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POLYMORPHISMOF ESTERASE ISOENZYMES OF RIPE SEEDS OF ACCESSIONS OF RADISH (*Raphanus sativus* L.)

A.S. RUDAKOVA[1], S.V. RUDAKOV[1], A.M. ARTEMYEVA[2], A.B. KURINA[2], N.V. KOCHERINA[3], Yu.V. CHESNOKOV[3] ✉

[1]*Moldova State University,* Republic of Moldova, MD-2009, Chişinău, Mateevich str., 60, e-mail rud-as@mail.ru, rudacov@yahoo.com;
[2]*Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources*, 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail akme11@yandex.ru, nastya_n11@mail.ru;
[3]*Agrophysical Research Institute*, 14, Grazhdanskii prosp., St. Petersburg, 195220 Russia, e-mail alle007@mail.ru, yuv_chesnokov@agrophys.ru (corresponding author ✉)
ORCID:
Rudakova A.S. orcid.org/0000-0001-9638-2151
Rudakov S.V. orcid.org/0000-0003-2591-6114
Artemyeva A.M. orcid.org/0000-0002-6551-5203
Kurina A.B. orcid.org/0000-0002-3197-4751
Kocherina N.V. orcid.org/0000-0002-8791-1899
Chesnokov Yu.V. orcid.org/0000-0002-1134-0292
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Abstract

A biochemical approach was used to assess the genetic variability of the seed radish (*Raphanus sativus* L.) accessions which are distinguished by a wide variety of morphological characters. It is known that the esterase complex in plants has intraspecific specificity; in addition, these enzymes are characterized by tissue specificity. Earlier, the accessions of the collections of the genetic resources of the radish were never evaluated for the presence of isozyme forms of esterases in mature seeds of this crop. The establishment of the general variability of isoenzyme systems and the identification of their genetic control make it possible to reveal the subtle mechanisms of the organism's relationship with the environment and homeostasis, which is especially important for long-term storage of accessions in genetic seed collections. The development of effective biochemical markers for the rapid assessment of collection, as well as genetically and breeding significant material is also essential. This work allows us to fill the gap that exists in relation to the accessions of genetic resources of the radish. From the collection of the Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources (VIR), 49 radish accessions were selected, belonging to three subspecies, divided according to geographic principle as Chinese, Japanese and European radish. All esterase isozymes of seeds were separated using native vertical electrophoresis in polyacrylamide gel followed by processing for nonspecific esterase. According to their esterase composition, all accessions were subdivided into 7 zymotypes, differing from each other by the presence or absence of certain zones. In total, in the esterase complex of radish seeds, 5 main isozymes with different molecular weights varying from 45.3 kDa to 35.0 kDa were found. All five zones were characterized by a high level of polymorphism among the accessions. Based on the composition of isozymes, all genotypes formed 7 zymotypes. Zymotype No. 1, represented by the maximum number of esterases (5 zones), comprised of 43 % of the total number of genotypes. Zymotype No. 2 constituted 33 % of all accessions. The rarest zymotypes No. 5 and No. 7 (4 %) differed in the minimum amount of esterase enzymes (2 zones each). Zymotypes No. 2 and No. 4 were characterized by 4 zones. Representatives of two groups, No. 3 and No. 6 had 3 zones in their esterase complex. The quantitative ratio of all esterase zones varied greatly in the studied accessions. The minimum content (4.78 %) was found for the B5 zone, the maximum amount (67.44 %) was found for the B1 zone. The prevalence of each zone among all studied accessions ranged from 13 to 23 %. Zones B3 (Mr = 39.7 kDa) and B4 (Mr = 37.1 kDa) were the most common among all esterase isozymes; these zones were observed in 23 % of genotypes. For 22 % of representatives, the B2 zone was characteristic (Mr = 42.9 kD). Zones B1 (Mr = 45.3 kD) and B5 (Mr = 35 kD) were less common, 19 % and 13 %, respectively. The average heterozygosity of isozygous forms of esterases of the studied radish accessions was H_{total} = 0.212, with variance for the same accessions $Var(H_{total})$ = 0.0007. Cluster analysis of esterase enzymes divided the studied set of radish accessions into European and Asian subspecies and varieties, and together with phenotypic traits, it allowed constructing a dendrogram corresponding to the botanical, agrobiological and geographical location of the accessions. It should be noted that the accessions of the European subspecies radish are located in two clusters, and the accessions of Russian origin form a separate group in the first

cluster, and the accessions of European origin are grouped in the third cluster which also includes Japanese radishes of European origin. Perhaps this division is associated with the peculiarities of the selection process in creating these accessions. Based on the data obtained, esterase enzymes are recommended as biochemical markers in genetic selection experiments.

