Original article

Analysis of the genetic diversity of wild, Spanish populations of the species *Elymus caninus* (L.) Linnaeus and *Elymus hispanicus* (Boiss.) Talavera by PCR-based markers and endosperm proteins

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Abstract – The use of wild species in plant improvement is greatly favored if their genetic variability and taxonomical relationships are known. A study was made of the genetic variability of fifteen Spanish populations of *E. caninus* and *E. hispanicus*. The relationships among these species and *E. panormitanus* were also investigated. Intra- and interpopulational variation was determined by electrophoresis of endosperm proteins. DNA polymorphisms generated by PCR amplification using arbitrary and specific primers were used to determine interpopulational variability and interspecific relationships. The variability observed was similar to that reported for other autogamous species. The reduced variability observed in some populations of *E. caninus* was attributed to increased distance from the species center of distribution and to the founder effect. Endosperm proteins and random amplified polymorphic DNAs (RAPDs) provided complementary results and differentiated most populations of *E. caninus*. The interspecific relationships observed were more in agreement with the classification of *E. hispanicus* as a different species to *E. panormitanus* than as a variant form of the same taxon.

Elymus / genetic diversity / endosperm proteins / PCR-based markers

Résumé – Analyse de la diversité génétique de populations sauvages espagnoles d'*Elymus caninus* (L.) et d'*Elymus hispanicus* (Boiss.) Talavera par des marqueurs PCR et des protéines de réserve. L'utilisation des espèces sauvages en amélioration végétale implique de connaître leur variabilité génétique et leurs relations taxonomiques. Une étude a été réalisée sur la variabilité génétique de quinze populations de *E. caninus* et *E. hispanicus*. Les relations entre ces espèces et *E. panormitanus* ont été ainsi examinées. La variation intra- et interpopulation a été déterminée par électrophorèse des protéines de réserve. Les polymorphismes d'ADN générés par amplification PCR à l'aide d'amorces aléatoires et

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spécifiques ont été utilisés pour déterminer la variabilité intraspécifique et les relations interspécifiques. On a observé une variabilité similaire à celle décrite pour d'autres espèces autogames. Dans quelques populations de *E. caninus*, on a observé une réduction de la variabilité qui peut être attribuée à un éloignement du centre de distribution de cette espèce ainsi qu'à un effet de fondation. Les protéines de réserve et l'ADN polymorphe amplifié aléatoirement (RAPD) apportent des résultats complémentaires et différencient la plupart des populations de *E. caninus*. Les relations interspécifiques observées sont concordantes avec la classification de *E. hispanicus* comme espèce différente de *E. panormitanus* et non comme une forme variante du même taxon.

Elymus / diversité génétique / protéines de réserve / marqueurs RAPD

1. Introduction

Owing to the importance of wild species as a source of genes for breeding cultivated species, international organizations and seed banks are very interested in their collection and conservation. The effective use of wild species in plant improvement is greatly favored if their genetic variability [24] and the taxonomical relationships between them is known.

According to the genomic classification of Löve (1984), *Elymus* is the largest genus of the tribe *Triticeae*, with more than 150 perennial species. It is also the most cosmopolitan, growing in Europe, Asia, North America, South America, New Zealand and Australia [9]. Many of the species of *Elymus* have good fodder qualities, are highly productive in favorable conditions, and tolerate different diseases.

The chromosome number of *Elymus* varies from $2n=4x=28$ to $2n=8x=56$. Some 75% of *Elymus* species are tetraploid with the genomic constitution SSHH. The S genome comes from *Pseudoroegneria* and the H from *Critiesion* [9]. Asiatic species such as *E. ciliaris* have a Y genome of unknown origin rather than the H genome, and are represented genomically as SSYY [18]. The majority of hexaploids are segmental autopolyploids $S_1S_1S_2S_2HH$ or $SSH_1H_1H_2H_2$, and in some cases such as *E. drobovii* their genomic formula is SSH-HYY [8]. Recently the P genome of *Agropyron* spp. Gaertn has been identified in *E. kengii* which has the genomic formula SSYYPP [13]. Similarly, the W genome of *Australopyrum* (an endemic genus of Australia and New Zealand) has been

found in *E. scabrus* (Löve) which is given the genomic formula SSYYWW [32].

