Russian Journal of Genetics, Vol. 31, No. 10, 1995, pp. 1155 -1161. Translated from Genetika, Vol. 31, No. 10, 1995, pp. 1358 -1364.

MOLECULAR GENETICS

Genetic Polymorphism in Barley Detected by PCR with Arbitrary Primers

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Received May 11, 1994; in final form, August 23, 1994

Abstract - Genetics and breeding studies require effective methods for polymorphism analysis that allow one to classify varieties and to determine phylogenetic interactions between plant species. A variant of the polymerase chain reaction for DNA amplification with arbitrary primers (RAPD) was used to determine genetic distances between *Hordeum vulgare* varieties and to integrate the varieties into groups. The dendrogram of relations between species from the genus *Hordeum* and species of some cultivated cereals was constructed on the basis of RAPD analysis.

INTRODUCTION

The DNA amplification technique, by means of a polymerase chain reaction (PCR), determined a new direction in methodology for identifying genome specificity. PCR allows one to reveal DNA polymorphism that may be used for analysis of inter- and intraspecies variability.

The two main variants of PCR (ASP and RAPD), differing in the primers used, received wide acceptance in molecular-genetic studies.

ASP (Amplified Sequence Polymorphism) addresses primers flanking genes with a known nuclear acid sequence. In this case, polymorphism of the known loci is studied.

RAPD (Random Amplified Polymorphic DNA) is a PCR product with arbitrary primers; this method was developed in [1, 2].

Primers used for RAPD are of relatively small size (8-12 nucleotides), have a random nuclear acid sequence, and have a [G + C] content exceeding 50% [1,3]. This method does not require one to identify the nucleotide sequence of the amplified DNA region; therefore, the analysis becomes simpler. Electrophoretic patterns of the amplified DNA (RAPDs) from different genetic sources may be used for comparative analysis to determine their genetic similarity [4-6].

The amplified DNA may represent unique genome structures [1] and have Mendelian inheritance [3, 7]. Since RAPD allows one to test many loci, this method shows considerable promise for genetic studies applied to plant breeding.

The barley *Hordeum vulgare* is one agricultural plant species that, genetically, is well studied. However, intense breeding of this culture requires effective new methods for quantitative evaluation and classification of genetic material.

The purposes of this work were to determine genetic distances between some species from the genus *Hordeum* L. and varieties from *Hordeum vulgare* and to construct dendrograms reflecting their relations on the basis of the PCR method.

MATERIALS AND METHODS

In this study, we used the following species from the genus *Hordeum* L.: *H. agriocrithon* A'berg., *H. spontaneum* C. Koch., *H. lagunculiforme* Bacht. [8], *H. vulgare* (Donetskii-4), *H. chilense* Roem. et Shult., *H. bulbosum* L., as well as *Triticum durum* L. (line 350/72), *T. aestivum* L. (Odesskaya polukarlikovaya), *Secale cereale* L. (Khar'kovskaya 60). The following varieties of *H. vulgare* were analyzed: Dzhau Kabutak, Odesskii-31, Odesskii-70, Odesskii-100, Donetskii-8, Donetskii-9, Istok, Tsiklon, Chernomorets, Viner, Riso-56, Riso-1508, Nutans-106, Pallidum 32, Pallidum 331/13, Betzes, Bomi, Bonus, Athos, Koral, Calsberg II, Productiv, and self-pollinated population no. 106/93 [the material was obtained from the collections of the Department of Genetic Bases of Breeding, the Department of Barley Breeding, and the Department of Genetics (Institute of Breeding and Genetics, Ukrainian Academy of Agricultural Sciences, Odessa)].

