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Genetic Diversity of Bulgarian *Phaseolus vulgaris* L. Germplasm Collection

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1. Introduction

The common bean, *Phaseolus vulgaris* (L.) *Savi*, is a member of the genus *Phaseolus*, belonging to subtribe *Phaseolinae*. It in turn is a member of the tribe *Phaseoleae* of the subfamily *Papilionoideae* and the family *Leguminosae (Fabaceae)* (van Eseltine, 1931; in Lackey, 1977; Maréchal et al., 1978). The neotropical genus *Phaseolus* includes about 50 to 60 species of wild bean (Toro et al., 1990), most of them distributed in Mesoamerica, five of which are cultivated: *P. vulgaris* (L.) *Savi* (common), *P. dumosus* Macfady (tepari), *P. coccineus* L. (multicolored, scarlet-coloured), *P. lunatus* L. (Lima) and *P. polyanthus Green* (Debouck, 1989; Caicedo et al., 1999; Delgado-Salinas et al., 1999; Gutiérrez Salgado et al., 1995). The last four species have much narrower commercial importance than *P. vulgaris*. In the USA less than five economically important varieties of common bean occupy half the area of this culture and at the same time it is from there that more than 10% of world production originates (Pachico, 1989). The vast narrowing of genetic variability is an indication of the risk of potential loss of a species and emphasizes the importance of its evaluation.

1.1 Increasing the genetic diversity in *P. vulgaris* L.

Various ways to increase the genetic diversity of *P. vulgaris* are known:

1.1.1 Interspecies hybridization

Studies of interspecies hybridisation have been carried out by Baudoin et al. (1985); Hucl & Scoles (1985); Federici & Waines (1988); Cabral & Crocomo (1989); Katanga (1989); Baudoin & Katanga (1990) and Smartt (1990), as cited in Maquet et al. (1997).

Harlan et al. (1971) with modifications (Smartt, 1990, as cited in Maquet et al., 1997) distinguish three main centres of origin:

- Primary gene pool, the equivalent to the concept of a species, whose members are free to cross-fertilize, and consists of cultivated and wild forms of the species.
- Secondary gene pool, the equivalent of all the species that can give viable or partially fertile hybrids with the primary one.
- Tertiary gene pool includes types among which artificial hybridization is possible, but the resulting hybrids are sterile, abnormal or lethal.

The genus Phaseolus is characterized by two main poles (Marshal et al., 1978). The first one includes the complex *P. vulgaris* - *P. coccineus*, and at the opposite end is *P. lunatus*. Interspecies hybridization has allowed to specify the proximity of a large number of species in this genus. The results in this aspect are summarized in several publications (Lorz, 1952; Dhaliwal et al., 1962; Fozdar, 1962, as cited in Baudoin, 1981 & Maquet, 1995).

Sources of genetic diversity of *P. vulgaris* are wild species of the genus *Phaseolus,* but also cultivated ones:

- *P. polyanthus* easily hybridizes with the other four cultivated species and is considered the ancestor of the common bean. Its value lies in its excellent taste.
- *P. coccineus* is difficult to hybridize with other *Phaseolus* species, but hybridizes successfully with *P. vulgaris*. It is of interest with its taste qualities, great potential for yield, resistance to low temperatures, viral, bacterial and fungal diseases. Because of these valuable traits, it is used in the breeding process in Bulgaria for gene transfer into *P. vulgaris* varieties, which enriches the Bulgarian working collection (Zagorcheva et al., 1983a, 1983b; Poryazov et al., 1988).
- *P. lunatus* is difficult to hybridize with the common bean. Lima beans are mainly grown for the green-coloured seeds. It is of interest for its excellent taste, great potential for yield and disease resistance.
- *P. acutifolius*, cultivated mainly for mature grain, is valuable for its resistance to bacterial blight and drought tolerance. It hybridizes with great difficulty with common bean (Poryazov et al., 1988, as cited in Kumanov et al., 1988). Successful hybridization has been achieved in Bulgaria using the *in vitro* methods for embriocultures (Nikolova et al., 1986; Rodeva, 1988).

1.1.2 The introduction of genotypes

The introduction from the primary centres of origin in the collections of crop plants is preceded by identification and characterization of sources and donors of valuable characters for selection, which is very important in organizing pre-breeding work (Konarev, 1998b). In targeted selection of accessions in breeding, it is especially important to establish and implement markers that are genetically linked to important economic traits.

1.1.3 Selection of local accessions

All data described support the American origin of the genus *Phaseolus*. The cultivated forms of beans were distributed around the world shortly after the discovery of America by Columbus in 1492. In Europe, a secondary gene pool, the path of introduction of bean species passes through Spain, Turkey, and only then to Bulgaria, about 300 years ago. The pathways of distribution of beans into and across Europe were very complex, with several introductions from the New World that were combined with direct exchanges between European and other Mediterranean countries. Currently, *P. vulgaris* has pantropical distribution, with a distinct genetic diversity for Europe, Africa and Asia.

The lack of homogeneity of the habitat determines the unevenness in the distribution of genetic diversity within species (Vavilov, 1987). There are many examples of differences in the variability distribution of specific traits, especially the resistance to diseases and stress. What matters is knowing the extent of diversity and the distribution of specific alleles

(Marshall et al., 1975; Maquet et al., 1999). Accessions introduced centuries ago have adapted to our latitudes and are thus regarded as Bulgarian. In the process of adaptation of the introduced forms to specific latitudes and altitudes, it is possible that spontaneous mutations, allowing better adaptability to the new environment of development, are retained. The presence of a vast genetic diversity adequately reflects the environmental conditions and guidelines for breeding.

1.1.4 Induced mutagenesis

Induced mutagenesis is a powerful source of genetic diversity. Mutational techniques lead to the improvement of important economic characters in *P. vulgaris* varieties useful in breeding. The induction of mutations for increasing the genetic basis or variability is not a new approach in agriculture, but it could become revolutionary if modern biological techniques are used, such as development of DNA-based molecular markers to map and/or clone these mutations (Fofana, 1995; Angioi et al., 2010). This could not only help molecular characterization of mutations, but also anable the marker-assisted selection (MAS). For many years and many efforts to increase natural genetic resources and to improve cultivated forms, breeding lines have been established in the working collection by applying these methods (Poryazov, 1990; Sofkova & Yankova, 2008; Tomlekova, 2010).

1.1.5 Genetically modified organisms

Genetic engineering employs techniques of targeted transfer of genes encoding valuable economic characters, among which the most frequent are resistance to pathogens and pests. Transformed genotypes are means of solving economically important breeding issues and a tool to increase the genetic diversity of a species. Molecular characterization and cloning of valuable genes is a source for the implementation of their transfer into other closely related or more taxonomically distant species. Based on studies, Shade (1994) and Ishimoto (1996) (as cited in Tomlekova, 1998) have conducted the transfer of a gene from *P. vulgaris* in *Pisum sativum* and *Vigna vulgaris*, which has a key role in determining resistance to the bruchid beetle *Callosobruchus maculatus*.

1.2 Levels of characterization of genetic diversity in *P. vulgaris* L.

Various data confirming variability can be collected at phenotypic level of the plant, as well as at various protein and nucleotide levels (electrophoresis of storage proteins and/or enzymes, total DNA restriction profile, chloroplast DNA and/or mitochondrial DNA, DNA sequencing) (Singh, 1991; Fofana et al., 2001; Tomlekova et al., 2009). Regarding the choice of marker used (morphological and/or molecular), there are ongoing disputes (Hills, 1987; Moritz et al., 1990; Donoghue et al., 1992; CIAT Report Information, 1990b).

1.2.1 Morpho-physiological characters

Material from the world collection of *Phaseolus*, stored in CIAT, has been characterized in detail and its genetic diversity has been calculated (Frankel, 1987). There have been observed special characters that allow to identify a given accession introduced. The data encompassed within the collection (the so-called "passport data") include basic information on the origin and place of collecting the accessions. The characterisation employs gene

markers which are mostly monogenic or linked genes, such as for the type of germination and vegetative appearance. The characters must be easy to identify and should be stable to the impact of a great number of external factors. In this way they promote the classification and description of the forms (Leakey, 1988).

The assessment includes identification of characters that are important for the potential of species, i.e. those that can be introduced into breeding programs. Most often they are polygenic and are influenced by environmental variations. The most sensitive of all characters are the ones related to the yield and the yield itself, tolerance to stress factors, e.g. tolerance to drought and high temperature stress (Nikolova et al., 2003; Krasteva et al., 2004; Sofkova & Petkova, 2007; Petkova et al., 2010), and resistance to pests and diseases (Poryazov, 1990; Yankova et al., 2011). To standardize the different characteristics and assessments, the International Plant Genetic Resources Institute (IPGRI) has published classifiers for many species (van Slotten, 1987). They can be improved according to the needs of a particular study (van Schoonhoven et al., 1987, as cited in Zoro, 1999). The observations must be coded and collected in a database in a way so as to facilitate their use in statistical analyses (Vanderborght, 1988). To study the structure of genetic diversity of *Phaseolus*, different statistical methods have been used (Singh et al., 1991a). The analysis of the major components allows to differentiate the accessions of *P. vulgaris* into two groups, Mesoamerican and Andean (Singh et al., 1991a), because they have different morpho-physiological characters. Singh et al. (1991b) grouped them into six races: three Mesoamerican races (Durango, Jalisco, Mesoamerica), and three Andean races (Chile, Nueva Granada, Peru). A prevalence of the Andean type (76%) was first detected by Gepts & Bliss (1988), and was then confirmed by Lioi (1989) in an analysis of a large collection from Italy, Greece and Cyprus (66%), by Logozzo et al. (2007) from abroad European collections (76%), and by others, from Portuguese and Spanish samples. Some researchers such as Adams et al. (1988) in Malavi, Castineiras et al. (1991) in Cuba and Gil de Rona (1992) in Spain have used statistical methods to estimate the variability and to explore the genetic structure of their national collections in order to give direction to their future breeding programs. The use of morpho-physiological characters is not without inconvenience. They respond to the environment and are often correlative, which reduces the number of discriminant characters. Moreover, morphologically similar varieties may differ at molecular level. Dominance in morphological characters of wild forms is a bottleneck in the search for polymorphism in a population. To know the exact genotype of an individual, proceeding from a certain morphological character, is difficult because it often depends on many genes that may have pleiotropic effects, of epistasis and/or can be influenced by the surrounding environment. These shortcomings have forced scientists to look for other markers to better characterize the genotype studied. It is assumed that the similarity or difference at the phenotypic level reflects similarity and difference at the genetic level. Protein and nucleic acid markers do not depend or rely less on the environment and the former often are codominant. According to Mayr (1992, as cited in Maquet, 1995), using only the morphological characters is helpless in cases such as "twin-species" (two different species with similar morphological traits) or in cases of multiple distinct intraspecies variants. The identification of accessions requires knowledge of the genetic basis of morphological diversity.

1.2.2 Variability of seed storage proteins

The most frequently used organ for conducting biochemical research is the seed as it is a well-defined stage in the plant life cycle and has a high concentration of proteins (Konarev,

1993). In bean seeds, globulins account for the largest proportion followed by albumins, and the lowest are glutelins. Electrophoresis on polyacrylamide gel (SDS/PAGE) in the presence of SDS clearly distinguishes between Lima bean and common bean (Manen et al., 1981; Baudoin et al., 1991). *P. lunatus* is indisputably different from *P. vulgaris, P. coccineus* and *P. acutifolius* on account of the absence of phaseolin established by immunoelectrophoresis (Kloz, 1962, 1971, as cited in Maquet, 1995) and by SDS-PAGE (Derbyshire et al., 1976, as cited in Maquet, 1995). In the seeds of *P. vulgaris* the globulin fraction predominates, with phaseolin being the most abundant. The albumin fraction is the second largest, including lectins and other albumins (Gottschalk et al., 1983). Two types of electrophoretic analysis are employed in beans. One is based on total proteins as a criterion for grouping, which is used with *P. polyanthus*, and the other on individual proteins like phaseolin and arcelin in *P. vulgaris.*

Our working collection includes later generations, breeding lines, developed, highly resistant to *Acanthoscelides obtectus* – a bruchid beetle that is widespread in the latitudes of Bulgaria (Yankova et al., 2004; Yankova, 2010; Sofkova & Yankova, 2011). The study of storage proteins is of interest in the fight against this pest, as the substance that has a major role in the mechanism of resistance – a lectin-like substance, located in the area of phytohemaglutinins, corresponds to arselin, which determines the resistance to *Callosobruchus maculatus* and *Zabrotes subfasciatus* and can be isolated from proteinograms (Dobie et al., 1990; Tomlekova, 1998).