According to the genomic classification of Löve (1984), two representatives of this genus are known in Spain: *E. caninus* (L.) Linnaeus (also known as *Agropyron caninum* (L.) P. Beauvois or *Roegneria canina* (L.) Nevski.) and *E. panormitanus* (Parl.) Tzvelev (also called *Agropyrum panormitanus* Parl. var. *hispanicus* Boiss. or *Elymus hispanicus* (Boiss.) Talavera).

Elymus caninus has the genomic constitution SSHH and grows in Europe and Asia. In Spain, it grows in the Cantabrian Range, the Pyrenees, Galicia (Northwest Spain) and the Central and Iberian Ranges (Central Spain). Its southern limit lies at 37° north. *Elymus panormitanus* (Parl.) Tzvelev is another tetraploid species with the genomic constitution SSYY, and grows in Europe and Asia [33]. In Spain it is found in the southern part of the peninsula [20].

The relationships among certain species of the *Elymus* genus have been clarified using different biochemical and molecular systems such as isozymes, DNA repeated sequences, microsatellites and random amplified polymorphic DNA (RAPD) markers. One variant of the polymerase chain reaction (PCR) technique used to obtain molecular markers consists of amplifying DNA fragments previously localized in the genetic map of a particular species. This technique has been used in certain *Triticeae*, such as *Triticum* spp. and *Hordeum* spp. [6, 22, 30].

The purpose of the present study was to analyze the genetic variability of fifteen Spanish populations of *E. caninus* and *E. hispanicus*. The relationships among these species and *E. panormitanus*

was also investigated using biochemical (endosperm proteins) and molecular (PCR) techniques. In the latter, primers with both arbitrary sequences (RAPDs) and with specific sequences were used.

2. Materials and methods

2.1. Plant material

The plant material consisted of fifteen wild, Spanish populations belonging to the species *E. caninus* and *E. hispanicus* (Tab. I). This material forms part of the living collection of wild relatives of cultivated *Triticeae* obtained from natural environments [25] which is maintained at the Plant Breeding and Biotechnology Department of the I.N.I.A (La Canaleja, Madrid, Spain). In order to conserve the original structure of the natural population, specimens were collected at random and each sample obtained following the method of Hawkes [11]. About 100 ears were picked from each population.

The Feulgen method was used to check the chromosome number of the species in each population.

For a better understanding of the relationships between the analyzed species, *E. panormitanus* (PI531646 from Iraq), *Pseudoroegneria spicata* (PI537387 from the USA), and *P. geniculata* (PI502271 from the Russian Federation), all provided by the Western Regional PI Station USDA-ARS, and *Hordeum chilense* (H80 from Chile), provided by the University of Córdoba (Spain), were included in the study.

2.2. Protein analysis

Endosperm proteins were analyzed according to the method of Payne et al. [23] using polyacrylamide gels in the presence of 10% SDS (SDS-PAGE). In order to perform this analysis without interfering with germination, a piece of embryo-free endosperm was separated out. In the majority of cases 30 individuals per population were analyzed. Individual samples were inserted into the gels. A molecular weight standard (Sigma 6H) was also used which contained myosin (205 Kd), galactosidase (116 Kd), phosphorylase B (97.4 Kd), bovine albumin (66 Kd), egg albumen (45 Kd) and carbonic anhydrase (29 Kd). Electrophoresis was performed at 20mA for 5 hours at room temperature.

Table I. Localities, provinces, mountain ranges and altitude in meters (m) of collection sites for populations (Pobl.) of *E. caninus* (L.) Linnaeus and *E. hispanicus* (Boiss.) species.