Analysis of inheritance and estimation of linkage between the amplification products C and F were performed according to [9] with self-pollinated population no. 106/93.

dATP, dGTP, dCTP, and dCTP (Pharmacia, Sweden) were adjusted to pH 7.0 by a 100 mM solution of Tris-OH. Tris-base, Na_2EDTA, KC1, H_3BO_3, and a light fraction of mineral oil (Bajol F) were received from Serva (Germany), $MgCl_2$ - from Merck (Germany), agarose and Tween-20 - from Sigma (USA), 10 TBE (0.89 M Tris-base, 0.89 M H_3BO_3, and 20 mM Na_3EDTA), and Tag-polymerase - from the All-Russian

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Primers used for DNA amplification

Primer	5-3' sequence	Ta * °C min - max
PI	CCATGGTACCCGGATCCTCG	57.17-58.51
P2	GACAGACAGACAGACA	50.18-51.52
P3	GATTTAGGTGACACTATAG	51.52-52.86
P4	ATTAACCCTCACTAAAGGGA	54.43 - 55.77
P5	AGGTCTTAACTTGACTAACAT	52.96 - 54.30
P6	GAGCAAGTTCAGCCTGG	53.77-55.11
P7	TAATACGACTCACTATAGGG	52.92 - 54.26
P8	CAGGAAACAGCTATGAC	52.02 - 53.36

is the optimal annealing temperature that results in products ranging in size from 300 to 2000 base pairs and [G + C] contents equal to 50%.

Center of Molecular Diagnostics and Treatment (Moscow). The instrument Biotherm (model 91) was used for DNA amplification.

Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems 380B automatic synthesizer.

Isolation of Plant DNA

Several etiolated seedlings (each less than 4 mm in size) were crushed in 0.5 ml of the lysing buffer (0.1 M Na_3EDTA, 100 mM Tris-HCl, pH 8.5 at 25°C, 100 mM NaCl, 1% SDS, 0.1% Triton X-100, and 100 μg/ml proteinase K) and incubated at 55°C for 3 hours. An equal volume of chlorophorm/isopentanol mixture (24:1) was added and thoroughly stirred. DNA was precipitated by adding 0.5 volume of propanol-2 to the water phase with subsequent centrifugation in a desktop Eppendorf centrifuge for 5 min at maximum speed. The DNA precipitate was dissolved in 400 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM Na_3EDTA) in the presence of 1 μg/ml RNase A. DNA concentration was determined using a TKO 100 fluorimeter (Hoefer Scientific Instruments).

Polymerase Chain Reaction

The reaction mixture for the polymerase chain reaction was 20 μl in volume and contained 50 mM KC1, 20 mM Tris-HCl, pH 8.4 (25°C), 4 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM each dNTP, 0.2 μM primer, 20 ng DNA, and 0.8 - 1 U *Tag* polymerase. A thin layer (50 μl) of mineral oil (Bajol F) was formed in each tube. Amplification was performed as follows: the first denaturation, at 94°C for 1 min; the first four cycles, at 94°C for 1 min, at 42°C for 1.5 min, and at 72°C for 2 min; in the subsequent cycles, the annealing was performed at the optimal conditions for each primer, usually at 52°C. Thirty-seven cycles were carried out. The final elongation cycle lasted for 10 min. The reaction was stopped by adding 40 μl of 10 mM Na_3EDTA in TE buffer. One-sixth of the reaction mixture was used for single electrophoresis. Amplification products were separated by electrophoresis in 2% agarose gel with 1 x TBE and visualized by ethidium bromide.

RESULTS AND DISCUSSION

The optimal temperature for hybridization between primers and DNA was calculated by the computer program ANNEALING applied in our laboratory (see the Table) according to the formulas [10, 11].

The program TREE [12] was used to construct schemes of genetic relations between the analyzed patterns on the basis of their electrophoretic spectra.

The program determines genetic distances *(D)* using the equation

$$F = 2N_{XY}/(N_X + N_Y),$$

where *NXY* is a sum of common bands for two patterns, and N_x and N_Y are number of bands in the patterns *X* and *Y* [13, 14].

The prerequisite for program execution is to create files with electrophoregram data. The presence of a band at a given level was designated as "1," and its absence, "0." If the compared patterns had bands of different intensity at the same level, we designated the most intense band as "2." Data from several files may be summarized and used for dendrogram construction based on averaged data. Fifty patterns may be processed.

Sequences of 8 - 12 nucleotides are usually used to study polymorphism of the amplified DNA by arbitrary primers. In our experiment, we used the longer sequences - from 16 to 21 base pairs. Thus, special conditions to carry out the reaction were required; we described these conditions in detail in our previous article [11]. Increase of primer size reduces the possibility of revealing completely complementary regions on mRNA. However, the use of such primers allows one to avoid the problem of reproducibility of results that often occurs in the case of short primers.