The vicilin-like globulin, called phaseolin, isolated from bean seeds, is well studied. It includes a group of related proteins whose genetic control has already been characterized (Chrispeels, 1978; Brown et al., 1981; Hall et al., 1977; Ma et al., 1978). In the sites of origin of *P. vulgaris*, the 'S' type has been found to prevail for the Mesoamerican (M) gene pool, initially described in the variety 'Sanilac', presented in cultivated and wild genotypes and 'M' phaseolin type in wild forms, where 'I' and 'J' types are also found (Gepts et al., 1986a; Koenig et al., 1989b). In the Andean (A) gene pool, 'T' phaseolin dominates, first described in the variety 'Tendergreen' (Gepts et al., 1986a; Gepts et al., 1986b), but 'H', 'C' and 'CH' types in wild and cultivated forms are also found (Koenig et al., 1989b). In the Mediterranean region (Gepts et al., 1988; Lioi, 1989) there are reports on 'T', 'S', 'C' and 'B' types, the latter being characteristic of wild and cultivated accessions. Along with the phaseolin variants such as 'Sb' and 'Sd', identified by two-dimensional electrophoresis (2D-SDS/PAGE/IEF), genetically similar to type 'S' (Gepts et al., 1986a); 'M_1', 'M_2', 'M_3', 'M_4', 'M_5', 'M_6' (Romero-Andreas et al., 1985); 'Ca', 'Nu', 'Ko' close to phaseolin 'T' (Tohme et al., 1995) and other variants representing its heterogeneity, so far, in total there are about 30 different known types of phaseolin in the cultivated representatives of *P. vulgaris*, while in the wild ones they are about twice as many (Debouck, personal communication). Research in molecular biology on phaseolin complements its characterization (Hall et al., 1977; Slightom et al., 1983; Talbot et al., 1984; Lawrence et al., 1990; Kami et al., 1995). The subsequencing analysis has shown that this division into two groups is at the level of nucleic acids, at which 'S' and 'T' types can be distinguished by a single polymorphism using Southern hybridization (Talbot et al., 1984; Nodari et al., 1992). Phaseolin DNA sequencing reveals that the gene phaseolin subfamilies are two, α and β, and that they differ by the presence or absence of a short direct repeat of the phaseolin stretch of DNA, consisting of 27 bp (base pairs); α and β are similar to each other in the 'S' and 'T' phaseolin types. They only differ in the α gene type by an additional repeat, made up of 15 bp, which

is found in 'T' phaseolin and is absent in 'S' (Slightom et al., 1985). They are responsible for the synthesis of protein complexes observed in the SDS-PAGE phoregrams. The presence of these repeats in combination with the post-translational glycosylation is responsible for the different 'T' phaseolin profile of gels in one-dimensional SDS-PAGE (Lioi et al., 1984; Sturm et al., 1987). In addition, the sequential analysis of the phaseolin type, characterizing the large groups according to the centres of origin of *P. vulgaris*, may enable the tracking of the potential sites for amino acid substitutions to enrich the methionine content of phaseolin (Gepts et al., 1984; Ma et al., 1978). By modifying phaseolin it is possible to achieve variability and because its proportion is significant among the total protein content of seeds, in the long run this approach would lead to overcoming the nutritional deficit present in the family *Leguminosae*. The amino acid content of protein determines its fraction composition. The nutritional value of beans depends on it. In general, dicarbonic amino acids bean varieties are present in the largest quantities, while the lowest are the sulfur-containing. The proportions of aspartic and glutamic acids, arginine, lysine, leucine and threonine are significant. A slight deficit of valine and isoleucine has been established in some bean species. The deficiency of methionine and cysteine is significant, their content being relatively higher in *P. lunatus* than in other representatives of the genus *Phaseolus* (Stoyanova, 1989). The phaseolin marker is most often used to characterize wild or cultivated populations by detecting new phaseolin types. Thus the identification of the phaseolin type is used as a geographic and evolutionary marker for studying variability, grouping of the germplasm of the type and studying the geographical distribution of groups. The phaseolin marker is also used to search for phaseolin recombinants whose existence could demonstrate combining the high productivity of the 'S' type with, for example, disease resistance or other characteristic of the 'T' type (Gepts et al., 1986b; Tomlekova et al., 2001; Genchev, 2011). The biochemical characterization of the accessions and above all the identification of their phaseolin type complete the overall characteristics of the breeding materials and the collections in the genetic banks. The domestication of the common bean was conducted in separate areas where accessions with small or large seeds prevail. The differentiation of the centres of origin of the species in seed size does not provide distinct differences, i.e. it does not always serve as a discriminant character. In contrast, the genetically determined biochemical character - phaseolin type of seeds is a reliable distinguishing factor.

1.2.3 Polymorphism of enzymes

Enzymes used as another type of protein markers, are characterized by catalytic power and specificity. Enzyme electrophoresis uses both the protein nature and specificity of enzymes. Contrary to storage proteins, enzymes should not be denatured. The ubiquity of enzymes and their role in metabolism is the reason for analysis of many tissues (cotyledons, roots, leaves, pollen, etc.). Isoenzymes are convenient markers in the study of plant taxonomy (Crawford, 1990; Murphy et al., 1990), evolution of cultivated plants (Doebley, 1990), genetics of populations (May, 1992) and conservation of plant genetic resources (Schaal et al., 1991; Gepts, 1995). The presence of genetic diversity is the basis of plant breeding. The description is also required for proper upbuilding the genebanks, for performing efficient selection of genotypes maintained in them, building a 'core collection'. Detailed studies have been conducted in predominantly autogamous populations of different species (Loveless et al., 1984). The first studies of *P. lunatus* were conducted with seven enzyme

systems of 20 populations originating from the central valley of Costa Rica by Maquet et al. (1996). At the level of populations, the studies with enzyme markers in this species were conducted by Zoro et al. (2003). The genetic diversity of the following species has been studied: *Arenaria uniflora* by Wyatt et al. (1992); *Bromus tectorum* by Novak et al. (1991); *Ceratophyllum demersum* and *C. echinatum* by Les (1991); *Datura stramonium* (Motten et al., 1992); *Eichhomia paniculata* by Barrett & Shore (1990); *Phaseolus acutifolius* by Schinkel & Gepts (1989); Koenig & Gepts (1989); *P. lunatus* by Wall et al. (1975, as cited in Brown, 1978); Baudoin (1991); Zoro (1994, 1997, 1998, 1999, 2003); *P. vulgaris* by Koenig & Gepts (1989); *Setaria glauca* by Wang et al. (1995b); *Sorghum bicolor* by Ollitrault, (1987); *Sphodromantis viridis* by Wang et al. (1995a).

The absence of natural areas in the distribution the species *Phaseolus vulgaris* and the transition from traditional to intensive agriculture in recent decades have contributed to the significant loss of genetic diversity. The threat of genetic erosion in common bean has become ever more real. Any analysis conducted to assess genetic diversity can help to solve the problems of species selection (Chase et al., 1991; Nienhuis et al., 1995; Forfana et al., 1997; Maquet et al., 1997).

Mendel's genetic basis of isoenzymes is a source for their numerous applications. The easy extraction of these proteins facilitates the research of a huge number of species, and the possibility of defining the homo- and heterozygote ones among them makes them irreplaceable markers in crop genetics. By using isozymes, intra- and inter- crop variability can be characterized and the degree of similarity among accessions can be evaluated (Hamrick et al., 1991; Schaal et al., 1991a).

In systematics, an enzymatic analysis defines the taxonomic relations in one group (generally the different species in a genus) by taking into account the percentage of common alleles (Crawford, 1990). West & Garber (1967) and Bassiri & Adams (1978) proved the existence of a close similarity between *P. vulgaris* and *P. coccineus* as well as the possibility to develop hybrids of these two species. Despite their cross-ability, there is a problem with rejecting the genome that originates from the male gametophyte (Wall, 1968; Wall & Wall, 1975; Guo et al., 1989). By using isozymes, a breeding program can be performed for hybrids that are close to common beans but in addition possess a part of the *P. coccineus* genome.

The greatest advantage of enzyme electrophoresis is the possibility to associate the observed phenotype (the profile of the bands on the gel) to a genetic basis. Proper analysis of the gels depends not only on the quality of enzyme expression but also on the genetic determination of the enzyme system. The aim is to identify how these different isozymes translate the genome variability. The differences derive from: the expression products of many alleles of one and the same gene with a single locus; the expression products of many genes located on equally many loci; the different molecules resulting from the changes in the conformation of one and the same protein molecule; the molecules synthesized by one gene or a group of genes, which have undergone various posttranslational modifications (Ryder et al., 1980, as cited in Maquet, 1995).

Morphological markers allow to classify wild and cultivated plant species, but when it is necessary to classify individuals of the same species, for example from different populations (as is the case of the below presented study), descriptive capacity of morphological markers is quite limited (Roux, 1987). The Mendelian genetic basis of the enzymes determines their

multiple applications. Their easy extraction allows a large number of individuals to be studied. The possibility to detect homo- and heterozigotes make isozymes essential markers in the genetics of populations, as the dominance of morphological characters impedes their characterization. Isozymes are useful in the characterization of intra- and interpopulation variability and the degree of similarity (Hamrick, 1990; Schaal et al., 1991; Sozinov, 1995). Through them the populations can be grouped to follow the dispersal and colonization of one species, to calculate their dynamics (Barrett et al., 1990). Isozymes markers enable the study of population variability depending on the surrounding environment (conservation *in situ*) or according to the conditions of multiplication and regeneration of the collections (conservation *ex situ*) (Konarev, 1998a, 1998b). By following the dynamics, it is possible to clarify the factors responsible for the maintenance and loss of polymorphism within populations. In taxonomy, especially for species in a genus, enzyme analysis determines the taxonomic relationships in a taxonomic group, based on the percentage of common alleles (Crawford, 1990). Lefort-Buson et al. (1985), Roux (1987) and Murphy et al. (1990) summarize the different uses of isozymes in breeding. The enzymatic analysis has allowed the identification of certain species of the *Phaseolus-Vigna* complex to be improved (West et al., 1967a; Bassiri et al., 1978a; Jaaska & Jaaska, 1988), and the study of the species *P. coccineus* (West & Gabet, 1967b; Bassiri et al., 1978; Pinero et al., 1988). Despite the possibility of a crossover between these two species, there is rejection of the genome of the male gametophyte (Wall, 1968; Wall et al., 1975; Guo et al., 1989). Isozymes allow that the program for selection of generations, closely related to common beans and possessing a part of the *P. coccineus* genome, not to be eliminated too quickly.

The number of enzymes that can be tested is about 100 of the 2000 that are known, which is a small number compared with the overall genome of the plant. Very often only 10-30 enzymes have good electrophoretic separation (Zoro, 1994; Zoro, 1999).

All this is indicative of the difficulties encountered in the introduction of new enzyme systems in a particular species. Enzyme electrophoresis does not fully establish variability. Of all substitutions at the gene level, only about 30% result in an amino-acid substitution with a change of the net charge of protein (Pasteur et al., 1987). Many triplets encode one and the same amino acid. The substitution of one amino acid by another with the same charge would not normally have any effect on the net charge of protein. Therefore, identical electrophoretic mobility does not necessarily mean the same DNA sequence. In all cases, however, the quantity of unmarked variability would be proportional to the amount of the marked one (Crawford, 1990). For a given species, the detection of rare alleles would not change significantly the frequency of common alleles and genes that were monomorphic and will probably remain such.