Keywords: *Raphanus sativus* L., morphological characters, phenological characters, seeds, esterases, isoforms, zymotypes, polymorphism, biochemical markers, clustering

Radish *Raphanus sativus* L. possesses a wide morphological variety of leaf rosette and root-crops. The existing intraspecific classifications used in Russia [1-4] divide the species geographically depending on the region of origin (Europe, China, and Japan). These classifications are based mainly on the highly variable and conditions-dependent morphological traits of the root-crop (color, shape) of heterogeneous and heterozygous cross-pollinated specimens. Therefore, it is important to find additional characters to more clearly determine the course of evolution and phylogenetic relationships within the species.

Over the past two decades, DNA markers [5, 6] and genetic maps [7-9] have been developed, and the entire genome of Japanese and Chinese radish has been sequenced [10-12]. A number of works note the effectiveness and prospects of using RAPD (random amplified polymorphic DNA) and ISSR (inter simple sequence repeats) molecular markers and some biochemical markers for assessing the genetic variability of radish varieties [13, 14]. Nonspecific esterases (NE, a complex of enzymes that hydrolyze ether bonds (EC 3.1.1.) can be such biochemical genetic markers [15-17]. It is known that the esterases complex in plants has intraspecific specificity, and, in addition, these enzymes are characterized by tissue specificity [18-20].

Due to the ability to hydrolyze cross-bounds of polysaccharides, NEs are important in the cell wall constructing and restructuring. The activity and isoenzyme composition of NEs play a significant role in certain mechanisms of interaction between the host plant and the pathogen [21-23], in the metabolism of fatty acids [24] and choline esters [25]. Nonspecific esterases are also involved in plant resistance to herbicides [26].

It has been shown that NE activity closely related to the physiological and metabolic states of the cell serves as an indicator of the toxic effect of pollutants [27, 28]. Environmental factors, in particular the temperature [29] and water stress [30], also affect the activity of NE, that is, esterases can be considered as potential stress markers.

In the collection of the Vavilov All-Russian Institute of Plant Genetic Resources (VIR), there are 1200 *R. sativus* radish accessions from 75 countries of all continents (including 573 accessions in the permanent catalog), representing all subspecies, varieties and types of the crop.

This paper is the first report on isozyme forms of esterases in mature seeds of the radish accessions from the VIR World Collection. Based on the obtained isozyme spectra, dendrograms of the phylogenetic relationships between the accessions were constructed which correspond to their botanical and agrobiological descriptions and geographic origin. The average heterozygosity of isozyme forms of esterases in the studied accessions and their variance were established, indicating the reliability of the results obtained.

The work aims to evaluate the polymorphism of esterases in mature radish seeds, its dependence on the origin and agrobiological traits of the accessions, and the suitability of esterases as biochemical markers of the *R. sativus* diversity.

Materials and methods. Mature seeds of eight *Raphanus sativus* varieties of various origins (49 genotypes in total, VIR World Collection) were ground in a porcelain mortar to flour. The100-mg flour specimens were placed into Eppendorf tubes, added with 2 ml of pre-cooled hexane, shaken periodically, and left

overnight in a refrigerator at 4-8 °C. The next day, the tubes were centrifuged (Eppendorf centrifuge 5410, Eppendorf AG, Germany) at 15000 rpm for 10 min, the supernatant was removed, and the fat-free flour precipitate was left under the fume hood to air-dry. Esterase enzymes from defatted and dried plant material were extracted with 0.05 M Tris-HCl buffer, pH 8.3 (flour: buffer ratio 1:4) at 4-8 °C for 14-18 hours. After centrifugation at 15000 rpm for 10 min, the enzyme extracts were poured off the sediment and frozen at –20 °C. Specimens were thawed before electrophoresis and loaded in the pockets of stacking gel.

Esterase isozymes were separated by native polyacrylamide gel electrophoresis [31] (the concentrations of resolving and stacking gel 11 and 5%, respectively, a Mini-PROTEAN Tetra Cell, Bio-Rad Laboratories, Inc., USA). A Prestained Protein Ladder marker (Thermo Scientific, USA) was pipetted into the last pocket of the gel. Protein concentration in enzyme extracts was measured as per Bradford [32]. Depending on the protein concentration, 15-20 μl aliquots were loaded into the gel pockets. Electrophoresis was conducted at 6-10 °C and 10 V/cm for 2.5 h. To visualize isoenzymes, the gel was exposed to a reagent detecting nonspecific esterase [33] for 10-15 min until the zones appeared. The gel was floated in a fresh solution of the dye and substrates, consisting of 100 mg α-naphthyl acetate and 120 mg β-naphthyl acetate (Sigma-Aldrich Chemie GmbH, Switzerland) dissolved in 10 ml of 70% ethanol, 500 mg of Fast Blue RR (Sigma-Aldrich Co., USA), 4 ml of propanol and 60 ml of 0.1 M phosphate buffer (pH 6.0). The excess dye was removed with 7% acetic acid.