The primers PI, P2, and P7 were species-specific. They showed interspecies polymorphism, but did not reveal differences between the varieties analyzed. The primers P3, P4, P5, P6, and P8 revealed polymorphism between the varieties. Electrophoretic patterns of amplification products obtained by these primers were identical in all individual plants within each variety.

Figure 1 *(a - d)* shows electrophoretic patterns of the amplified DNA fragments of the studied species with four (PI, P3, P7, and P8) of the eight primers used. The primers have a different number of mRNA complementary sites and, accordingly, show a different number of bands (from 24 to 36) on the electrophoregrams. It appeared that at least 167 loci might be tested for interspecies polymorphism.

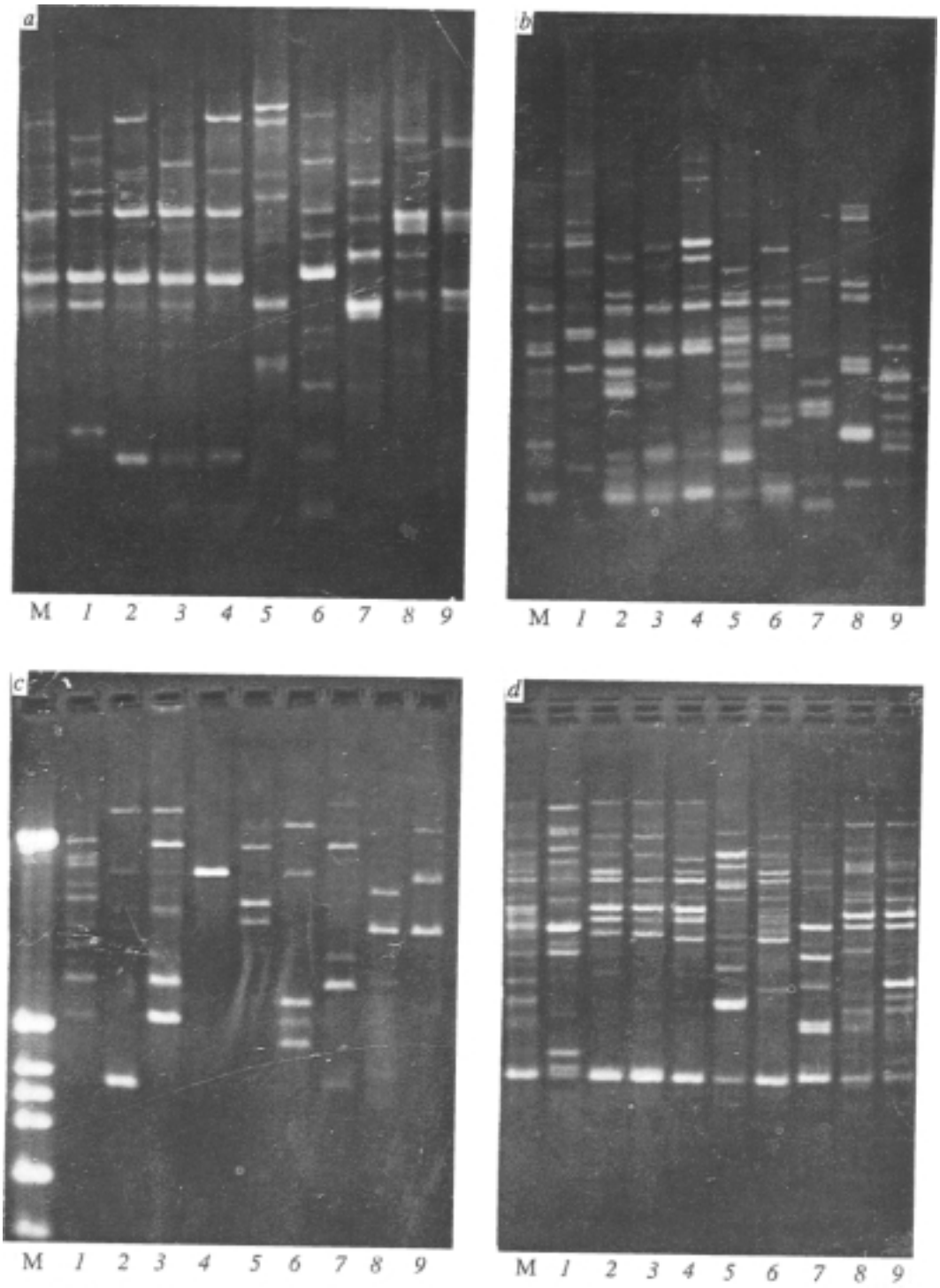


Fig. 1. DNA amplification patterns of different species: *1 - H. chilense; 2-H. agriocrithon; 3-H. spontaneum; 4-H lagunculi-Jorme-5 - H. bulbosum; 6-H. vulgare; 7-5. cereale; 8-T. durum; 9-T. aestivum;* with primers: *a-Pl; b-P3; c- PI d* - P8 Line M is marker: in *a-b,* and *d* is total PCR product for all the patterns; in *c* is pBR 322 digested with *Hinfl*

Figure 2 presents the dendrogram of phylogenetic relations between the species studied. The analyzed species were grouped into two big clusters according to their genetic distances. The first cluster includes most of the *Hordeum* species, the second, *T. aestivum, T. durum, S. cereale,* and *H. chilense.* The lowest genetic distances were between *H. agriocrithon* and *H. lagunculiforme.* These species of wild six-rowed barley are very similar in morphology, and differ only in the presence of peduncles on lateral spikelets in

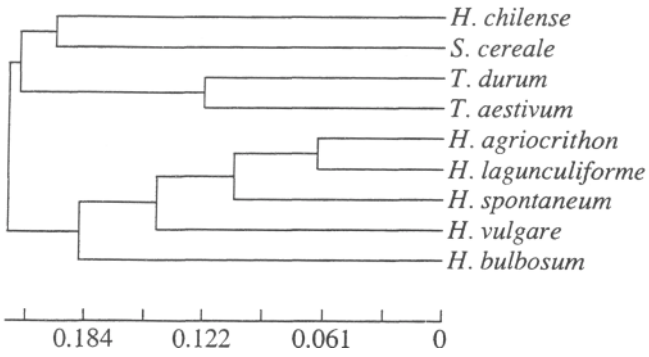


Fig. 2. Dendrogram of interspecies relations based on genetic distances.

H. lagunculiforme [8]. *H. spontaneum* is closer to *H. vulgare.* This fact correlates with the phylogenetic scheme of Nevskii presented by Bakhteev [15]. According to this scheme, *H. vulgare* and *H. spontaneum* are assigned to section VI, *H. bulbosum,* to section V, and *H. chilense,* to section III.