Biochemical and morphological data have supported the existence of two gene pools in *Phaseolus vulgaris* species. In recent years, combinations of phaseolins and various morphological characters have been intensively used to analyze the structure of the European common bean and the presence of representatives of both gene pools. A prevalence of the Andean type (76%) in the European collections was first detected by Gepts & Bliss (1988), and was then confirmed by Lioi (1989) in an analysis of a large collection from Italy, Greece and Cyprus (66%). It was also confirmed in an analysis of a large collection from Portuguese and Spanish samples by Rodiño et al. (2001), Ocampo (2002), and Rodiño et al. (2003); and in representative European collections by Logozzo et al. (2007).

In some self-pollinating plants, genetic variability can be too low to characterize a species. The number of polymorphic loci and the number of alleles per locus are limiting in the case of restricted gene flow. The low level of polymorphism in these autogamous species makes these markers inadequate to identify the genetic structure of the species. The search for allozymes to be used as molecular markers makes it possible to trace the variability between individuals within the populations and between the populations, irrespective of environmental factors. The fact that in other genotypes or related species of *P. vulgaris* there are allozyme variants of the enzymes described is a reason for conducting this study. This evaluation would allow to better understand the factors responsible for the availability or loss of polymorphism. In connection with the expected results, we cannot but mention the disadvantages of isozymes as markers of genetic diversity. In cases of restricted gene flow in some autogamous plants, genetic variability may be too low to characterize a particular species. The absence of polymorphism in self-pollinating species can render these markers inadequate to identify the genetic structure of the populations studied. To avoid difficulties associated with the use of biochemical and morphological markers, researchers have turned to DNA markers in order to obtain a direct evaluation of the genetic variability.

Koenig & Gepts (1989a) suggest the use of DNA-markers to characterize the genetic diversity of common beans as a consequence of the low variability assessed using isozymes. Enzyme electrophoresis does not fully establish variability. Only about 30% of the substitutions at the gene level result in an amino acid substitution with a change in the net charge of protein (Pasteur et al., 1987). Many triplets encode one and the same amino acid. The substitution of one amino acid by another with the same charge would normally have no effect on the net charge of protein. Therefore, the identical electrophoretic mobility does not necessarily mean the presence of the same DNA sequence. In all cases, however, the quantity of unmarked variability would be proportional to the amount of the marked one (Crawford, 1990). Considering above all the high cost of consumables and equipment needed to perform DNA analyses for a large-size population and the fact that so far in Bulgaria enzyme systems have not been applied in studying the variability of *P. vulgaris* they were used for the purpose of the present study.

1.2.4 DNA molecular markers

The direct use of DNA ensures accuracy and speed of the techniques due to their specificity, while there is no requirement to wait for the manifestation of a property at a certain age, and also is not affected by the surrounding conditions. With the development of new techniques for DNA testing, the study of the genome is achieved in its entirety. Techniques are not intended to detect characters and to draw conclusions on the activity of genes. They aim to isolate DNA from samples that are representative of the studied populations. Therefore, they consist in determining the differences in the compositions of the DNA samples analyzed that are to serve as markers for variability. They reveal the genetic diversity at the level of genetic information. Once DNA has beens isolated, it can be analyzed either by fragmentation by enzymes, involving restriction fragment length polymorphism (RFLP - Restriction Fragment Length Polymorphism), or by amplification (PCR-based techniques) and DNA sequencing. For example, in studies using *Phaseolus*, based on the use of Randomly Amplified Polymorphic DNA - RAPD (Nienhuis et al., 1995; Fofana et al., 1997), Inter-Simple Sequence Repeats - ISSR, Amplified Fragment Length Polymorphism - AFLP (Svetleva et al., 2006), diversity is calculated not only according to

geographical origin and botanical status, but also depending on the cultivated group. Based on the organization of genetic diversity observed in *P. lunatus*, Fofana et al. (1997) offer a hypothesis on the process of domestication of this species.

A summary of molecular marker capabilities indicates that the molecular markers have various applications and utilities, being indicated for different objectives and/or populations. It is advisable that markers that have low cost are applied properly, i.e. in fingerprinting and/or variability studies, suggestions point favourably to microsatellites and AFLP, or RFLP when synteny information is available (Park et al., 2000; Svetleva et al., 2003; Torres et al., 2004).

Depending on species and related breeding problems for solving, additional techniques may be used, such as DNA microarrays, single nucleotide polymorphisms (SNPs) and ELISA. General analytical approaches such as MAS, tagging, genotyping for germplasm surveys, heterosis groups, cytogenetic stocks, pedigree analysis, gene bank inventory/collections are useful for gaining further information about the characters of interest. The development of such data will provide support for utilization of more powerful techniques for physical mapping such as bacterial artificial chromosomes (BACs), tranformation-competent artificial chromosomes (TACs) and gene isolation (Working material, 2001).

International organizations such as the International Plant Genetic Resources Institute (IPGRI), el Centro Internacional de Agricultura Tropical (CIAT) and the International Institute of Tropical Agriculture (IITA) have undertaken numerous studies to establish and maintain gene banks, compiling catalogues and formulae of database, based on up-date and dissemination of information for the genetic resources, input and information analysis, curation database through data collection. To increase the efficiency of utilization of plant genetic resources in improving the existing and developing new varieties, the study of genetic diversity has an important role, and so does the proper organization of the crop collection (Konarev, 1998a). An important stage in the process of formation of the collection is the identification and registration of the accessions and the molecular markers are very helpful. As noted, for the identification and registration of genetic diversity DNA and protein markers are used. For a number of reasons (co-dominant nature of inheritance, and also inexpensive and simple techniques, well reproducible results), the latter are employed with priority in studies of *P. vulgaris*.

Part A

A study of genetic diversity of *P. vulgaris* L. by total storage proteins

The aim of the present study was to investigate the *P. vulgaris* genetic diversity in the Bulgarian collection through identification of phaseolin types, with a view to assist gene bank management and plant breeding.

2. Materials and methods

In this study, 409 common bean genotypes were investigated. Among them 235 local accessions from the gene bank of the Institute of Plant Genetic Resources in Sadovo, collected by 21 expeditions since 1977, were included. The accessions were sampled from 69 locations (Fig. 1) in order to cover the whole territory of the country and to be representative of the

geographical and ecological areas of Bulgaria. Another set of genotypes consisted of 127 breeding lines and varieties resulting from breeding programmes and available in the working collections of the Maritza Vegetable Crop Research Institute, Plovdiv, and of the Dobroudza Agricultural Institute, General Toshevo. The remaining 47 recently introduced accessions during the last ten years came from different gene banks outside the country; such accessions are integrated as parental genotypes in hybridization programmes with local accessions and breeding lines and varieties from the Maritsa and Dobroudza Institutes working collections. In order to compare the electrophoretic profiles of the Bulgarian accessions, controls ('witnesses') for the phaseolin types 'S', 'T', 'Ca' and 'C', were obtained from CIAT, Cali, Colombia, recognized as the worldwide repository bank of *Phaseolus* genus.

2.1 Basic method - 1D-SDS/PAGE

Identification of phaseolin type was performed in 10 single ripe seeds from randomly selected plants of each accession by one-dimensional SDS/PAGE (Koenig et al., 1970). Two protein molecular weight markers, purchased in 1998 from Merck, MMW–1 – Standard mixture IV (Art. No.15791) and MMW-2 - Standard mixture VIII (Art. No.11536), were used for phaseolin zone determination on the proteinograms. Protein types were identified thanks to referential accessions: ICA Pijao - for 'S' type phaseolin, CDRK - for 'T' type, G2362 - for 'Ca' type, and G21194 - for 'C' type.

2.2 Assessment the intensity of protein fragments

For the phaseolin identification of variants with close electrophoretic phenotypes, a separation to 1D-SDS/PAGE proteinograms was performed. Proteinograms were photographed with a gel documenting system using the Ultra Violet Products Gel Documentation System, Version 7.00 Science Park, Milton Road, Cambridge CB4 4FH, England. Consecutively Gel Work-1D-Advanced, Version 3.01 (http://www.statsoft.com) software product was applied. The intensity of each fraction of the phaseolin zone on the proteinograms obtained from different accessions was measured and compared. Intensity measuring is a method developed by the authors and has not been published yet. This was also useful to distinguish the phaseolin types 'T' and 'Ca'.

The calculations for each fraction of the proteinograms were made including:

- Rf relative mobility of the molecules (distance from start to band relative to length of phoregram)
- RV (Raw Volume – intensity expressing in relative units protein levels)
- The molecular weight was measured by the method of Weber & Osborne (1967, in Acquaah, 1997)
- Factor (Kn) to equalize protein levels for each proteinogram (calculated by the RV of a band with the same intensity at individual starts (reference) and their overall RV)
- Construction of graphs for comparing the intensity of phaseolin fractions.

2.3 Mathematical procedures

To be grouped in clusters data from the proteinogram analysis, the following procedure was applied: the Euclidean distances between genotypes were calculated using the data from the analysis of storage proteins with the formula:

$$E(X,Y) = ((X_1 - Y_1)^2 + (X_2 - Y_2)^2 + \ldots (X_K - Y_K)^2)^{0.5}$$

The matrix was formed from the distances and the distances thus determined were classified according to proximity. Statistica, Version 6.0. software was applied. Each genotype was involved in the assessment of the intraspecies diversity through a representative profile (> 90%) in an analysis of 10 single seeds.

To calculate the diagrams representing the proportion of accessions from the Andean and Mesoamerican gene source, in each climate zone the standard procedures by using Microsoft Excel Help were implemented.

3. Results and discussion

The accessions available in our collections were found in four different climatic zones (Fig. 1). The most appropriate climatic zone, i.e. the Highlands zone, was represented by the lowest number of accessions in Bulgaria. This can be explained by a different adaptation of common bean accessions (only domesticated) in a secondary diversity centre, compared with the adaptation of accessions (both wild and domesticated) occurring in the primary diversity centre. The breeding "pressure" (both natural and anthropic) is different between Bulgaria and the neotropical regions. More details on the geographical distribution of these localities are given in Tomlekova et al. (1999).

3.1 Phaseolin types

The screening of 409 common bean local accessions, breeding lines and varieties from the Bulgarian collection allowed us to identify four phaseolin types: 'S', 'C', 'Ca' and 'T'. Phaseolin 'S' corresponds to the Mesoamerican gene pool while 'C', 'Ca' and 'T' types correspond to the Andean gene pool (Fig. 1).

The proteinograms of the total seed storage proteins include the fractions corresponding to the phaseolin types, which identification was described in Tomlekova (1999) and Tomlekova et al. (2009). No difference in phaseolin type was observed from single ripe seeds belonging to each Bulgarian accession except few cases within the breeding lines and varieties. The different phaseolin types identified in plants from the group of the breeding lines and varieties corresponded to the same types of phaseolin observed in the parents. The phaseolin fractions cover all types assessed in the national collection. Phaseolin fractions on the proteinograms are characterized by molecular weights between 45 000 D and 51 000 D (Brown et al., 1981; Osborn, 1988).

In Fig. 2 and Fig. 3, the three groups of Bulgarian common beans (local accessions; breeding lines and varieties; and introductions) are compared according to phaseolin types. The percentage of the phaseolin types is differently distributed in each one of the groups. 'T' type is prevalent in local accessions while 'Ca' type prevails over the 'C' and 'T' in breeding lines and varieties. The 'C' type proportion is low, especially for the breeding lines and varieties from the Andean gene pool (7%), while in the group of local forms, it represents 25%. 'S' phaseolin type covers 40% and 43%, respectively of the collection made of local accessions on one part, and of breeding lines and varieties, on the other part. The remaining material belongs to the Andean gene pool. The accessions from the A predominate in both local accessions (60%) and breeding lines and varieties (58%). 'T' type predominates in local

forms from the Andean gene pool, while 'Ca' type is the most frequent among breeding lines and varieties from the same gene pool. The difference in the predominating phaseolin types is obvious and the difference of distribution frequencies of all the phaseolin types in the two groups (local accessions, and breeding lines and varieties), reflects the preferences of farmers and breeders for genotypes bringing specific characters. The 'T' type is associated with the white seed coat colour that is preferred by the Bulgarian consumers. The introduced accessions of common bean are characterized with their high proportion of 'S' type. The common bean introductions had a stronger restricting effect on the genetic variability: the meso-American group was presented from an only 'S' phaseolin type. A greater diversity of the accessions from the Andean gene pool, in the introduced collection was established 'T', 'C' and 'Ca', however it was presented mostly from phaseolin 'T' type.