Species	Pobl.	Locality	Province	Range	Altitude (m)
<i>E. caninus</i>	53	PAJARES	Oviedo	Cantabric	1370
<i>E. caninus</i>	484	PUEBLA	Granada	Betic	1450
<i>E. caninus</i>	513	CANENCIA	Madrid	Central	1400
<i>E. caninus</i>	514	CANENCIA	Madrid	Central	1300
<i>E. caninus</i>	515	DURATON	Segovia	Central	950
<i>E. caninus</i>	1054	BENASQUE	Huesca	Pyrenees	1400
<i>E. caninus</i>	1058	CAMPO-SEIRA	Huesca	Pyrenees	700
<i>E. caninus</i>	1061	BIELSA	Huesca	Pyrenees	1050
<i>E. caninus</i>	1071	BROTO	Andorra	Pyrenees	760
<i>E. caninus</i>	1091	ANDORRA	Andorra	Pyrenees	1400
<i>E. hispanicus</i>	475	CAZORLA	Jaén	Betic	1280
<i>E. hispanicus</i>	485	BAZA	Granada	Betic	1820
<i>E. hispanicus</i>	488	EL MOLINILLO	Granada	Betic	1210
<i>E. hispanicus</i>	489	BEJAR	Granada	Betic	1430
<i>E. hispanicus</i>	523	ZAFARRAYA	Granada	Betic	1200

Coomassie Brilliant Blue R-250 was used to stain the gels and 6% acetic acid used for counter-staining.

2.3. Analysis of PCR fragments

DNA was extracted from leaves of 4–6 week old plants as described by Dellaporta et al. (1983), using 15 to 20 plants per population. Amplifications were carried out in a PTC-100 thermocycler (M.J. Research, Inc.). Each reaction and corresponding electrophoretic analysis was repeated at least twice. The amplified products were separated in 1.5% agarose gels at $4.5 \text{ V} \cdot \text{cm}^{-1}$ for 2–3 h in TBE buffer and stained with ethidium bromide. DNA fragment size-testers of 154 kb to 2176 kb were used.

The primers used in the RAPD analysis were obtained from kits M, R, S and T (Operon Technologies) and from the University of Alcalá de Henares, and were 10 bp in length (Tab. II). PCR reactions were performed using the method of Williams et al. [34], with modifications by De Bustos et al. [4].

The primers used in the amplification of specific sequences are given in Table III. The first ten oligonucleotides were kindly provided by Dr. Thomas K. Blake of Montana State University, and belong to the set of markers used in the elaboration of the wheat and barley genome maps. The design of the last three primers was based on well characterized DNA sequences [12, 17, 21] and they were produced using a Beckman Oligo 1000 synthesizer. The composition of the mixture for each reaction volume of 100 μl was: 100–200 ng from the DNA template, 100 μM from each of the dNTPs, 150 ng from each of the primers, 10 μl of BioTaq buffer, and 5 U of BioTaq (Bioprobe Systems). The reaction conditions for the primers Pst340, ABG356, WG181, ABA003, A1, D2, and G12 consisted of one 5 min cycle at 94 °C, 32 cycles of 1 min at 94 °C, 1 min at 45 °C, 1 min 20 s at 72 °C, and one cycle of 2 min at 72 °C. The annealing temperature was increased to 55 °C when Rrn5S2 was used. For primer aMST108 the conditions were: one cycle of 4 min at 94 °C, 30 cycles of 45 s at 94 °C, 60 s at 15 °C, and one cycle of 5 s at 60 °C.

Table II. Primers from Operon Technologies (OP) and the University of Alcalá de Henares (UA) used to obtain number of phenotypes (P), number of total bands (TB) and percentage of polymorphic bands (% PB) in populations of the species *E.caninus* and *E. hispanicus*.