H. chilense cannot be crossed, in practice, with the other species from *Hordeum* genus, and, to some extent, this fact is reflected in the partial isolation of this species on the dendrogram. Genetic distances between *H. chilense* and the other *Hordeum* species are comparable to the distances between *H. chilense* and rye *S. cereale.*

Figures 3-5 present the intraspecies polymorphism of the amplification products. One hundred and ninety-six loci were analyzed in 21 varieties studied. The number of bands per pattern varied from 20 to 30 and depended on the genotype and the primer used.

As expected, the interspecies range of genetic distances based on RAPD is higher than the range between the varieties. Genotypes of the original varieties and the mutants produced from them have slight differences (Fig. 6). This was shown for Riso-1508 and Bomi, as well as Riso-56 and Calsberg II. The dendrogram shows that cv. Dzhau Kabutak, which considerably differed from the European varieties by its morphological traits (e.g. naked caryopsis and six-row), is far from the other varieties.

In published data, most RAPD-bands are non-allelic dominant markers [16 - 18]; however, sometimes they are codominant [17].

We studied the inheritance of PCR products with the P3 primer in 52 individual plants from the barley variety Productiv and with the P6 primer in 135 plants from the self-pollinated population no. 106/93. ($F_{\to\infty}$).

Using the P3 primer, we revealed that seven of 17 electrophoretically separated PCR products of DNA from the variety Productiv were polymorphic (Fig. 7). Two bands with monogenic inheritance, 510 bp (C) and 250 bp (F), were linked with each other (recombination value 0.9 ± 0.9%). The same values of their frequencies in populations allowed us to conclude that these products were inherited as codominant.