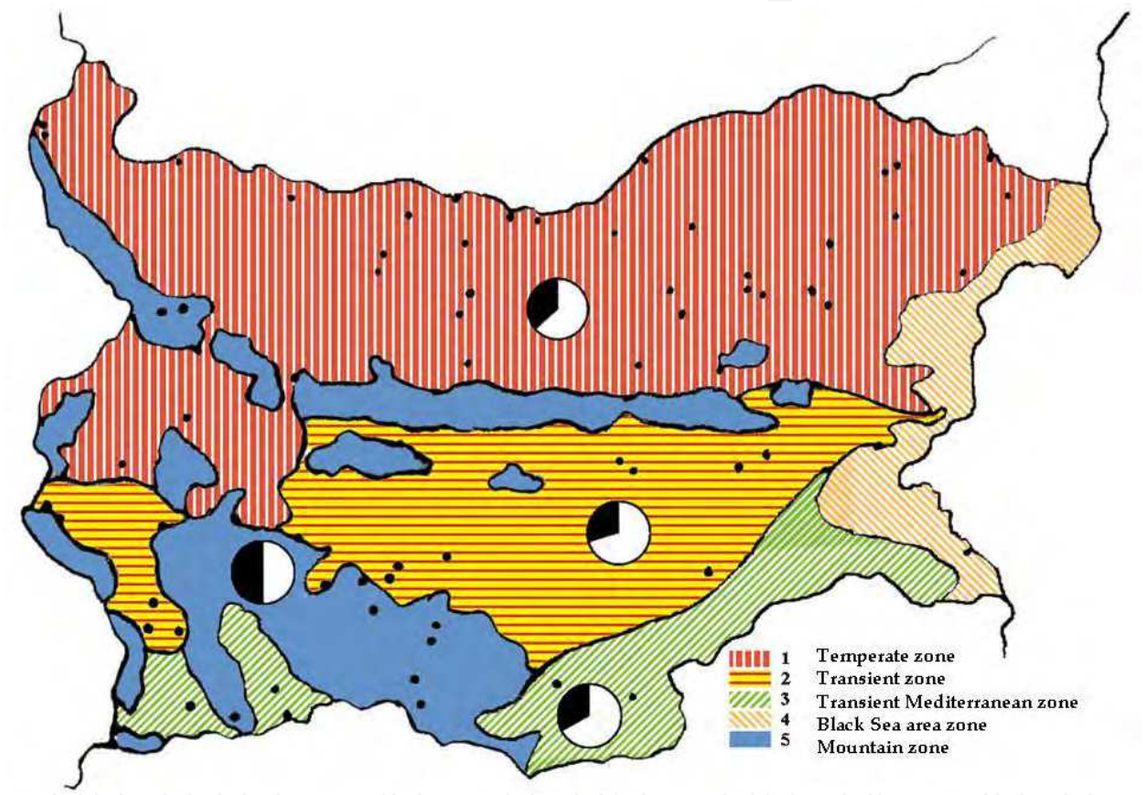

Fig. 1. Map of Bulgaria with marked climatic zones and localities of collection of *P. vulgaris* accessions and diagrams of the share allocation of the phaseolin types (Andean and Mesoamerican groups, white and black colour, respectively).
* The points mark the locations of collecting sample in the territory of Bulgaria.

Classification of the accessions in two basic groups, corresponding to the established both gene pools - Andean (from the region of the Andy) and meso-American (from middle-America) was made on the basis of phaseolin type identification from the electrophoregrams of storage proteins of the dry seeds from common bean accessions (Fig. 2). Data from our study showed that 60% of the Bulgarian accessions came from the Andean gene pool (A) while 40% came from the Mesoamerican one (M). According to a preliminary study (Tomlekova, 1999), the domination of the Andean representatives over

the Mesoamerican ones was expected. However, the results of this study conducted approximately 15 years later later showed a relatively fair proportion of accessions from the Mesoamerican group, which can be explained by the recent introduction of accessions characterized predominantly by 'S' phaseolin type (66%). Among the accessions introduced mostly during the last 10-15 years into our working collections, the Andean phaseolin types represented a lower proportion. Presence of 'S' phaseolin is frequently associated with resistances to important diseases which explain their introduction in the country for breeding purpose and hybridization with local materials (Koenig et al., 1989b). The breeding efforts towards desease and pest resistance to overcome this major problems in crop species (i.e., in common bean), have altered the ration between A and M groups.

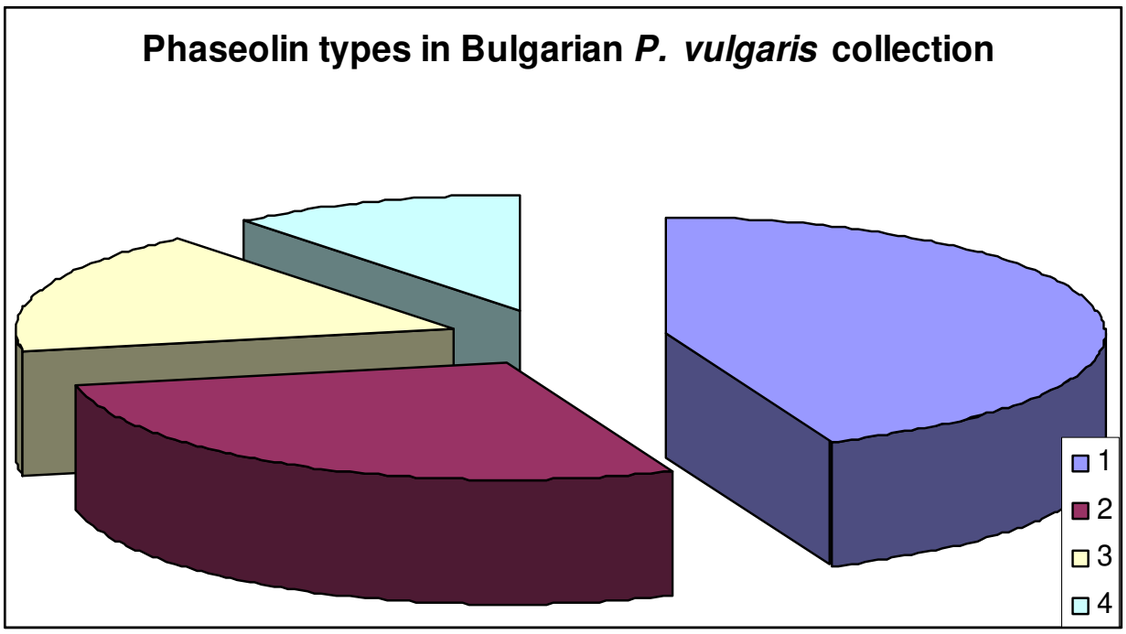

Fig. 2. Proportion of phaseolin types in Bulgarian *P. vulgaris* collection;
* 1- 'S', 2- 'T', 3- 'Ca', 4- 'C'

On the basis of phaseolin determination, results showed a low variability level in the analyzed Bulgarian accessions. The latter represent a very limited genetic diversity, by comparison with the total variability displayed among the material distributed in the primary centre of common bean, i.e. the New World. The low level of genetic diversity is a result of the significant "founder effect", a term introduced by Ladizinsky (1985), in the secondary domestication centres of *P. vulgaris*, such as Bulgaria. Common bean was introduced more than 300 years ago in the country and this food legume was converted as a traditional crop. At the beginning, the level of genetic variability was low due to the limited number of introduced accessions. This reduction of genetic diversity was reinforced because of the lack of adaptation in some materials to the new ecological conditions. Additional factors might have strongly restricted the genetic variability of Bulgarian accessions, such as a specific consumer preference (for large and white seeds), occurrence of diseases (*Bean Common Mosaic Virus*, *Xanthomonas campestris* pv. phaseoli (Common

Bacterial Blight), *Pseudomonas syringae* pv. phaseolicola (Halo Blight) and insects, such as *Acanthoscelides obtectus* (Bean weevil) and the breeding systems of the species (autogamous floral biology).

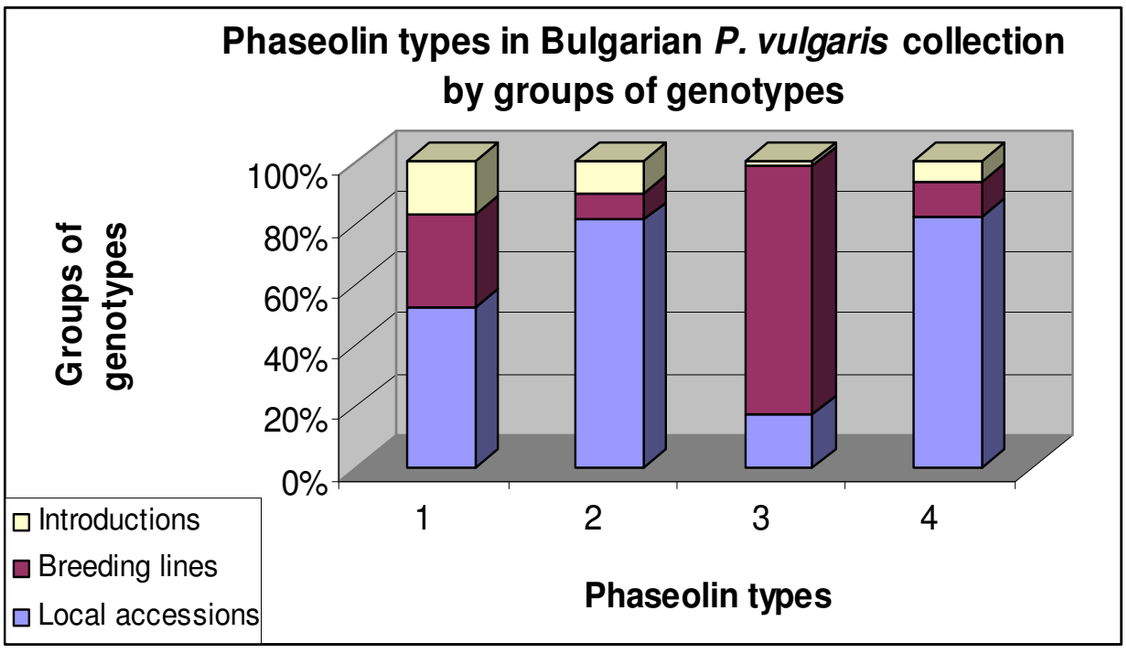

Fig. 3. Proportion of phaseolin types established in Bulgarian *P. vulgaris* collection by groups of genotypes; * 1- 'S', 2- 'T', 3- 'Ca', 4- 'C'

In this context, it might also be relevant to consider the domestication phases in *P. vulgaris*. First, there was a decrease in genetic diversity, but accompanied by an increase in morphological variability (Gradinarov (1939), Vishnevsky (1940), Rachinsky (1968), Ganeva (1983), Stoilova (1998), as cited by Genchev, 2007). This restricting effect was, on the basis of our own results, stronger among the Mesoamerican group represented by one phaseolin type, compared with the Andean group represented by three different phaseolin types. This confirms the data published by Gepts (1990) for the primary domestication centre of the species that can also be used as a plausible explanation for the larger genetic diversity of the Andean accessions in Bulgaria. In a second phase, there was a reduction in genetic diversity explained by the linkage occurring between the phaseolin locus and other loci, influencing the phenotype and submitting the genotype to breeding "pressure" (Maquet, 1995).

3.2 Patterns of storage proteins

To facilitate the analyses, the fractions of the total protein patterns were grouped into zones (Fig. 4):

Zone A; ~ 200 kD of MMW**.** This zone includes the slowest moving fractions of the phoregrams which have different Rf and RV (Fig. 4). Three variants were observed: Ax - in accessions having 'S' phaseolin (Fig. 4c, i); Ay - in accessions with 'T' phaseolin (Fig. 4a, b, d,

e, f, g, h, j, m; Az – rarely found in some breeding lines (Fig. 4l, m) (not clearly visible in the figure because of low intensity).