Primer	Sequence 5' ——— 3'	<i>E. caninus</i>			<i>E. hispanicus</i>		
		P	TB	% PB	P	TB	% PB
OPM-02	ACAACGCCTC	3	8	38	1	7	0
OPM-12	GGGACGTTGG	4	11	36	1	12	0
OPM-15	GACCTACCAC	4	5	40	1	5	0
OPM-17	TCAGTCCGGG	1	5	0	1	5	0
OPM-19	CCTTCAGGCA	7	14	64	1	8	0
OPM-20	AGGTCTTGGG	2	6	33	1	4	0
OPR-01	TGCGGGTCCT	6	10	50	1	10	0
OPR-07	ACTGGCCTGA	10	13	69	3	9	22
OPR-08	CCCGTTGCCT	4	7	43	1	5	0
OPR-10	CCATTCCCCA	7	8	75	2	8	13
OPR-12	ACAGGTGCGT	1	7	0	1	6	0
OPS-03	CAGAGGTCCC	2	7	14	1	7	0
OPS-11	AGTCGGGTGG	1	4	0	1	5	0
OPS-14	AAAGGGGTCC	1	5	0	1	6	0
OPS-19	GAGTCAGCAG	2	9	11	1	8	0
OPS-20	TCTGGACGGA	2	5	20	1	10	0
OPT-01	GGGCCACTCA	1	7	0	1	6	0
OPT-05	GGGTTTGGCA	1	5	0	1	5	0
OPT-06	CAAGGGCAGA	10	13	77	1	4	0
OPT-11	TTCCCCGCGA	1	5	0	1	7	0
OPT-19	GTCCGTATGG	1	1	0	1	1	0
UA91	GTGCATGCCA	1	1	0	1	1	0
		72	156	36	25	139	2

“Touchdown” was performed with the primers ITS-1, gf-2.8, RIP30 and ABG458 [10]. This consisted of 20 initial cycles of 1 min at 94 °C, 1 min at 62 °C and 2 min at 72 °C. During this phase the annealing temperature was decreased 0.5 °C per cycle to 52 °C. The second period started at 52 °C and consisted of 20 cycles without modification followed by a final incubation cycle of 5 min at 72 °C.

2.4. DNA digestion

The amplified products were digested with six restriction endonucleases that recognized 4 bp targets: *AluI*, *MspI*, *HaeIII*, *HinfI*, *HhaI*, and *TaqI*

Table III. Designed sequence-specific primers.

Primer set	Species	Primer sequences L: 5'-3' R: 3'-5'	Reference
A2	Wheat	L: CAACAGAGATATTGCCGTAG R: AAGATTGTCAACAAGTGCC	Talbert et al. 1994
D2	Wheat	L: CGAATGTTTCTACTGCGCTGT R: CTCCCTGTTTGTGGAAAGCT	Talbert et al. 1994
G12	Wheat	L: CCAGTGTGTAGTTCTCTAT R: TATACTTCTGAGCTGCCGAG	Chen et al. 1994
ABA003	Barley	L: GCTGCGCGCTTCAGCT R: GACCTCCACGAGTTGC	Kleinhofs et al. 1993
ABG458	Barley	L: AGTCTTGCGCATGGTGACAC R: CACCAATTGCATCAAAGCTC	Talbert et al. 1994
ABG356	Barley	L: TCAACTGAGGTAGAATACTA R: CCAACAATAAAGAATCAAAT	Blake et al. 1996
aMST108	Barley	L: ATGGCCCGCAC(C/G)AAGCAGAC R: GACTTCCT(C/G)GCCGCCTGCAA	Talbert et al. 1994
Pst340	Barley	L: TAGCATCGGTAATCTCTCGC R: CCCTTTATATACTGCCGA	Talbert et al. 1994
Rrn5s2	Barley	L: TGGGAAGTCCTCGTGTGCA R: TTTAGTGCTGGTATGATCGC	Kanazin et al. 1993
WG181	Barley	L: AGATACAACCAGCGTCAGT R: CGGTCACTCTACTCAGTTTT	Talbert et al. 1994
RIP30	Barley	L: TGACGACGCTGCTCCTC R: CCATGCGAGTGGTGGA	Leah et al. 1991
gf-2.8	Wheat	L: TTCTCGTCCAAGTTGTCC R: GTCTCCTTCAACAGCCAG	Lane et al. 1991
ITS-1	Wheat	L: CGTGACCCTTGACCAAAAC R: GACTCTCGGCAACGGATAT	Hsiao et al. 1994

(Boehringer Mannheim). Amplified and restricted DNAs were electrophoresed in 6% acrylamide gels in TBE buffer for 2 hours at 180V and stained with ethidium bromide.

2.5. Statistical analysis

The NTSYS package was used for the statistical analysis of data. Intrapopulation variability as shown by endosperm proteins was determined by calculation of Jaccard's similarity coefficient. The presence or absence of the same protein band was considered as a discriminating factor. In order to determine interpopulational variation with endosperm proteins, individual band frequencies

were calculated for each population. Estimations of similarity between populations were then made by calculation of Pearson's product-moment correlation coefficient. Interpopulational variability with PCR markers was determined by calculation of Jaccard's similarity coefficient. Bands of identical size amplified with the same primer were considered to be the same DNA marker.