The obtained zymograms were scanned (Epson Expression 10000XL, GE Healthcare, USA). The proportion of zones found in the track and the determination of molecular weights according to the corresponding standards based on the Rf value were calculated using the Phoretix 1D Advansed software (Total Lab, Ltd., UK).

Heterozygosity H_l of the population for each locus and the average (total) heterozygosity of $H_{tot.}$ were calculated as per by the formulas [34, 35]:

$$H_l = 2n(1 - \sum_k x_k^2)/2n - 1\ ,$$
$$H_{tot.} = \sum_{l=1}^{r} H_l/r,$$

where l is the ordinal number of the locus, n is the population size, x_k is the frequency of the k-th allele of the l-th locus, and r is the total number of loci.

The variance of heterozygosity $Var\ (H_l)$ for each locus and the variance of the average heterozygosity within the population $Var\ (H_{tot.})$ were calculated as follows [36]:

$$Var(H_l) = H_l(1 - H_l)/n\ ,$$
$$Var(H_{tot.}) = \frac{1}{nr^2}\sum_l H_l(1 - H_l) + \frac{1}{nr^2}\sum_l \sum_{l \neq \hat{l}} (H_{l\hat{l}} - H_l H_{\hat{l}}).$$

The morphological and agrobiological descriptions of the accessions were performed according to Sazonova et al. [37] (Pushkin and Pavlovsk laboratories of VIR, St. Petersburg).

The main morphological and phenological markers for the *R. sativus* intraspecific attributing to varieties and types were the shape and color of the root-crop and the length of the growing season [1, 37]. When constructing a matrix for cluster analysis, the following quantitative and qualitative characteristics were used: the root-crop length and diameter, the root-crop index (the length to diameter ratio), the color of the root-crop bark (1 — white, 3 — green, 5 — pink, 7 — red, 9 — black), the root-crop shape (2 — conical, 3 — cylindrical, 4 — elliptic, 5 — round, 6 — flat round, 7 - cylindrical with a run up), leaf type (1 — solid, 3 — lyre-like), the leaf pubescence (0 — no pubescence, 1 — pubescent), the length of the growing season. Qualitative characteristics were assessed in points according to the descriptor [38].

Cluster analysis based on the profiles of seed esterases only and on a complex of traits (morphological and phenological features and esterase profiles in seeds) was performed by the UPGMA method using the PAST program (http://sonraid.ru/past/), including bootstrap analysis.

Results. Table 1 lists the radish accessions studied in the work.

1. *Raphanus sativus* L. accessions (VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **involved in the study**

VIR-number	Name	Origin	Convariety (convar.)	Variety (var.)	Number on zymogram
		R. sativus subsp. *sativus* (L.) Sazon.			
k-1675	Belaya Adzharskaya	Georgia	*sativus* Sazon.	*sativus* Sazon.	3
k-1833	Odesskaya 5	Belarus	*sativus* Sazon.	*sativus* Sazon.	7
k-2163	Maiskaya belaya	Russia	*sativus* Sazon.	*sativus* Sazon.	44
k-1778	Zimnyaya krukglaya chernaya	Germany	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	4
k-1892	Dazwish ali	Egypt	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	9
k-1971	Round Black Spanish	USA	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	15
k-1764	Local	Russia	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	26
k-2115	Chernaya	Russia	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	35
k-2124		Turkey	*hybernus* (Alef.) Sazon.	*niger* (L.) Sinsk.	36
k-1914	Zimnyaya krukglaya belaya	Russia	*hybernus* (Alef.) Sazon.	*hybernus* (Alef.) Sazon.	28
k-2025	Skvirovskaya belaya	Ukraine	*hybernus* (Alef.) Sazon.	*hybernus* (Alef.) Sazon.	32
		R. sativus subsp. *sinensis* Sazon. et Stankev.			
k-698		Asia Minor	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	1
k-1805		Middle Asia	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	5
k-1902	Belaya zelenigolovaya	China	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	11
k-1978	Local	Kyrgyzstan	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	16
k-2101	Chinese White Winter	Chile	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	21
k-2074	Local	Egypt	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	33
k-2151	Altari mu	South Korea	*lobo* Sazon. et Stankev.	*lobo* Sazon. et Stankev.	39
k-1815	Margilanskaya	Uzbekistan	*lobo* Sazon. et Stankev.	*virens* Sazon.	6
k-1865	Wei-syan	China	*lobo* Sazon. et Stankev.	*virens* Sazon.	8
k-2000	Local	Uzbekistan	*lobo* Sazon. et Stankev.	*virens* Sazon.	17
k-2148	Local	Kazakhstan	*lobo* Sazon. et Stankev.	*virens* Sazon.	38
k-725		Asia Minor	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	2
k-1895	Hun-dyn-lun	China	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	10
k-1903	Krasnaya	China	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	12
k-1935	Nerima Pointed rooted	Japan	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	13
k-1857	Chan-shun-lobo	China	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	27
k-1967	Local	Afghanistan	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	30
k-1983	Nexhnaya	Russia	*lobo* Sazon. et Stankev.	*rubidus* Sazon.	31
		R. sativus subsp. *acanthiformis* (Blanch) Stankev.			
k-1958	Hakata haruwaka	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	14
k-2033	Turnip	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	18
k-2063	Unzen shigatsu	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	20
k-2111	Minotoki No. 2	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	22
k-1946	Unsen-4-gatsu	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	29
k-2134	Eifuku 2	Japan	*minowase* (Kitam.) Sazon.	*minowase*	37
k-2154	Mikura gross F_1	The Netherland	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	40
k-2155	Local	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	41
k-2159	Yasato riso F_1	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	42
k-2161	Horiyou	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	43
k-2184	Cheng sugeng zung	South Korea	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	47
k-2335	April cross	France	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	48
k-2336	Spring Feller	Japan	*minowase* (Kitam.) Sazon.	*minowase* Kitam.	49
k-2034	Miyasige Oonaga	Japan	*acanthiformis* (Blanch) Stankev.	–	19
k-2133	Eifuku	Japan	*acanthiformis* (Blanch) Stankev.	–	23
k-2136	Shinuchi Sobutori F_1	Japan	*acanthiformis* (Blanch) Stankev.	–	24
k-2177	Back-ok	South Korea	*acanthiformis* (Blanch) Stankev.	–	25
k-2093	Mijshige long pointed rooted	Japan	*acanthiformis* (Blanch) Stankev.	–	34
k-2175	Sodam	South Korea	*acanthiformis* (Blanch) Stankev.	–	45
k-2178	Shinmyeong	South Korea	*acanthiformis* (Blanch) Stankev.	–	46