Zone B; MMW from ~ 114 to ~ 130 kD. Two variants were observed: A 2 options: Bx - in accessions having 'S' type phaseolin (Fig. 4c, i, k, l, m; By - in accessions with 'T', 'Ca' and 'C' type (Fig. 4a, b, d, e, f, g, h, j, n, o). There is a well-defined correlation, without exception, between Bx profile and 'S', and between and By 'T', 'Ca' and 'C' phaseolin type. Its visualization depends to the greatest extent on the proper application of the method. (The more intense stain in the sample in Fig. 4e, in zone B, marked with an arrow, is an artefact.) Bx and By fractions have the same Rf and differing RV, i.e. the differences in both variants originate from their different expression expressed by the intensity of staining of the bands. The intense staining of the area shows its relatively high share of the accessions having 'S' phaseolin.

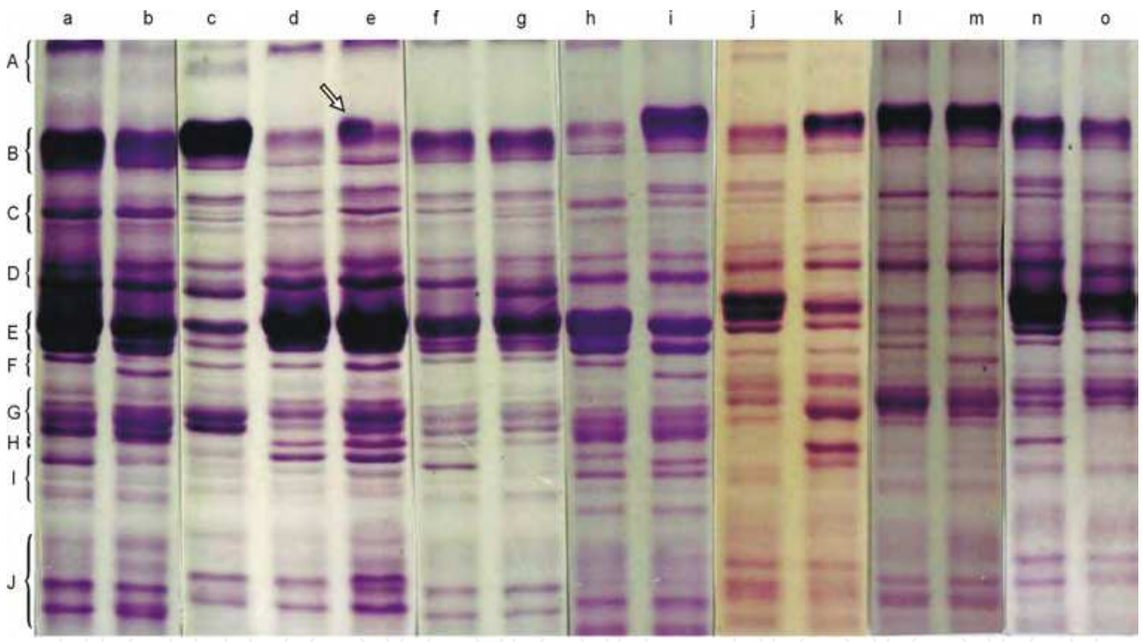

Fig. 4. Proteinograms of *P. vulgaris* accessions in the Bulgarian collection: a) predominant profile of the variety 'Oreol' and b) a rare profile of the variety 'Oreol'; c) 'Vulkan'; d) predominant profile of the variety 'Thrakiiski' and e) a rare profile of variety 'Thrakiiski'; f) predominant profile of line 'Mastilen 11b'and g) a rare profile of line 'Mastilen 11b'; h) accession E6091; i) line 213-17-1; j) line 536; k) line 538; l) line 563-6; m) line 563-5; n) line 564-3; o) line 5642. * As the phoregrams does not represent a stereotype, differences of the order of millimeters are quite permissible.

The difference in the significantly higher intensity of the Bx area in the accessions with 'S' type is obvious and can be used to distinguish them from those with 'T', 'Ca' and 'C' type (By). This area can help identify the type of phaseolin.

Zone C; MMW ~ 75 - ~ 97 kD. In Rf, RV and lower band number 5 variants were distinguished. When comparing genotypes with the reference accessions, no relation with the phaseolin type was found. For example, Fig. 4c, k, l, m (variety 'Vulcan', and lines 538-S, 563-6-S, 563-5-S) shows the configuration of zone C, typical of the 'S' phaseolin type. The profile of C zone is the same in the variety 'Mastilen 11b' (the rare profile) that is with 'Ca' phaseolin (Fig. 4g), and in E6091 (Fig. 4h) with 'C' phaseolin. The characteristic profile of zone C is not associated to the phaseolin type.

Zone D; MMW from ~ 60 to ~ 70 kD. It was observed in two variants: Dx and Dy vary in intensity of the last two bands in it. In types 'S' and 'C' (the fastest line is intense) and Dx variant (Fig. 4c, g, o); in 'T' and 'Ca' variant Dy is presented, where the most intense is the penultimate band (Fig. 4a, b, d, e, f, h, i, j, k, l, m, n). Furthermore, the Dx and Dy configurations cannot be associated with a phaseolin type or centres of origin.

Zone E; Phaseolin zone; MMW - 45-51 kD. The variants (phaseolin types) identified in the studied material are four: 'S' (Fig. 4c, i, k, l, m), 'T' (Fig. 4j), 'Ca' (Fig. 4a, b, g, h, n, o), 'C' (Fig. 4d, e). (The designation used was introduced in studies of species in centres of origin.)

Area F; MMW - 41-42 kD. Immediately after the phaseolin zone two bands were observed. Their different intensity divides the material into two groups. They migrate close one after another and the accessions are present in the following two variants: One of the fractions is much more intense than the other and in some accessions it is the one that is more high-molecular. In others the fraction with lower molecular weight has a stronger expression. Variation in this zone is independent of the phaseolin type as well as of other bands.

Zone G; Zone of lectins (phytohemagglutinins); MMW - ~ 33,5 - ~ 41 kD. Variation is expressed by differences in Rf and RV of the fractions. In most cases it is associated with characteristic electrophoretic phenotype of the zone, which depends on phaseolin. This correlation is strictly observed in the accessions from the local germplasm. But in the breeding lines the phaseolin type and lectins combine more independently. There are cases of such a profile of zone G, which are not observed in the used witnesses. They cannot be associated with phaseolin. As can be seen in Fig. 8k, l, m, o, these configurations are present in the accessions with 'S' and 'Ca' phaseolin. It is characteristic of some of the groups of lines numbers: 556, 564, 566 and all studied groups of lines 561, 563, bred for resistance to bacterial diseases. The gene families that encode phaseolin and phytohemagglutinin are not linked (Brown et al., 1981). But in the cultivated forms there are characteristic profiles of the fractions of the lectin group which are associated with a particular profile of phaseolin (Lioi, 1991).

Zone H; MMW ~ 23 - 24 kD. It usually consists of one line, which varies in Rf in different accessions. The cases where the band is the least mobile were denoted with variant H1 (Fig. 4a, b, c), with H3 where it is the fastest moving (Fig. 4d, e, f, g, i, j), H2 with medium mobility is shown in Fig. 4h, l, m, n, o. In one and the same phoregram two versions are rarely seen: H12 and H23, in which there are two bands simultaneously.

Zone I; MMW - ~ 25 - ~ 28 kD. The first band from this zone in some accessions has strong intensity (Fig. 4a, d, e, f, h, i, k, n), while in others the intensity is weak (Fig. 4b, c, g, j, l, m, o). The intensity of this band is not related to the phaseolin type or to other band of the phoregram. Beside the described variants that are found in the reference accessions,

phoregrams are observed that are different from them, such as breeding line 538 (Fig. 4k) in zones G and I.

Zone J; MMW - 21,5 - 14 kD. It is composed of a great number of bands. Three different configurations of the bands Jx, Jy and Jz were observed. They differ in the absence (in Jx) or presence of one band with a different Rf value in accessions with Jy and Jz variants. Most often, the Jx profile is associated with 'S' phaseolin (Fig. 4b). In the breeding materials, Jx was observed in accessions containing 'C' as well as 'T' and 'Ca' phaseolin (Fig. 4a, d, f, g, k, n). For the accessions of Andean origin the Jy profile is typical (Fig. 4b, h, i, j, k, l, m). Although less frequent, it is found also in 'S'. The third Jz profile was observed in a small number of breeding forms possessing different phaseolin types (Fig. 4e).

In the variation of zones C, F, H, J, intrapopulation polymorphism was observed (variety 'Oreol': a predominant profile (Fig. 4a) and a rare profile (Fig. 4b); variety 'Trakiiski': predominant profile (Fig. 4d) and rare profile (Fig. 4e); breeding line 'Mastilen 11b': predominant profile (Fig. 4f) and rare profile (Fig. 4g).

The examples shown in Fig. 4 cover all different configurations of zones established. In the phoregrams of the accessions a much large number of combinations of the zones described are found.

Grouping of accessions

The screening of the electrophoretically fractionated total SDS-PAGE profile of storage proteins enabled to established polymorphism in the accessions and to group them. In 409 *P. vulgaris* accessions analyzed 116 groups were defined. This means that with regard to the researched spectrum of storage proteins, a third of the proteinograms differed from each other. Data from the identification of the phaseolin type of the accessions was the first grouping criterion and from the analysis of the overall profile of their storage proteins was the second grouping criterion (Table. 1). In column 2 are accessions indicated in the graphs of the clustering, presented in the same section (Fig. 5). (The choice of accessions in participating in the graphs is random within all presenting the same profile. The names and numbers of the accessions, varieties and lines in the dendrogram are abbreviated, while in Table 1, column 2 they are written in full). These accessions represent groups comprising different numbers of accessions specified in column 3. In each of these groups the accessions have completely identical phoregrams, i.e. they do not differ among themselves by any of the fractions of their storage proteins. Between the groups with the same phaseolin type there is diversity between other fractions of the proteinograms. The variability in these fractions does not correlate with any other character (or more precisely, there is no evidence of research of such dependency in the available literature and from the authors' observations). This means that in the grouping in this study, an independent (neutral) character is used - a marker of diversity, which is important when developing a core collection in a genetic bank. It can be used in so-called hierarching of the material is therefore important for the pre-breeding work. The polymorphism described in the analysis of proteinograms according to zones can serve as a basis for future research, which will look for correlation between the electrophoretic phenotype of the bands and characters that are target for the breeding of common beans.

No	Accession presented in dendrogram	Accessions with identical proteinograms to the representative one
Groups of accessions with 'T' phaseolin type divided by identical profiles:		
1	Ruets	E1426, 90E126, 90E298, 90E596-1, 90E596-7, 90E596-8, 90E596-9, 90E596-10, 3E96008, 3E96009, 3E96018, 90E297-4, 90E297-5, CDRK (witness)
2	3E96017	
3	190A-3	4E96068
4	3E96039	90E060, 91E048, 91E050, 91E053
5	E6779	E6823, 90E192, 190a-5, 83E1-200-1, 83E1-200-2, 83E1-220-1, 83E1-220-2, 83E1-220-3, 83E1-220-4, 83E1-220-5, 83E1-220-10
6	90E169	90E283
7	91E137	91E153, 3E96010, 3E96011
8	Hitovo 1	4E96078, 4E96086
9	Ilinden	Dunav-1, 90E139, 3E96027
10	89E085	E6192, 90E128, 90E129, 91E297, 3E96013, 3E96021, 3E96022, 3E96025, 3E96029, 4E9692
11	90E688	
12	566-1-1-1-2-1-3-2(7)	
13	566-1-1-1-1-1-1-1(1)	566-1-1-2-1-2-3-3(2), 566-1-1-1-4-2-1-1(4), 566-1-1-1-2-1-3-2(3)
14	566-1-1-1-4-2-2-1(5)	
15	566- 1-1-1-4-2-2-1(6)	
16	BP-1	
17	90E191	
18	83E229	90E303, 91E055, 91E209, 91E212, 91E282, 84-34-11
19	536-1-1-1-3	83E120, 90E222, 90E248, 90E250, 91E060, 4E96072
20	559-2-1-4-1-3-2-1(6)	
Groups of accessions with 'Ca' phaseolin type divided by identical profiles:		
21	Fiesta	
22	551-1-1-1-5-1-2(126)	90E116, 85-20-14, 551-1-1-1-5-1-3(127)
23	522-1-2-2-1	564-2-5-2-1-4-2-1(2)
24	Zornitsa	
25	Oreol	Perun
26	521-1-1-1-2	
27	529-1-1-1-1	
28	551-1-1-1-4-1-1(123)	551-1-1-1-4-1-1(124)
29	612	E7585, 90E125, 90E312, 92E050, 92E056, 91E299, 3E96014, 3E96015, Trudovets
30	534-1-1-1-3	
31	550-1-4-2-3-3-1-1(141)	
32	Padesh 1	E6087, 89E502, 4E96070, 559-2-1-1-2-3-2-1(2), 559-2-1-1-2-3-2-1(4), 559-2-1-4-1-3-2-1(5)
33	90E673	
34	E6122	90E679-1, 90E679-2, 90E679-3, 90E679-4, 90E679-5(X-5)
35	554-1-5-1-1-2-3-3(131)	
36	90E602	90E713, 91E171, 91E220, 559-2-1-4-1-3-2-1, 559-2-1-1-1-1-2-3-2, 559-2-1-1-2-3-2-1, 559-2-1-4-1-3-2-1-1, 559-2-1-4-1-3-2-1-2, 559-2-1-4-1-3-2-1-3, Cher Starozagorski