Jaccard's similarity coefficient and Pearson's correlation coefficient were used to produce a dendrogram using the unweighted pair-group method (UPGMA). Means and variation coefficients of the similarity indices were determined with the STATGRAPHICS PLUS program (Manugistics Inc. and Statistical Graphics Corporation).

3. Results

Intrapopulation variability was analyzed using endosperm proteins. Interpopulation variability and interspecific relationships were analyzed using endosperm proteins and PCR markers.

3.1. Intrapopulation variability

The protein bands analyzed were those found in the areas of high (116–97 Kd) and average (97–50 Kd) molecular weight because these were the best visualized with the SDS-PAGE technique. The total number of bands was 20: 14 in *E. caninus* and 10 in *E. hispanicus* (Tab. IV). An example of these markers is given in Figure 1.

The degree of variation of each population was analyzed in two different ways. First, the phenotypes in each population were studied. Two phenotypes were considered different when variation was found in at least one band. A population was considered polymorphic when at least one individual

Figure 1. Polyacrylamide gel electrophoresis of different individuals of population 0485 of *E. hispanicus*. M: standard proteins of known molecular weight (kDa).

showed a phenotype different from the rest. *Elymus caninus* showed 3 monomorphic and 7 polymorphic populations; *E. hispanicus* showed 4 monomorphic and 1 polymorphic populations (Tab. V).

Table IV. Bands shared by *E. caninus* (Eca), *E. hispanicus* (Ehi), *E. panormitanus* (Epa), *Hordeum chilense* (Hch), *Pseudoroegneria spicata* (Psp) and *P. geniculata* (Pge) using proteins, RAPDs, specific sequences, and specific sequences digested with endonucleases.

Proteins							RAPDs					
Species	Hch	Eca	Ehi	Epa	Psp	Pge	Hch	Eca	Ehi	Epa	Psp	Pge
Hch	10						132					
Eca	6	14					39	156				
Ehi	5	4	10				27	91	139			
Epa	6	5	4	7			20	59	82	119		
Psp	4	6	2	5	10		16	34	36	41	102	
Pge	5	8	4	3	7	15	12	32	31	31	31	88
Specific sequences							Specific sequences digested with endonucleases					
Species	Hch	Eca	Ehi	Epa	Psp	Pge	Hch	Eca	Ehi	Epa	Psp	Pge
Hch	24						56					
Eca	14	25					25	65				
Ehi	14	25	29				29	61	85			
Epa	11	19	22	24			26	51	65	77		
Psp	8	14	14	12	22		23	52	52	53	66	
Pge	8	15	16	15	14	21	20	47	47	41	49	54

Table V. Intrapopulation variation in the species *E. caninus* and *E. hispanicus* using endosperm proteins, mean and coefficient of variation (C.V.) of the similarity index (Jaccard), and number of individuals with an specific phenotype.

Specie	Population	Mean	C.V.	Phenotype (number of individuals)
<i>E. caninus</i>	53	1	0.00	0053.1 (30)
<i>E. caninus</i>	484	1	0.00	0484.1 (30)
<i>E. caninus</i>	513	1	0.00	0513.1 (30)
<i>E. caninus</i>	514	0.9	16.7	0514.1 (24), 0514.2 (5), 0514.3 (1), 1054.5 (2)
<i>E. caninus</i>	515	0.9	13.98	0515.1 (17), 0515.2 (13)
<i>E. caninus</i>	1054	0.59	27.73	1054.1 (5), 1054.2 (3), 1054.3 (2), 1054.4 (2), 1054.6 (2), 1054.7 (2), 1054.8-1054.18 (1)
<i>E. caninus</i>	1058	0.6	37.8	1058.1 (12), 1058.2 (6), 1058.3 (3), 1058.4 (3), 1058.5 (2), 1058.6 (1), 1058.7 (1), 1058.8 (1)
<i>E. caninus</i>	1061	0.94	13.23	1061.1 (25), 1061.2 (4), 1061.3 (1)
<i>E. caninus</i>	1071	0.85	16.5	1071.1 (14), 1071.2 (11), 1071.3 (4)
<i>E. caninus</i>	1091	0.9	15.94	1091.1 (22), 1091.2 (5), 1091.3 (3)
<i>E. hispanicus</i>	475	0.91	13.28	0475.1 (30)
<i>E. hispanicus</i>	485	1	0.00	0485.1 (30)
<i>E. hispanicus</i>	488	1	0.00	0488.1 (30)
<i>E. hispanicus</i>	489	1	0.00	0489.1 (30)
<i>E. hispanicus</i>	523	0.8	23.17	0523.1 (16), 0523.2 (11), 0523.3 (3)