N o t e. Dashes mean that varieties are not classified.

Native electrophoresis detected five main esterase isozymes, the B1 (45.3 kDa), B2 (42.9 kDa), B3 (39.7 kDa), B4 (37.1 kDa), and B5 (35.0 kDa) (Fig. 1) in seeds of the 49 studied radish genotypes. All five bands were

characterized by polymorphism among the accessions (Table 2). We did not find monomorphic zones present in all accessions.

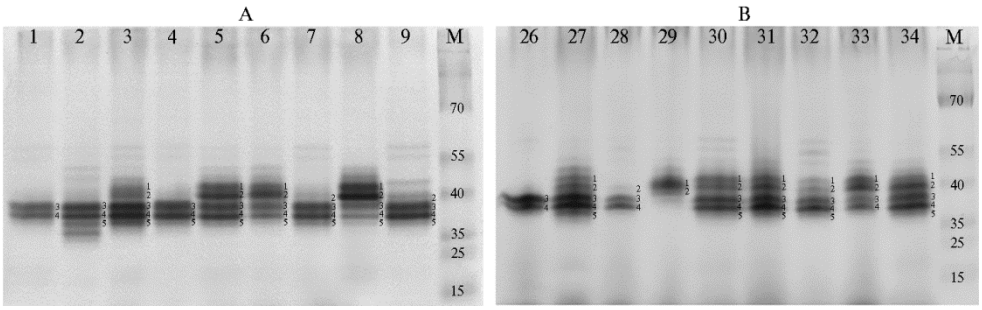


Fig. 1. Zymograms of esterases from mature seeds of *Raphanus sativus* L. (accessions of the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources). Esterase zones are indicated along the tracks on the right, accessions numbers are above the lanes For line numbers, see Table 1. M — molecular weight markers (15-70 kDa; Prestained Protein Ladder, Thermo Scientific, USA).

2. Esterase zymotypes in mature seeds of *Raphanus sativus* L. (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources)

Zymotype	B1 (45,3kDA)	B2 (42,9kDA)	B3 (39,7kDA)	B4 (37,1kDA)	B5 (35,0kDA)	Total number of zones
No.1	+	+	+	+	+	5
No. 2	+	+	+	+	−	4
No. 3	−	+	+	+	−	3
No. 4	−	+	+	+	+	4
No. 5	−	−	+	+	−	2
No.6	−	−	+	+	+	3
No. 7	+	+	−	−	−	2
Total	38	45	48	48	26	205
Frequency of zone occurrence, %	19	22	23	23	13	100

By the esterase profiles, all accessions were attributed to seven winter types differing from each other by the presence or absence of certain zones (Tables 2, 3).