37	Zarya	
38	559-2-1-1-1-1-2-3-2(7)	
39	559-2-1-1-1-1-2-3-1(3)	
40	525-1-3-1-1	
41	558-2-2-1-3-2-5-1	559-1-1-1-1-1-1-3(1)
42	556-1-3-3-5-1-3-2(11)	Pokrovnik 7, 556-1-1-1-2-1-1-1(12)
43	556-1-1-1-2-1-1-1-2(5)	556-1-1-1-2-1-1-1-2(24), 556-1-1-1-5-2-2(25)
44	564-2-5-2-5-3-3-2(3)	564-2-5-2-5-3-3-1(4)
45	564-2-5-2-1-4-2-1(9)	
46	3E96016	564-2-5-1-3-3-1-1(1), 564-2-5-1-3-3-1-3(7)
47	Mastilen 11б	90E092-1-1-4, 90E092-4-4-1, 90E092-4-4-2
48	Veritsa	
49	564-2-5-2-5-3-3-1(5)	564-2-5-2-5-3-3-3(10), 564-2-5-1-3-3-1-2(11)
50	564-2-3-2-2-5-2-1(6)	564-2-3-2-2-5-2-2(8)
51	216-73-1	216-77-1
52	554-1-5-1-4-2-3-2(129)	85-20-60
53	554-1-5-1-4-2-1-1(128)	
54	554-2-2-1-3-1-3-1(132)	
55	Hotovo-2	
56	614	608
57	90E255-2	90E255-4, 90E255-8, 90E255-10
58	91E289	4E96074
59	93E007	90E609, 91E139, 91E205, 4E96091, 91E307
60	93E009	
61	89E185	
Groups of accessions with 'C' phaseolin type divided by identical profiles:		
62	Radomir 2	E5780, 91E054, 91E278, 91E280, 91E285, 91E316, 93E003, 93E011, 3E96032, 3E96034, 3E96035, 4E96082, 4E96083, 4E96084, 4E96085, 4E96089, Samuranovo
63	213-42	213-43
64	95E002	
65	Nikos	
66	4E96081	
67	Trakiiski	
68	E6195	
69	E6091	
70	Contender (witness)	
71	4E96079	
72	555-1-2-6-2-2-4-2	
73	620	
Groups of accessions with 'S' phaseolin type divided by identical profiles:		
74	194-1	194-2
75	556-1-3-3-4-3-2-1(19)	556-1-3-3-4-5-2-3(20), 556-1-3-1-4-5-2-2(21)
76	563-1-1-3-5-3-3-2(6)	
77	563-1-2-2-1-2-1-3(3)	
78	556-1-3-1-3-2-1-3(16)	

79	556-1-3-1-5-4-1-1(28)	
80	561-1-3-3-4-4-3-3(1)	561-1-3-3-1-2-3-2(2)
81	563-1-1-1-2-1-1-3(2)	563-1-2-2-1-2-1-3(4), 563-1-2-2-1-3-3-3(5), 563-1-2-1-4-4-2-1(7), 563-1-2-1-4-4-3-1(8), 563-1-2-3-2-1-2-1(9)
82	190A-4	
83	556-1-1-1-5-5-1-1 (26)	556-1-3-2-3-2-2-1(27)
84	556-1-3-1-5-4-1-3(14)	556-1-3-1-3-2-1-3(15)
85	556-1-3-3-4-5-2-1(13)	
86	556-1-3-1-5-4-1-1(1)	556-1-3-1-3-2-1-3(2), 556-1-3-3-4-5-2-1(3), 556-1-3-3-1-2-2-1(4), 556-1-3-3-4-5-2-1(6), 556-1-3-1-5-4-1-3(7)
87	556-1-3-4-2-2-1-1(22)	
88	550-1-4-3-1-3-2-1(143)	
89	550-1-4-2-3-3-1-3(142)	
90	550-1-4-1-4-3-1-1(32)	
91	563-1-2-1-4-1-3-1(1)	
92	556-1-3-2-3-2-2-1(17)	
93	Tarnovo 13	
94	556-1-1-1-2-1-1-1(18)	
95	556-1-1-1-5-5-1-2(8)	556-1-3-1-4-5-2-3(9), 556-1-3-1-5-4-1-1(10)
96	556-1-3-3-1-2-2-1(23)	
97	92E053	92E055
98	90E198	190A-1, 190A-2, 190A-6,
99	Prisad	90E243, 90E247, 90E597, 90E604, 90E617, 90E635, 90E687, 90E274, 92E054, 3E96019, Dobrudzhanski 5, Rusenski ran, Dobrudzhanski 7, Plovdiv 164, Planinets
100	Ludogorie	
101	Prelom	90E231, 90E240, 90E270, 90E277, 90E282, 90E288, 90E296-1, 90E296-2, 90E296-3, 90E296-4, 90E305, Garmen, Ruse-17
102	Balgari	90E187, 90E644-1, 90E644-2, 90E644-3, 91E314, 3E96038, 82-11-16
103	Abritus	
104	538-3-3-1-4	
105	Vulkan	E6083, 90E100, 90E138, 90E245, 90E715, 3E96020, Bistrenski, ICA Pijao (witness)
106	Dunavets	E6085, E7380, E7381, 90E228, 90E286, 90E301, 90E309, 90E319, 90E390, 91E281, 91E288, 95E003, 93E008, 4E96075, 4E96076, Samokov-3, E6787, Kristal-137, Ruse-2, 82-7-11-12
107	4E96071	
108	91E292	89E120, 89E405, 89E504, 90E251, 90E257, 93E005, 4E96067, 4E96080, 4E96087
109	Desislava	
110	91E304	
111	89E129	
112	91E044	90E107, 90E659, 3E96040, 4E96088
113	213-17-1	213-27-1
114	95E001	83E118, 89E116, 89E560, 91E072, 91E129, 3E96036, 4E96069, 4E96073

*№ - serial number of the group

Table 1. Groups of proteinograms of total storage proteins in Bulgarian *P. vulgaris* accessions

For proteinograms of the various accessions a hierarchical structure was observed that is illustrated with a dendrogram (Fig. 5). The representatives of the 114 groups listed (Table 5, column 2) are given in the overall dendrogram, composed of all accessions tested. The grouping carried out on the basis of this analysis provides mathematical explanation of the degree of similarity and difference. In the dendrogram the accessions are at different distances from one another depending on the number of differing fractions.

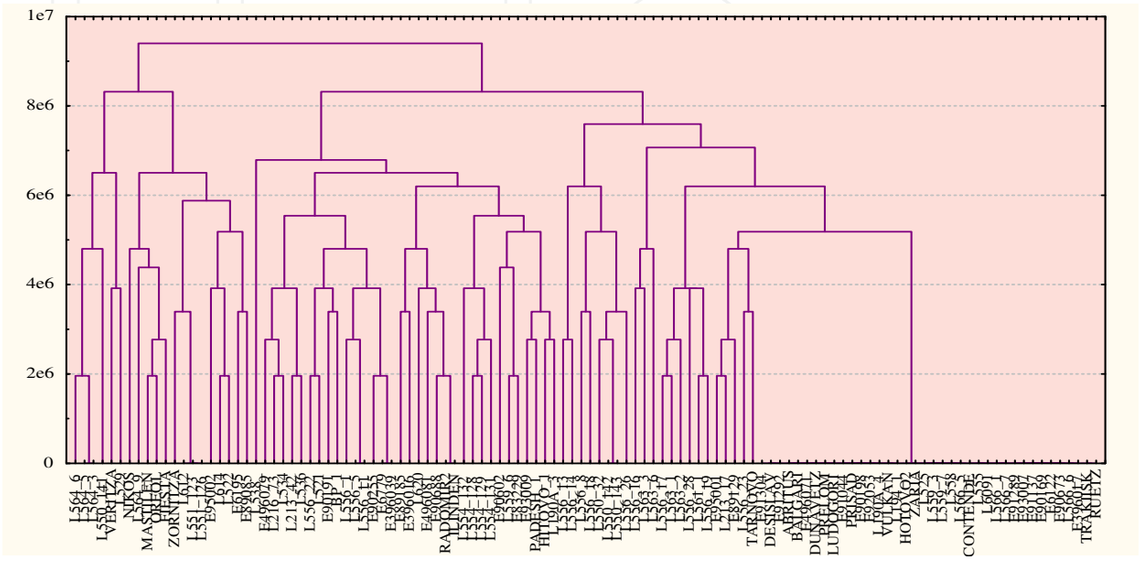

Fig. 5. Graph of cluster analysis on data from phoregrams of *P. vulgaris* L. accessions from the Bulgarian collection

According to the genetic potential they have, proteinograms can be grouped into two main clusters (Fig. 6).

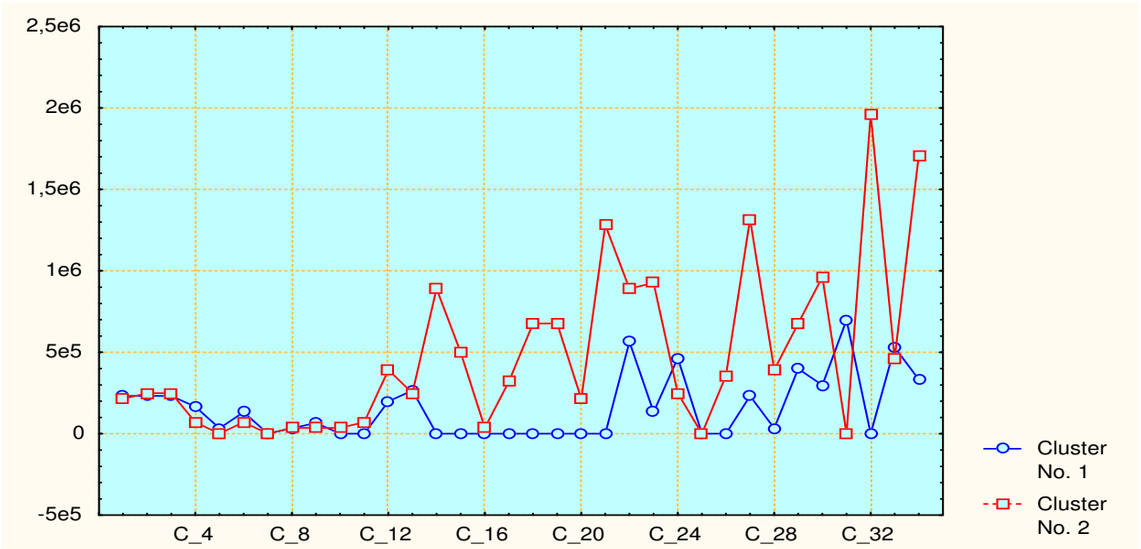

Fig. 6. Clusters in *P. vulgaris* L. accessions in the Bulgarian collection based on data from analysis of phoregrams of storage proteins

The graphs in Fig. 5 and 6 give an idea of the distance between clusters. In each basic cluster, there is distribution in subgroups. In each group, they have similar properties, but in the subgroups the accessions are united in strict differentiation. The presence of clusters means that there is a reason for grouping the accessions (objects), which is assumed to be genetically determined. Otherwise, the distribution of the objects would be totally chaotic, which means that the hierarchical structure, observed in Fig. 5 and Fig. 6, would be entirely absent. If grouping is done on the basis of data only on the phaseolin type and the zones correlating with it, it should be expected that the only cluster is composed of accessions containing 'S' type, and the other one of 'T', 'Ca' and 'C ' type, as their distribution according origin centre is. This dependence is followed, though not strictly, in the native accessions in which typical configurations of the zones corresponding to phaseolin type are observed. In most of the breeding material the dependence is not obeyed. In the cluster composed mostly of accessions with 'S' type, which attributes them to the Mesoamerican origin centre, there are included some breeding forms containing phaseolin typical of the Andean origin centre. The opposite case is also observed. The lack of genetic link between zones A, C, E, G, J and the phaseolin type explains the peculiarities in the grouping in two clusters.