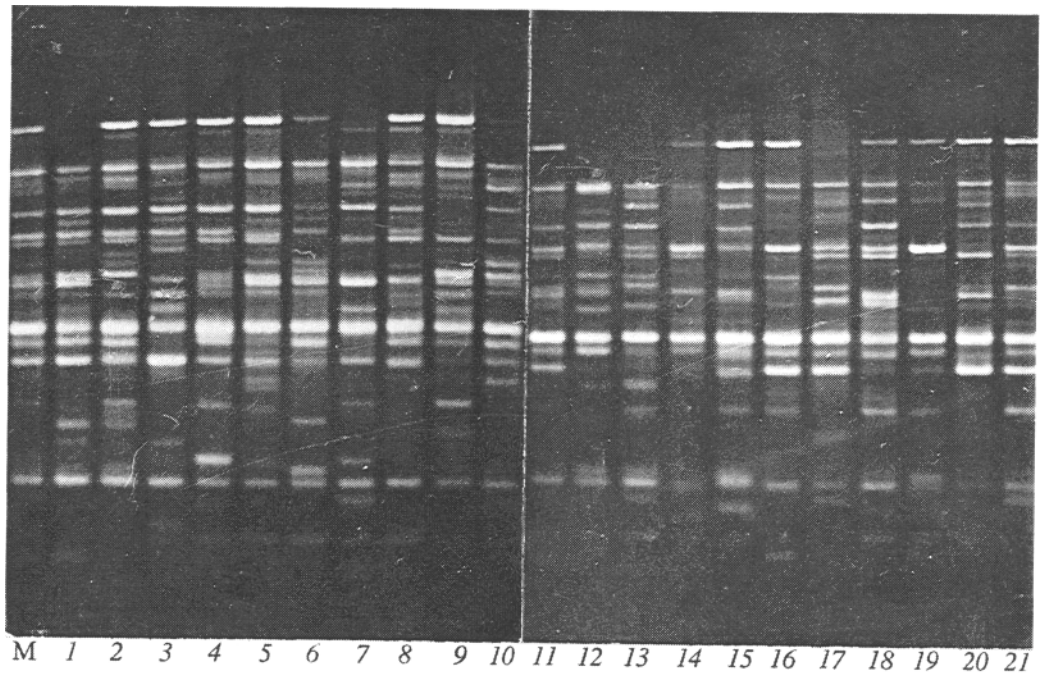


Fig. 3. Patterns of amplified DNA from barley varieties with P4 primer. M is marker (total PCR product in all patterns)' (7) Dzhau Kabutak, (2) Odesskii-31, *(3)* Odesskii-70, *(4)* Odesskii-100, (5) Donetskii-9, *(6)* Donetskii-8, *(7)* Istok, *(8)* Tsiklon (9) Chernomorets, *(10)* Viner, *(11)* Riso-56, *(12)* Riso-1508, *(13)* Nutans-106, *(14)* Pallidum-32, (75) Pallidum-331/13, (76) Betzes (77) Bomi *(18)* Bonus, (79) Athos, *(20)* Koral, (27) Calsberg II.