The mean and coefficient of variation (CV) of the similarity indices among the individuals of each population were then calculated using Jaccard's similarity coefficient (Tab. V). The similarity index provides an idea of the homogeneity of the individuals in a population. The coefficient of variation of the index provides a standardized measurement with reference to the mean distance between individuals. This is a global measurement of the variability of a population, independent of the number of phenotypes [5]. According to this criterion, populations 1058 (CV = 37.80) of *E. caninus* and 0525 (CV = 23.17) of *E. hispanicus* were the most variable.

3.2. Interpopulational variability

The phenotypes of the individuals of the different populations, as determined by endosperm proteins, were compared. In *E. caninus* it was observed that the most frequent phenotype of population 0514 (24 individuals) was identical to that of all the individuals of population 0513. It also occurred that the most frequent phenotype of popu-

lation 1058, with 12 individuals, was identical to that presented by 13 individuals of population 0515. In *E. caninus*, three of the five populations were identical for their endosperm protein patterns (Tab. V).

In the study of all populations, one band was considered variable when it was absent in at least one population. Following this criterion, 50% of protein bands were variable in *E. caninus* and 20% in *E. hispanicus*.

The frequencies of bands in each population were used to generate a data matrix. This was then used to calculate Pearson's product-moment correlation coefficient. Finally, the similarity coefficients were used to produce a dendrogram by the unweighted pair-group method (UPGMA). The index of Pearson's coefficient varies from -1 to 1. In *E. caninus* the values of this index ranged from 0.99 between the two closest populations, to 0.76 between those furthest apart. In *E. hispanicus*, the three identical populations had an index of 1.00 and the furthest apart an index of 0.65 (Fig. 2A).

Screening with RAPD markers provided 22 primers which gave reproducible results in all

Figure 2. Dendrograms obtained with *E. Caninus* (ECA), *E. Hispanicus* (EHI), *E. panormitanus* (Epanor) and *Pseudoroegneria spicata* (Pspicat), *P. geniculata* (Pgenicu) and *Hordeum chilense* (Hchilen), with the UPGMA method using (A) endosperm proteins, (B) RAPDs, (C) fragments of specific sequences, and (D) fragments of specific sequences digested with restriction endonucleases.

Figure 3. RAPD profiles using primer R7 in *Hordeum chilense* (1) *E. Caninus* (2-11), *E. Hispanicus* (12-16), *E. panormitanus* (17) and *Pseudoroegneria* (18) and M: molecular weight marker (base pairs).

analyzed populations. An example of amplification with RAPDs is given in Figure 3. The number of bands provided by each primer varied from 1, with UA91 and OPT-19, to 14 with OPM-19 (Tab. II). The size of the amplified products in base pairs (bp) ranged from 100 to 1230. The total number of analyzed bands were 156 in *E. caninus* and 139 in *E. hispanicus*. The percentage of polymorphic bands was 37% in *E. caninus* and 2% in *E. hispanicus*. The oligonucleotides with the highest degree of polymorphic bands were OPT-06 (77%) in *E. caninus* and OPR-07 (22%) in *E. hispanicus*. The oligonucleotides with highest number of different phenotypes per population were OPR-07 in *E. caninus* and OPT-06 in *E. hispanicus* (Tab. II). The data obtained with molecular techniques (the band presence or absence data) was used to generate a data matrix and to calculate Jaccard's similarity coefficient. The UPGMA method used in the protein analysis was also used with the similarity coefficients to produce a dendrogram. The similarity index of this coefficient varies from 0 to 1. The dendrogram originated with RAPD markers is given in Figure 2B. The similarity index gave values of 0.98 and 0.81 for maximum and minimum separation respectively between two populations of *E. caninus*, and of 1.00 and 0.99 for the same in *E. hispanicus*.