3. Attribution of mature seeds of *Raphanus sativus* L. (accessions of the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **to esterase zymotypes**

Zymotype	Number of zones in zymotype	Number on zymogram	Number of genotypes	
			total	of total number, %
No. 1(B1-B5)	5	3, 5, 6, 8, 10-14, 16, 17, 23, 24, 27,30-32,34,38,40,48	21	43
No. 2(B1-B4)	4	18-22,25,33,36,37,39,41, 42, 44-46,49	16	33
No.3(B2-B4)	3	28,35,43,47	4	8
No.4(B2-B5)	4	7,9,15	3	6
No.5(B3-B4)	2	1,26	2	4
No. No.6(B3-B5)	3	2,4	2	4
№ 7(B1-B2)	2	29	1	2

N o t e. For description of the accessions, see Table 1, for esterase profiles of zymotypes, see Table 2.

4. Esterase is enzyme contents in mature seeds of *Raphanus sativus* L. (accessions of the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources)

Indicator	B1	B2	B3	B4	B5
M_r, kDa	45.3	42.9	39.7	37.1	35.0
Min, %	7.06	7.78	16.77	8.74	4.78
Max, %	67.44	39.91	54.22	52.10	25.96
Mean, %	20.11	25.16	29.28	26.60	11.48

N o t e. M_r— molecular weight, Min — minimum amount, Max — maximum amount.

The amount of esterase within each zone varied greatly (Table 4). The prevalence ranged between zones from 13 to 23%, the minimum was characteristic of B5 zone (4.78%), the maximum of B1 zone (67.44%). The average statistical

value of the content of esterase isozymes varied from 11.48 for zone B5 to 29.28% for zone B3.

Zymotype No. 1 (5 zones) accounted for 43% of the total number of genotypes. The main part of this group was represented by specimens of the Chinese subspecies green (var. *virens* Sazon.), pink-red (var. *rubidus* Sazon.), and white (var. *lobo*) varieties from China and Central Asia. In addition, this group included six accessions of Japanese radish of European and Japanese origin and an accession of summer European radish. Zymotype No. 2 (4 zones) was typical for 33% of the studied accessions. This group consisted mainly of accessions of Japanese subspecies from Japan and South Korea, several genotypes of the white variety from Egypt and Chile, and two accessions of European summer and winter radish. Zymotype No. 3 (3 zones) was characteristic of two accessions of winter European radish from Russia and two accessions of daikon of autumn type and made up 8% of all genotypes. Three accessions (6%) of European radish demonstrated zymotype No. 4 (4 zones), each of the two zymotypes, No. 5 (2 zones) and No. 6 (3 zones) was represented by two accessions (4%) of local lobo radish from Asia Minor and winter black radish. One accession of the daikon Unzen-4-gatsu (k-1946, Japan) of zymotype No. 7 (2 zones) was the least common, that is, having the rarest esterase profile with 2%frequency of occurrence.

The frequency of heterozygotes is one of the most important characteristics of a population, since each heterozygote carries different alleles and provides variability. It should be noted that the smaller the difference between the values of the allele frequencies per locus, the higher the obtained value of heterozygosity for this locus. Our calculation showed the highest heterozygosity (H_l= 0.503) for the isoform B5 (Table 5). For B3 and B4, only one polymorphic allele was identified and, therefore, the heterozygosity was the lowest (H_l= 0.039). Variance is a quantity dependent on heterozygosity, and therefore the patterns identified for heterozygosity are similar to those for variance. The application of the formula for calculating the variance of average heterozygosity [36] is due to covariations between heterozygosities at loci l and $\hat{l}$ determined by the frequencies of double heterozygotes $H_{l\hat{l}}$of these loci.

5. Population heterozygosity and its variance among *Raphanus sativus* L. accessions (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **as calculated based on isozyme analysis data**