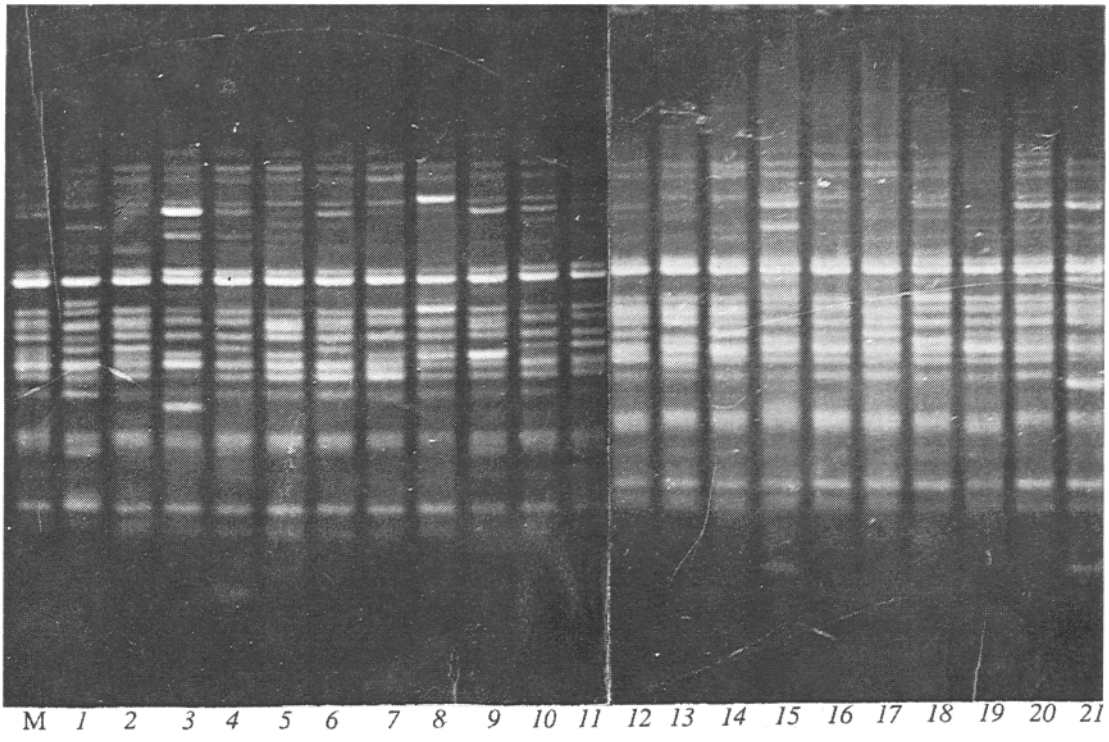


Fig. 4. Patterns of amplified DNA from barley varieties with P6 primer. M is marker (total PCR product in all patterns). Designation of the lines (7) - (27) as in Fig. 3.

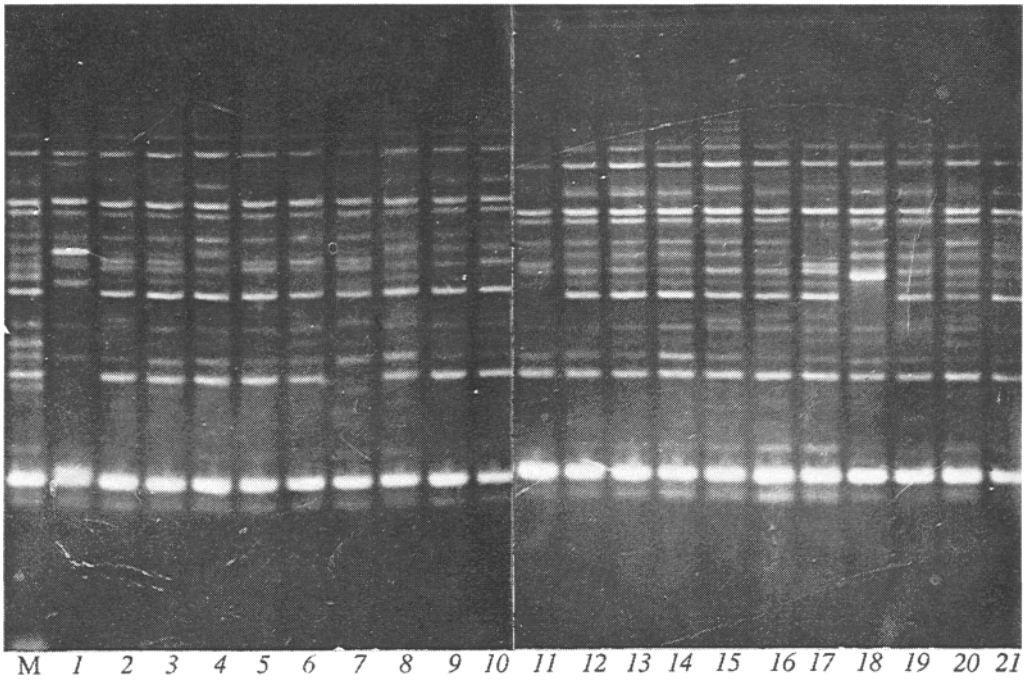


Fig. 5. Patterns of amplified DNA from barley varieties with P8 primer. M is marker (total PCR product in all patterns). Designation of the lines (7) - (27) as in Fig. 3.