The analysis of interpopulational variability with molecular markers developed from specific sequences gave 13 primer sets which provided reproducible results. The total number of bands were 25 in *E. caninus* and 29 in *E. hispanicus*,

none of them polymorphic (Tab. III). The sizes of these bands varied from 154 to 1230 bp.

The PCR products amplified with ITS-1, ABG356, ABG458, Pts340, and Rrn5S2 were digested with the restriction enzymes that recognized targets of 4 bp: *AluI*, *HhaI*, *HaeIII*, *TaqI*, *MspI* and *HinfI*. An example of these kinds of markers is given in Figure 4. The number of bands obtained were 65 in *E. caninus* and 85 in *E. hispanicus*. The percentage of polymorphic bands was

Figure 4. Electrophoretic profiles using the primer set Rrn5S2 digested with the restriction endonuclease *AluI* in *Hordeum* spp. (1-4), *E. Caninus* (5), *E. Hispanicus* (6-10), *E. panormitanus* (11) and *Pseudoroegneria* spp. (12-13). M: molecular weight marker (base pairs).

5% in *E. caninus* and 12% in *E. hispanicus*. The dendrogram corresponding to these markers is given in Figure 2D. In the *E. Caninus* group only one population was separated from the rest with a similarity index of 0.96. In the *E. hispanicus* group there were three identical populations. The most separated population had a similarity index of 0.9.

3.3. Interspecific relationships

The species *E. panormitanus*, *P. spicata*, *P. geniculata* and *H. chilense* were included in the study of interspecific relationships. The dendrograms in Figures 2B, 2C and 2D (obtained from the molecular marker study) show that *Elymus* species are closer among themselves than they are to *Pseudoroegneria* and *H. chilense*. However, endosperm proteins failed to group the three *Elymus* species together.

The maximum values of the similarity index for the most separated species of *Elymus* were 0.42, 0.68, and 0.62, for RAPDs, specific sequences, and specific sequences after digestion respectively.

In the dendrogram shown in Figure 2C the separation between *E. panormitanus* and the other two *Elymus* species is observed because of polymorphisms detected with primers D2, G12, WG181, aMST108 and ABA003. In the dendrogram of Figure 2D, polymorphisms were generated with primers PST340, ABG458, Rrn5S2, ITS-1, and ABG458. Both dendrograms indicate that of the three species *E. caninus*, *E. hispanicus*, and *E. panormitanus*, the first two are more closely related. In the RAPD dendrogram, *E. panormitanus* and *E. hispanicus* are grouped together with a similarity index of 0.48 and are separated from *E. caninus* with a similarity index of 0.42. Tables IV B, IV C and IV D, obtained from the molecular marker study and showing the common bands among *Elymus* species, show that *E. panormitanus* always shares more bands with *E. hispanicus* than with *E. caninus*, and that *E. caninus* always shares more bands with *E. hispanicus* than with *E. panormitanus*. The results of the chromosome counts showed *E. hispanicus* to be hexaploid.

4. Discussion

4.1. Variability analysis

The degree of variability obtained with different techniques at different taxonomical levels indicates the most appropriate choice of method should be made for each particular analysis. In the present study, endosperm proteins provided information on intra- and inter-populational variability. In interspecific studies they failed to group *E. panormitanus* with the other *Elymus* members (Fig. 2A), probably because of the small number of bands obtained from only one accession of this species (Tab. IV). This did not occur with the DNA markers (Figs. 2B, 2C and 2D). Specific sequence markers that detected only a low level of interpopulational polymorphism were informative in the study of interspecific relationships, providing sufficient bands to be of interest (Tab. IV).