The presence of clustering can be interpreted as a direct indication of the general behavior of the studied storage proteins, i.e. each of the analyzed fraction is in some relation with the other, which is the reason for the more detailed study of the experimental material. That is why within the four groups of accessions, differentiated by phaseolin type, grouping was carried out according to the fractions of the total pattern of storage proteins.

Grouping of material simultaneously according to both attributes facilitates the selection of accessions to participate in hybridizations, as well as the selection of accessions to be maintained in genebanks, avoiding duplication and reducing the amount of breeding work, costs and time. Inside the cluster, the accessions have a similar genetic potential and origin. Using accessions from one cluster in a possible breeding, differences will occur in terms of the investigated character, but they will not be statistically significant. Therefore, out of each group the most typical representative is selected, e.g. the most widely used and well-established variety.

Farmer's preferences and consumer requirements were the two reasons explaining the occurrence of a specific seed character. During the 20th century, breeding efforts concentrated on market varieties which were important to the region. Hybridizations were made between closely related genotypes resulting in homogeneous breeding lines. Gene pools and groups within them can be recognized in cultivated common bean on the basis of the protein marker. Our investigation confirmed that phaseolin, a useful indicator of genetic origin, has been a breeding-neutral character during the process of introduction and adaptation of bean accessions in Bulgaria.

4. Conclusions

Four phaseolin types are distinguished in the investigated material from the Bulgarian common bean collection: 'S', 'T', 'Ca' and 'C'. 'T' phaseolin type is the most predominant among the group of the local Bulgarian accessions, which represents 66% from the Andean gene pool. 'Ca' type is the most frequent in the breeding lines and varieties, covering 79%

from the Andean gene pool. The introductions have 'S' phaseolin from the Mesoamerican group; and the Andean one is presented mostly from phaseolin 'T' within 'T', 'C' and 'Ca' types.

The analyses carried out allow the formation of groups and clusters, because of variability in the germplasm. Divided according to phaseolin types and according to the other fractions from the phoregrams of the storage proteins, the accessions are grouped in different number of clusters: in type 'T' the clusters are 3; in type 'Ca' the clusters are 4; in type 'C' - 4, in type 'S' - 3. The accessions in different clusters have the same potential of the studied character, therefore the breeding work should include one representative of each cluster. It is advisable that the genebanks maintain one representative from each group that participates in the clustering.

Part B. A study of genetic diversity of *P. vulgaris* L. by isoenzyme markers

The aim of this study was to establish a methodology for characterizing the genetic diversity of large common bean collection by analysis of enzyme systems. Another aim was to analyse the genetic aspects of allozyme variation in genotypes cultivated in the Plovdiv agricultural region of Bulgaria, which has appropriate conditions for growing green bean (French bean) including the linkage relationships for certain loci to other species.

5. Material and methods

5.1 Plant material and sampling

The studied common bean *P. vulgaris* genotypes - 7 varieties ('Fiesta', 'Oreol', 'Perun', 'Veritsa', 'Starozagorski cher', 'Trakiiski', 'Nikos'), 3 accessions (Acc.6091, Acc.6787, Acc.82201206) and 4 breeding lines (BL) (BL620, BL614, BL612, BL608), were grown for green bean in the experimental field of Maritsa Vegetable Crops Research Institute, Plovdiv. Of the above genotypes, an average of 50 cotyledons of 4 to 6-day-old germinated mature seeds were analyzed in order to identify polymorphic loci at species level and between individual plants of the selected genotypes. The breeding lines were obtained by crosses: 608 ='Oreol' x 82201206; 612 ='Fiesta' x 6091; 614 ='Fiesta' x 6787; 620 ='Perun' x 6091.

5.2 Enzyme assays

For electrophoretic variation, 31 readable and reproducible enzyme loci resolved from 15 enzyme systems assayed by electrophoresis, were analyzed: aconitate hydratase (ACOH, E.C. 4.2.1.3. lyase, alcohol dehydrogenase (ADH, E.C. 1.1.1.1 oxidoreductase), cytosol aminopeptidase (CAP, E.C. 3.4.11.1), dihydrolipoamide dehydrogenase (DDH, E.C. 1.8.1.4), a non specific enzyme endopeptidase (END, E.C. 3.4.-.-, hydrolase), colorimetric and fluorimetric esterases (cEST and fEST, E.C. 3.1.1.-, hydrolases), non specific, glucose-6-phosphate isomerase (GPI, E.C. 5.3.1.9, isomerase, glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49, oxidoreductase), glutamate dehydrogenase (GDH, E.C. 1.4.1.2, oxidoreductase), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42, oxydoreductase), malate dehydrogenase (MDH, E.C. 1.1.1.37, oxidoreductase), malate dehydrogenase (NADP+) (MDHP, E.C. 1.4.1.40, oxidoreductase), phosphoglucomutase (PGM, E.C. 5.4.2.2, isomerase), shikimate dehydrogenase (SKDH, E.C. 1.1.1.25, oxidoreductase), and superoxide dismutase (SOD, E.C. 1.15.1.1, oxidoreductase. The choice of enzyme systems for analysis was dictated

by published data (Zoro et al., 1999, 2003) about high variability in different plant species that can serve as genetic markers for polymorphism, as well as for identification of genotypes. Loci were labeled sequentially, with those migrating closest to the anodal end designated as number 1. In the procedure of manifestation of the enzyme activity a 'witness' was placed in the initial and final positions of each gel. The 'witness' was one of the analyzed accessions used as a referent one, so that differences were standardized and laboratory errors were eliminated. The allozyme from this genotype was designated 100, and all other allozymes were assessed according to their relative migration distance.

The extraction of the material was conducted according to the procedure introduced by Maquet (1995) to study the *P. lunatus* species. The extraction buffer, consisting of potassium phosphate (Sigma-Aldrich # P-2222) with pH 7.0, containing 3% of sucrose (Sigma-Aldrich # S-8501), 5% PVP-40, 0.1 M KH_2PO_4, 0.05% Triton X-100 (Sigma-Aldrich # T-8532) added *ex tempore* and 14 mM 2-mercaptoethanol (Sigma-Aldrich # M-6250) also added *ex tempore*, allows the retaining of whole catalytic activity of the enzyme and inhibition of the interfering substances such as phenol, toxins and phenoloxidases. The ratio between the cotyledonic plant tissue and the extraction buffer was 1:2. Stored at -20 ^{o}C, the extract remained active for one year.

The solid carrier for the horizontal electrophoresis was 10% starch (Sigma-Aldrich # S-4501) gel, prepared by a procedure described by Wendel & Weeden (1990) and Maquet et al. (1993), and optimized by Zoro (1994).

The electrophoretic separation, procedure of loading the gel, migration of the fragments was performed in three different electrode systems, presented in the Results part, Table 1 according to the procedures previously described:

i. 0.065 M histidine-0.019 M citric acid (H-C), pH 6.1 (Kazan et al., 1993) prepared in a ratio of 1:10 mixture of solution buffer system H-C:H_2O.
ii. 0.030 M lithium-0.19 M borate (Li-Bo/T-C), pH 8.1/0.05 M Tris-0.008 M citrate, pH 8.4 (Murphy et al., 1990) prepared in a ratio of 1:9 mixture of solution buffer system Li-Bo/T-C:H_2O.
iii. 0.040 M citric acid-0.068 M morpholine (M-C), pH 6.1 (Wendel & Weeden, 1989) prepared in a ratio of 1:20 mixture of solution buffer system M-C:H_2O.

The chemicals were purchased by Sigma-Aldrich: L-histidine (# H-8000) and citric acid (# C-7129), boric acid (# B-0252) and lithium hydroxide (# L-4256), Tris (# T-1503) and citric acid (# T-8657).

Preferably, the organ is always separated at the same physiological stage for maximum avoidance of any possible modifications of enzymes. The organ used is young, since the enzymatic activity is at its peak. The preparation of gels, electrophoresis buffers, the loading of samples and the conditions for separation were developed by Bushuk et al. (1978), Bassiri et al. (1978), Aebersold et al. (1987), Pasteur et al. (1987), Stuber et al. (1988), Charlionet (1990), Hames (1990), Murphy et al. (1990), Wendel & Weeden (1990), Acquat (1993). To make an enzyme visible, the gel is immersed in a liquid reaction medium with a specific substrate for the enzyme system studied and various chemical compounds whose activity is needed (a coenzyme, ion, etc.). The staining recipes are provided in the work of Shaw et al. (1970), Harris et al. (1977), Trouslot et al. (1980), Gottlieb (1981, 1982), Soltis et al. (1983), Hussain et al. (1988), Guo et al. (1989), Weeden (1990), Gabriel et al. (1992), Hussain (1988), Waugh et al. (1992).

Different technical parameters for carrying out electrophoresis were tested: currency voltage and duration of fraction movement in the electric field. Of all voltage and amperage values tested for fraction movement, a constant voltage of 4-10 $V.cm^{-1}$ and duration of electrophoresis 5-17 h were accepted (Table 2).

6. Results and discussion

6.1 Introduction of the methods for enzyme assays

As a result of the systems tested, in the migration of the protein extract the best separation by the optimal buffers and conditions for electrophoresis of the enzyme systems studied in cotyledon tissues of germinated seeds in genotypes from the Bulgarian collection of *P. vulgaris* are shown in Tables 1 and 2.

	Electrophoresis buffer system		
Enzyme	Histidine-Acid Citric	Litium-Borate/Tris-Citrate	Morpholin-Acid Citric
ACOH		**	-
ADH	*	***	-
CAP	***		
DDH	***	***	*
END		***	
F., C. EST		**	
GDH		***	
G6PDH		-	**
GPI	***		
IDH	***		
MDH	***		**
MDHP		*	***
PGM	***	***	***
SKDH		-	***
SOD		**	

Table 1. Electrophoresis buffer systems of studied enzyme systems
Notes: *** - perfect resolution (optimal conditions), ** - very good resolution, * - good resolution, 0 – no resolution (conditions for resolution are not created).

The buffer systems employed in the assays were: continuous histidine-citrate (pH 6.1) for ADH, CAP, DDH, GPI, IDH, MDH, PGM, and PGDH, and discontinuous lithium-borate (pH 8.1)/Tris-citrate (pH 8.4) for ACOH, ADH, DDH, END, cEST, fEST, GDH, PGM, SKDH, and SOD. Morpholin-citrate (pH 6.1) and lithium-borate (pH 8.1) buffer systems were also tested and presented the best resolution for G6PDH, MDH, MDHP, PGM, SKDH. The techniques for gel electrophoresis and histochemical staining procedures are those reported elsewhere (Zoro Bi et al., 1999).

The combination of factors coupled for the selected buffers that allowed good resolution of the isoenzymes in cotyledon tissue of common bean are summarized in Table 2.