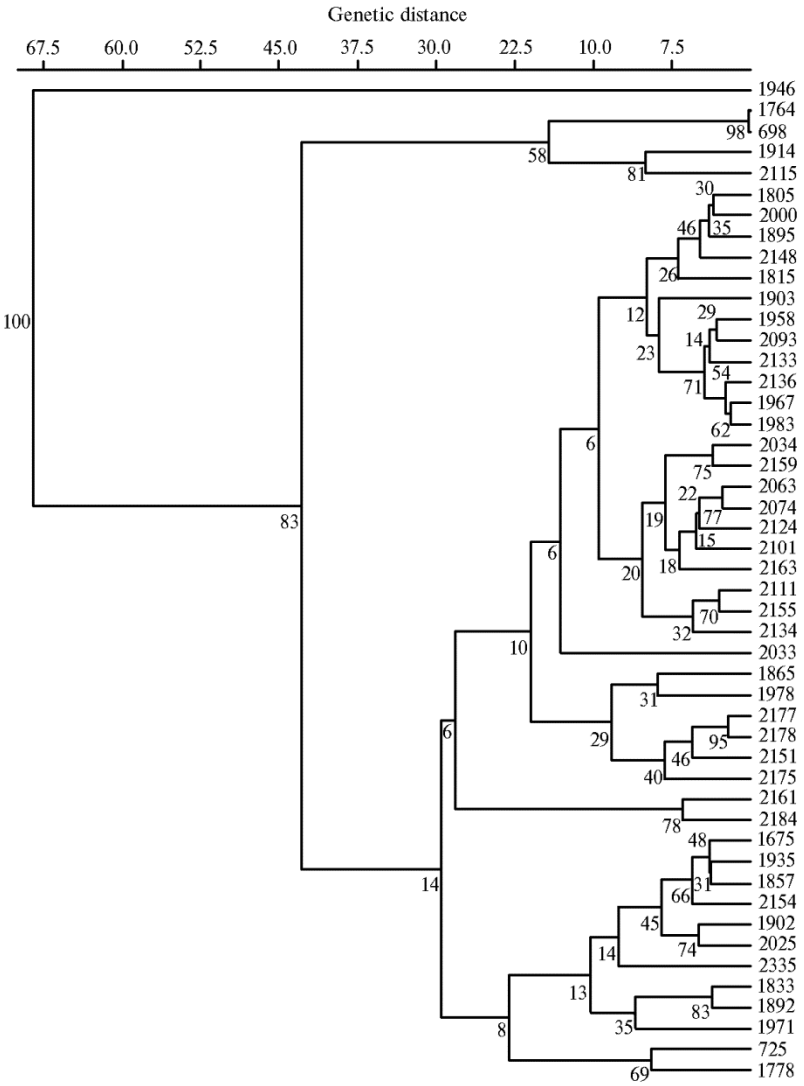
Statistical parameter	Isozyme loci (esterase zones)				
	B1	B2	B3	B4	B5
Heterozygosity H_l	0.328	0.152	0.039	0.039	0.503
Variance $Var(H_l)$	0.004	0.002	0.001	0.001	0.005

N o t e.Average heterozygosity $H_{tot.}$ is 0.212, the variance of the average heterozygosity with in a population $Var(H_{tot.})$ is 0.0007.

The formulas we used in our work allow any polynomial in a set of variables distributed multinomially to be solved, and the calculated heterozygosity is regarded as a measure of polymorphism information which is actively used in genetic research and selection programs.

The isozyme analysis revealed a total of 205 electrophoretic bands of esterase isoforms which were used to construct a dendrogram (Fig. 2). The radish accessions were found to form one large and two small clusters. An accession of the daikon Unzen-4-gatsu (k-1946, Japan) was an out-group. The first small cluster grouped accessions of European winter radishes from Russia (var. *niger* (L.) Sinsk.; var. *hybernus*) and a local white lobo from Asia Minor (k-698). The second large cluster was mainly Chinese and Japanese radish accessions divided into four subclusters. The lobos from Russia, Afghanistan, China (var. *rubidus* Sazon.), Uzbekistan and Kazakhstan (var. *virens* Sazon.), and of spring and autumn daikons

were in the first subcluster. The Japanese and European radishes (var. *sativus*; var. *niger* (L.) Sinsk.), lobos from Egypt and Chile (var. *lobo*) were in the second subcluster. An accession of daikon k-2033 was located outside the first two subclusters. The third subcluster grouped lobo accessions from South Korea, Kyrgyzstan, China (var. *lobo*; var. *virens* Sazon.) and daikons from South Korea and Japan. Note, the genotypes from South Korea and Japan formed two separate groups within the subcluster. Two daikons from South Korea and Japan were the fourth subcluster.



Fir. 2. UPGMA-dendrogram of genetic similarity among *Raphanus sativus* L. accessions (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **based on esterase profiles of mature seeds.** The bootstrap values on the branches indicate the linkage distances. For description of genotypes (numbers on the right), see Table 1.

In the third cluster, mainly accessions of the European subspecies radish, and the Japanese subspecies but of European origin were located. The cluster was divided into two subclusters, the first was the European winter radishes from Canada and Egypt (var. *niger* (L.) Sinsk.) and summer radishes from Belarus (var. *sativus*), the second was daikons from France and the Netherlands, white European radishes from Ukraine and Georgia (var. *hybernus*; var. *sativus*), and Chinese

radishes from China and Japan (var. *rubidus* Sazon.; var. *lobo*). Two accessions remained outside the subclusters, i.e., the winter black radish from Germany and lobo from Asia Minor.

By phenotypic traits, the studied radish accessions were grouped into five clusters (the dendrogram we obtained is not shown, since it corresponded to their botanical and agrobiological attribution). European winter and summer radishes grouped into separate clusters. Asian radishes were represented by two clusters (accessions of the Japanese and Chinese subspecies), and there were no significant differences between the accessions of different ecological and geographical origin. The last cluster was several accessions of lobo from Central Asia and Asia Minor and Chile and a daikon of a local variety population from Japan. That is, the phylogenetic pattern obtained using only phenotypic characters did not fully reflect the peculiarities of origin and evolution of the studied radishes.