The analysis of interpopulational variability using endosperm proteins divided the populations of *Elymus* into monomorphic and polymorphic groups. Polymorphic populations possessed a major band pattern and several low-frequency band patterns. This distribution of variability is similar to that observed in other endosperm protein studies of autogamous species [5]. This agrees with the relationship proposed by many authors between interpopulational variability and reproductive method [1, 31]. The data also agrees with the studies of Jensen et al. [13] who tested the high degree of autocompatibility in *Elymus* species including *E. caninus* and *E. panormitanus*.

In autogamous species, several authors have described the existence of monomorphic populations through the action of the so-called founder effect [2, 3]. Through this effect, monomorphic populations develop when a limited number of individuals of one species are introduced into a new area. This effect produces similar phenotypes in the surrounding area due to random factors rather than selective environmental factors.

The most polymorphic populations of *E. caninus* were found in the Pyrenees (Tab. I). This may be because the north of Spain is closer to its center of

distribution, which, according to Vavilov, is the location which shows most variation. Taking into account the morphological characteristics of *E. caninus*, Flora Europaea [20] described this species as very variable and as having multiple local forms. Sun et al. [27], who used molecular markers to investigate variability observed the existence of differences among accessions of *E. caninus*, some of which were from different countries. The analysis performed in this report with endosperm proteins and RAPDs allows the differentiation of all the studied populations of *E. caninus*.

The studies with endosperm proteins and RAPDs were complementary and jointly investigated the true variability of these populations. Endosperm proteins produced a lower number of total bands than RAPDs, but a higher percentage of variable bands. This could be due to the different origin of these markers. Endosperm proteins belong to very polymorphic multigenic families. This gives them the advantage of detecting a high number of variations. RAPDs provide a high number of bands for study and the largest sample of the genome. Some authors, such as Spooner et al. [26], indicate that the resolving efficiency of RAPDs in genetic diversity studies is due to the great number of bands produced compared to other techniques.

4.2. Taxonomy of *Elymus hispanicus*

By means of cytogenetic studies, Jensen and Hatch [14] deduced that *E. panormitanus* was an autotetraploid with the genomic formula $S^pS^pY^pY^p$, since the two genomes were modified forms of the S and Y genomes found in the Asiatic polyploids. The analyzed populations of *E. hispanicus* in the present study were hexaploid. This disagrees with the reported tetraploid constitution of *E. panormitanus* (Parl.) Tzvelev var. *panormitanus*. One hexaploid variant of *E. panormitanus*, denominated *E. panormitanus* (Parl.) Tzvelev var. *heterophyllus* Bornm. ex Meld., has been reported in Israel with the genomic constitution $SSSSYY$ [15]. These authors found little morphological variation between the variants *panormitanus* and

heterophyllus, which hindered their distinction. *Elymus hispanicus* is morphologically different from the two variants of *E. panormitanus*. In 1986, Talavera, using morphological characteristics (length of glumes, number of nerves on the glumes and some features of the glume awns), considered *E. hispanicus* to be a distinct species. *Elymus panormitanus* grows in forests and swales of the middle mountain belts of Southern Europe, the Mediterranean, Asia Minor and Northwestern Iran [33]. Talavera [29] described the geographical separation of these two species belonging to the calcareous mountains of the Mediterranean: *E. hispanicus* is found in Southern Spain and Northern Algeria and *E. panormitanus* in the Eastern Mediterranean (Italy, Greece, Turkey, Lebanon, Syria, etc.).

The dendrograms of Figures 2B, 2C, and 2D show that *E. caninus* is always in a different subgroup to *E. panormitanus*. This agrees with the results of Svitashv et al. [28] and Sun et al. [27]. *Elymus hispanicus* is closer to *E. caninus* than to *E. panormitanus* in the dendrograms obtained with two different groups of specific sequence markers (Figs. 2C and 2D), and *E. hispanicus* is in the same subgroup as *E. panormitanus*, but it is not far from *E. caninus* in the dendrogram obtained using RAPD markers (Fig. 2B). This seems to better support the classification of Talavera [29] which considers *E. hispanicus* as a different species to *E. panormitanus*, than the classification of Flora Europaea which records *E. hispanicus* as a variant of *E. panormitanus*.

These new genetic and molecular studies of the *Elymus* species and their variants bring us closer to the knowledge required for a revision of this genus.

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