Two polymorphic products of DNA amplification in the sample no. 106/93, 550 bp (A) and 450 bp (B) (Fig. 8), had the same frequencies. This fact proves there allelism and confirms the codominant type of inheritance.

Revealing codominant RAPD-markers is of great interest for experiments on genetics and breeding, the studies performed show the possibility of using RAPD for systematics of cereals and for classification

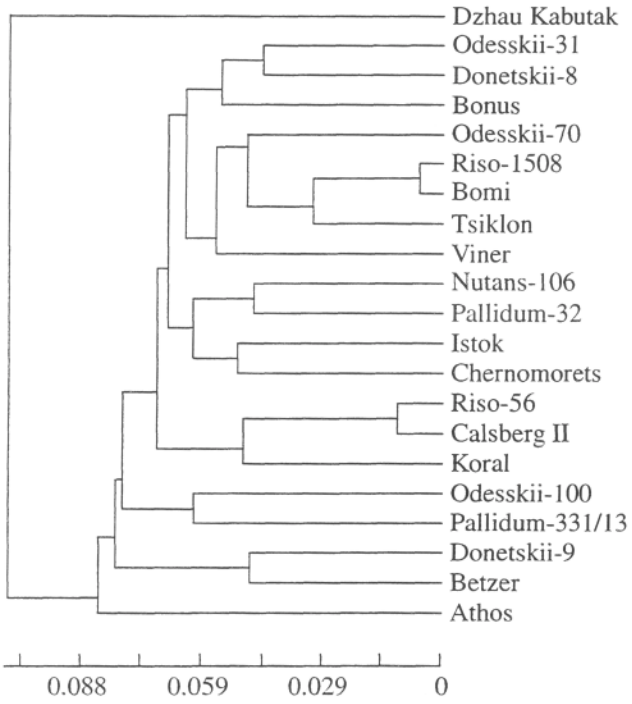


Fig. 6. Dendrogram of relationships between 21 varieties of *H. vulgare* based on genetic distances.

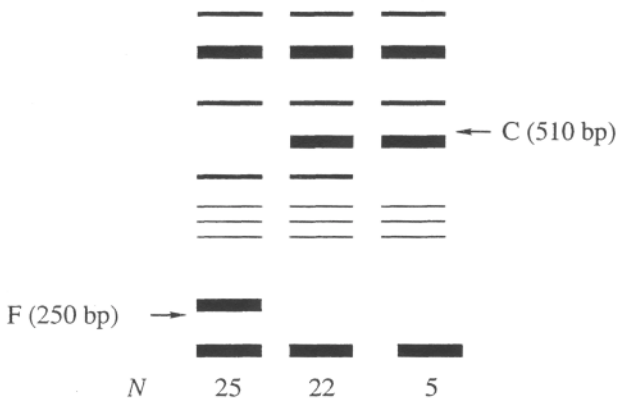
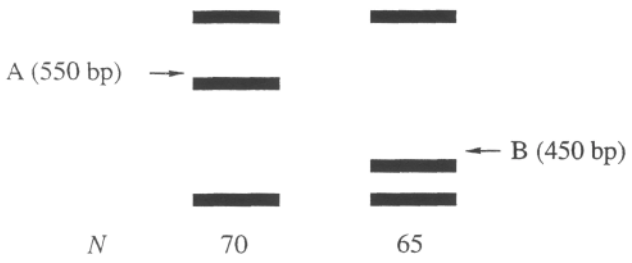


Fig. 7. Diagram of amplified polymorphic PCR products



with P3 primer in individual lines cv. Productiv. N is number of plants with represented phenotype.

Fig. 8. Diagram of amplified polymorphic PCR products with P6 primer in self-pollinated population no. 106/93. N is number of plants with represented phenotype.

of *H. vulgare* varieties according to the degree of their genetic relationships.

ACKNOWLEDGMENTS

We thank Dr. V.P. Netsvetaev (the Department of Genetic Bases of Breeding, the Institute of Breeding and Genetics, Ukrainian Academy of Agricultural Sciences, Odessa) for supplying barley seeds and giving helpful comments on the work and preparation of the manuscript.

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