similarity within this population, thus suggesting that gene flow through pollen dispersal was extensive enough in past generations to prevent local differentiation, or micro-ecological factors to have significant effects on the genetic differentiation.

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Fig. 2. Dendrogram of the 119 individuals based on the UPGMA method, using the matrix of the Jaccard coefficient from microsatellite data. The first one or two digits represent the no. of maternal plants, the last digit represents no. of seeds from each maternal plant

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