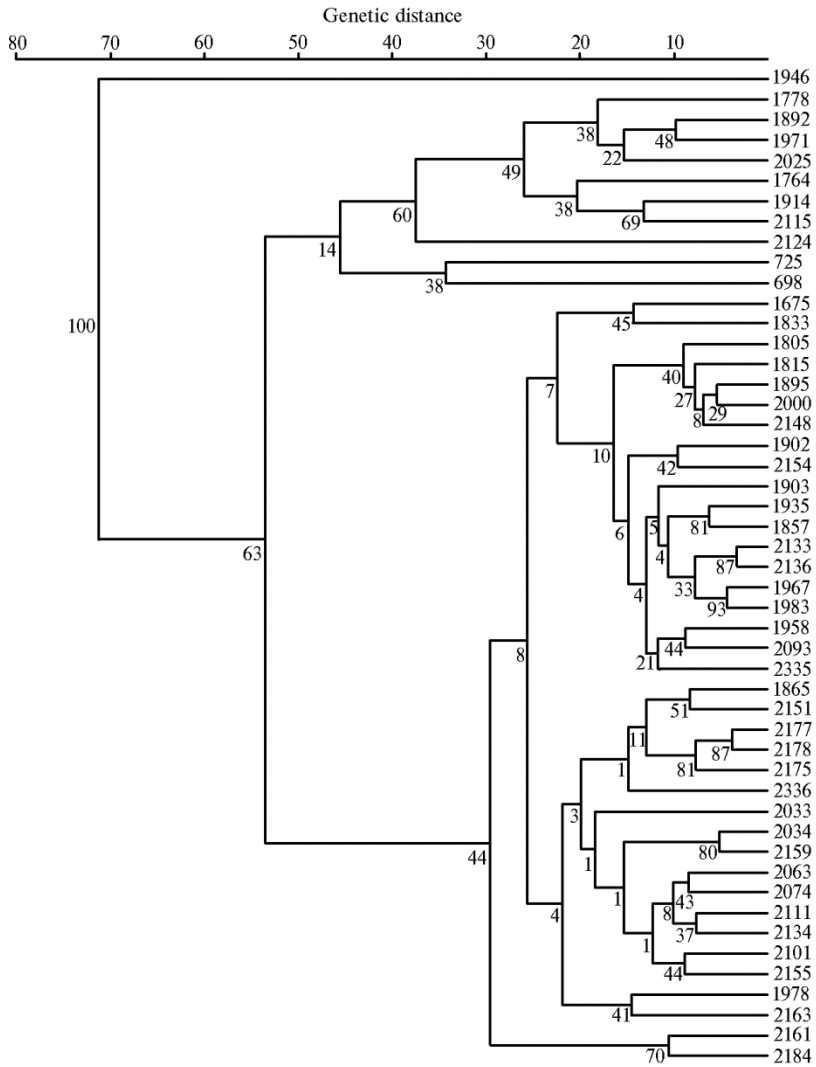


Fig. 3. UPGMA-dendrogram of genetic similarity among *Raphanus sativus* L. accessions (the VIR World Collection, the Vavilov All-Russian Institute of Plant Genetic Resources) **based on** morpho-phenological traits and **esterase profiles of mature seeds.** The bootstrap values on the branches indicate the linkage distances. For description of genotypes (numbers on the right), see Table 1.

The dendrogram based on morpho-phenological trait sand esterase profiles

of seeds (Fig. 3) consists of a large and two small clusters. Outside, as in Figure 2, there was a Japanese accession of the Unzen-4-gatsu daikon (k-1946). European radish subspecies were in the first cluster divided into two subclusters. The first subcluster was winter radish of black (var. *niger* (L.) Sinsk.) and white (var. *hybernus*) varieties, the second subcluster was Chinese radish genotypes from Asia Minor (k-725, k-698) which were local variety populations.

The second large cluster comprised the Chinese and Japanese subspecies and the European summer radish. The cluster was divided into four subclusters. Two European summer radishes (var. *sativus*) from Belarus and Georgia were in the first subcluster. The second subcluster consisted of a separate group of lobos from Central Asian, a group of lobos of a pink-red variety (var. *rubidus* Sazon.) from China, Russia, and Afghanistan, and daikons from Japan, France, and the Netherlands. In the third subcluster, there were two large groups of accessions, the first group of the lobo and daikon genotypes from South Korea with a daikon from Japan (k-2336) close to them and the second group of daikons from Japan and two accessions of the white lobos from Egypt (k- 2074) and Chile (k-2101). The fourth subcluster comprised only two accessions, the white lobo from Kyrgyzstan (Local, k-1978) and the summer European radish from Russia (Maiskaya, k-2163). The third small cluster contained two daikon accessions from Japan and South Korea.

A comparative isozyme profiling of the accessions revealed an intraspecific polymorphism and divided the accessions into seven zymotypes different in quantitative ratios of the isozyme zones of esterases. The appearance of all five esterase zones (zymotype No. 1) was more characteristic of the Chinese subspecies, which indicates large intervarietal differences within the subspecies. Four esterase zones (zymotypes Nos. 2 and 4) were found mainly in the Japanese and European subspecies. Three (zymotypes Nos. 3 and 6) and two (zymotypes Nos. 5 and 7) esterase zones were found in European winter radish from Russia, daikons from Japan and South Korea, and lobo from Asia Minor. The zymotypes Nos. 3 and 6 were cultivars presumably resulted from individual selection from populations or through hybridization followed by selection. The accessions of local origin which expressed zymotypes Nos. 5 and 7 are highly homogenous within the cultivar, possibly, their selection was localized in a certain area.

Consequently, the rare esterase zones in these accessions are due to their selection or agrobiological affiliation, which is consistent with other reports [15-17, 39]. We for the first time have shown [15-17] that the assessment of esterase isozyme polymorphism is reliable to evaluate genetic polymorphism not only in radish (*R. sativus*) and lines of *Brassica rapa* L. doubled haploids, but also in hexaploid spring wheat (*Triticum aestivum* L.). Similar results were obtained in studying the polymorphism of various wheat varieties (*Triticum* L.) [39]. In all these studies, polymorphism of the isozyme profile of esterases isolated from mature seeds was revealed in accessions of varietal, linear and collection breeding material. A wide variety of electrophoretic profiles of esterase isozymes of mature seeds has been shown and the possibility of determining the polymorphism of esterases in hybrid generations has been established. Along with the results we obtained in this study, this allows us not only to select promising starting material for breeding, but also to recommend this type of biochemical markers for solving practical problems as a means that can accelerate and simplify selection of breeding material. The fact that similar work on radish, small radish, *B. rapa*, wheat has not been carried out before, once again emphasizes the prospects of using the approach we have proposed.

Cluster analysis of the esterase profiles of seeds showed that the

accessions were grouped mainly by origin and partly due to their botanical affiliation. Accessions of the European subspecies radish were located in two clusters, and the accessions of Russian origin formed a separate group in the first cluster, and the accessions of European origin were in the third cluster which also includes Japanese radishes of European origin. Perhaps this division is due to the peculiarities of the selection of these accessions.

The second large cluster of Asian radishes grouped not only accessions of the Chinese and Japanese subspecies, but also several accessions of the European subspecies of summer and winter varieties, which could be associated with the peculiarities of their origin or with an error in reproduction. Interestingly, South Korean accessions, regardless of their botanical affiliation, formed a separate group within the third subcluster, which made an important addition to intraspecific differentiation.

Thus, European and Asian accessions were distributed in separate clusters, which confirm the origin of the radish diversity from two primary geographic centers, the Mediterranean and Asian [2, 4].

Clustering of accessions by a set of characters (morphological, phenological traits, and seed esterases) showed the results most consistent with botanical and agrobiological division. The first cluster included all accessions of European winter radishes and two variety populations of the lobo. The second large cluster grouped accessions of the Chinese and Japanese subspecies, as well as European summer radish. Summer European radishes are considered the intermediate forms between European winter radishes and small radishes, and lobos are the original forms of Japanese radishes and Chinese small radishes. Perhaps this clusters these two groups of varieties together. The third cluster contained a group of lobo and daikon accessions from South Korea, as in the first dendrogram. This refinement was revealed due to the analysis of esterase enzymes and, probably, indicates a multiple origin of Japanese radishes [3].

The daikon Unzen-4-gatsu (k-1946, Japan) was distinguished by the rare esterase zones (zymotype No. 7) and occupied the out-group position. It belongs to a morphologically sharply different cultivar Ninengo, a characteristic feature of which is a long thin root-crop (50-55 cm in length, 5-6 cm in diameter) and a large rosette of leaves (25-30 cm in height, 35-40 cm in diameter). The cultivars of this group are the most resistant to frost and stemming [40].

So, the performed biochemical analysis of esterases of mature seeds of radish accessions of different origin revealed the isozyme polymorphism. Calculation of heterozygosity H_l for each locus and total heterozygosity of the population $H_{tot.}$ revealed the most (B5) and least (B3 and B4) heterozygous esterase isoforms. The formulas used in this paper make it possible to solve any polynomial in a set of variables distributed multinomially. The calculated heterozygosity $H_{tot.} = 0.212$ and the variance for the same accessions $Var\ (H_{tot.}) = 0.0007$ can be regarded as an effective measure of informational polymorphism to be used in breeding programs. In addition, the cluster analysis based on the seed esterase profiles coupled with phenotypic traits are consistent with botanical and agrobiological classification by origin from two primary geographic centers. Consequently, esterase profiles of mature seeds are convenient biochemical markers in physiological, biochemical, genetic and breeding studies of the crop.

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