Parameters	Buffer system		
	Histidine-Acid Citric	Litium-Borate/Tris-Citrate	Morpholin-Acid Citric
Voltage (constant)	10 V.cm^{-1}	4 V.cm^{-1}	10 V.cm^{-1}
Electrophoresis duration	5 h	17 h	5 h

Table 2. Conditions for electrophoresis buffer system for separation of isozymes in cotyledons of *P. vulgaris*

6.2 Monomorphic enzymes

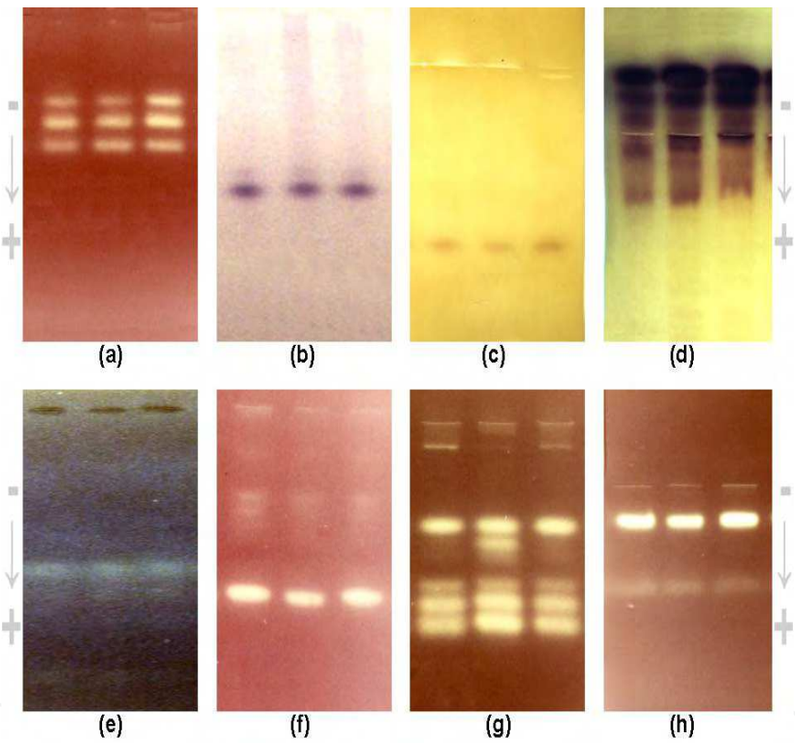

Fig. 1. Zymogrammes of the monomorphic enzyme systems in French bean genotypes grown in the region of Plovdiv:

a. Alcohol dehydrogenase (ADH). Profiles 1, 2, 3: ADH-1$^{100/100}$; ADH-2$^{100/100}$.
b. Cytosol aminopeptidase (CAP). Profiles 1, 2, 3: CAP$^{100/100}$.
c. Endopeptidase (END). Profiles 1, 2, 3: END$^{100/100}$.
d. Esterases - colorimetric (cEST). Profiles 1, 2, 3: cEST$^{100/100}$.
e. Esterases - fluorimetric (fEST). Profiles 1, 2, 3: fEST$^{100/100}$.
f. Isocitrate dehydrogenase (IDH). Profiles 1, 2, 3: IDH$^{100/100}$.
g. Malate dehydrogenase (MDH). Profiles 1 & 3: MDH-1$^{100/100}$; MDH-2$^{100/100}$; MDH-3$^{100/100}$. Profile 2: MDH-1$^{100/100}$; MDH-2$^{100/84}$; MDH-3$^{100/100}$.
h. Malate dehydrogenase (NADP+) (MDHP). Profiles 1, 2, 3: MDHP$^{100/100}$.

6.2.1 Alcohol dehydrogenase (ADH)

The ADH zymograms in Fig. 1a reveal three zones of activity. Each of the anode (ADH-$1^{100/100}$) and cathode (ADH-$2^{100/100}$) bands shows one allelic variant. The band in an intermediate position comigrates between ADH-1 and ADH-2. In the analyses this band was accepted as a product of inter-gene interaction. This is confirmed by the more intense colouration manifested by this band on the gels. A change in the electrophoresis conditions (sequential electrophoresis) can be expected to cause the intermediate band to be divided into two parts. After the analysis of the enzymograms, there were established two active loci encoding ADH, manifested in *P. vulgaris*. In the studied genotypes, a single allozyme phenotype was observed for both loci. Alcohol dehydrogenase in the studied accessions grown in the region of Plovdiv is a monomorphic isoenzyme. In neither case was polymorphism detected. Profiles in *P. vulgaris* were similar to those observed in *P. coccineus* (Wall & Wall, 1975), *P. lunatus* (Zoro, 1999), *Pennisetum glaucum* (Banuett-Bourrillon & Hague, 1979), *Camellia* (Wendel & Parks, 1984), *Ciser arietinum* (Tuwafe et al., 1988). According to literature data on the genus *Phaseolus*, the natural hybrids observed allow to assume the existence of a dimeric quaternary structure of the isozyme (Zoro, 1999). The results for these species showed that two loci, *Adh*-1 and *Adh*-2, encoded ADH. Each locus encoded codominant alleles which were dimeric and viable. The studies of *P. vulgaris* revealed monomorphism in the zone controlled by *Adh*-1 (polymorphism in *P. coccineus* and *P. lunatus*) and polymorphism in the zone of the *Adh*-2 products. Polymorphism of alcohol dehydrogenase was observed in wild and cultivated populations.

In many plant species where different allelic variants were identified, the distribution of genotypes was traced in their disintegration with generations. It has been established that in some species there is a genetic linkage between the *Adh* loci (Banuett-Bourillon & Hague, 1979; Brown, 1980), while in others they were not related (Freeling & Schwartz, 1973; Torres, 1974; Tanksley & Jones, 1981).

The identified phaseolin types of the accessions included in this study proved that they belong to different centres of origin of the species *P. vulgaris*. Andean and Mesoamerican representatives have different ADH alleles, which can be used to determine their affiliation to the respective centre of origin (Koenig & Gepts, 1989; Maquet et al., 1993). The absence of heterozygotes observed in the studied material could be due to various reasons: strong autogamy, crossbreedings aimed at homogamy, breeding favouring homozygous individuals. Since similar studies of Bulgarian material have not been performed until now, there is no possibility that breeding by enzyme systems was previously made. However, ADH may correlate with an economic character used in the selection of the studied forms, which has kept an allelic variant of the enzyme analyzed.

The genetics of alcohol dehydrogenase has been studied in a much greater number of higher plants than the genetics of other enzymes (Tanksley, 1983; Weeden, 1983). In the species *Zea mays, Triticum aestivum, Hordeum vulgare, Pennisetum typhoides, Solanum lycopersicum, Helianthus annuus, Stephanomeria exigua, Lupinus angustifolius* two *Adh* genes have been described. Under aerobic conditions, ADH isoenzymes can be found in seeds (embryo and/or endosperm) and pollen. In mature seeds, most frequently either of the two alcohol dehydrogenases prevails. The gene responsible for most of its activity in mature seeds is usually the same one that encodes ADH in pollen. Different *Adh* genes can be induced or not by anaerobiosis. In monocotyledonae species, all *Adh* genes are induced by

environmental stimuli. It has been established that in dicotyledonae species only one of the two genes can be activated by anaerobic stress. In maize, both genes are independent, but in other species they are closely related due to their origin by tandem duplications.

6.2.2 Cytosol aminopeptidase (CAP)

With electrophoretic separation the activity of cytosol aminopeptidase was demonstrated (Fig. 1b). Following interpretation of the zymograms obtained from the studied material, monomorphism was established for the enzyme CAP. There is a single band with the same mobility (Rf) of the gels in all investigated seeds. The zymograms are characterized by a single locus called *Cap*. Similar results for *P. lunatus* were found by Zoro (1994), who also established one monomorphic locus in the study of wild populations of this species. In later investigations of the polymorphism of *P. lunatus*, Maquet (1995, 1997) found four different alleles in the aminopeptidase cytosol locus. Some of the alleles are endemic for the accessions of the Andean gene pool and may serve to distinguish them from accessions belonging to the Mesoamerican gene pool. The natural hybrids observed in the genus *Phaseolus* allow to assume the existence of a dimeric quaternary structure of CAP isozyme (Maquet, 1995). The dimeric nature of this enzyme system is shown in *Pisum sativum* (Scandalios & Espiritu, 1969) and *Abies balsama* (Neale & Adams, 1981). In studying the species *P. acutifolius*, Schinkel & Gepts (1989) identified three isozymes of leucine aminopeptidase. Koenig & Gepts (1989) established two isozymes of the same enzyme system in *P. vulgaris*.

6.2.3 Endopeptidase (END)

The gels treated for manifestation of endopeptidase activity display a single band (Fig. 1c). The identified locus has migrated towards the anode. The identical electrophoretic phenotype shows that the species are homozygotic at the locus analyzed, which controls the expression of endopeptidase End^{100}/End^{100}. In the examined *P. vulgaris* crop one allele END^{100} with monomorphic pattern is detected. The researched natural hybrids of other types of the genus *Phaseolus* enable the evidence of a monomeric quarterly structure of the isozyme (Maquet, 1995). Maquet (1995) has detected four codominant alleles of the locus of END, which he relates to the different origin sources of the *Lima* bean accessions. Zoro (1999) examined a locus that possesses three codominant alleles that control the endopeptidases which represent monomeric proteins in wild types of *P. lunatus*. Similar results have also been achieved with soya (Griffin & Palmer, 1987).

The selected accessions are distinguished by morphologic and economic criteria, which vary according to the environmental conditions. The search for alozymes, aiming to use them as molecular markers, allows tracing the variability among species within a crop frame and among different crops, regardless of the environment factors. The fact that alozyme variants of endopeptidase are detected in other species close to *P. vulgaris* gives grounds for carrying out this research. This judgment allows better understanding the factors responsible for the loss of polymorphism. The breeding in these cultivated genotypes by certain agricultural characters has been done for many generations and this might have indirectly influenced the limitation of the alozyme variants of endopeptidase. An analysis of such a relation with the present work and available literature sources cannot be made, because the END locus does not feature in published gene maps of the species. On the other hand, the number of the

polymorphic loci as well as the number of the alleles for each locus is restricted in case of a narrowed gene flow. In some autogamous plants the genetic variability can be too low to characterize a certain pattern. The absence of polymorphism in self-pollinating species makes these markers inadequate to be able to define the genetic structure of the species. The detected lack of polymorphism in endopeptidase, as well as in the above discussed alcohol dehydrogenase and cytosol aminopeptidase enzyme systems, confirms their narrowed genetic base. These results in the chosen accessions do not exclude the chance of finding variability in the analysis of other isoenzyme systems. Literature references on endopeptidase are scarce. From the complex of the *Phaseolus* genus, which has five crop species, the endopeptidase enzyme system was analysed in *P. lunatus*, in which it can serve to establish the inter- and intra- polymorphism of crops (Maquet, 1995; Maquet et al., 1996; Maquet & Baudoin, 1997; Maquet et al., 1997; Zoro, 1999).

6.2.4 Esterases (EST)

Nonspecific esterases were manifested - colorimetric (cEST) (Fig. 1d) and fluorimetric (fEST) (Fig. 1e). Their use as markers, as is this case, does not require prior knowledge what type of esterases the analysis refers to. They are divided into two types according to the method for their determination. In the analyses conducted polymorphism was not established. In cEST, a double band migrating to the cathodic side of the gel is observed. This picture shows the second band, which was accepted to be a degradation product, i.e. one locus was determined whose activity in the studied genotypes is manifested through one allele ('100'). In the samples obtained at the witness, allele '84' was obtained, which was interpreted as "effect of positioning". A second zone of activity was expected, migrating to the anode and representing the activity of another locus. However, it was not well visualized on all gels and because the absence of good reproducibility it was not interpreted. In such analyses in *P. vulgaris* (Wall & Wall, 1975) as well as other species of the family *Leguminosae,* such as *P. coccineus* (Wall & Wall, 1975), *P. lunatus* (Maquet, 1995; Zoro, 1999), *Ciser arientum* (Kazan et al., 1993) two loci in two allelic variants of the second locus were identified, which produced two allozyme phenotypes. In the locus near the anode, heterozygous individuals were identified that showed the monomeric nature of the quaternary structure in *P. lunatus* (Lima bean) (Zoro, 1999).

Fluorimetric (fEST). Two zones of activity were observed, but only one was reproducible. The only one band on Fig. 1e) was considered as a product of one locus (f*Est*). One allele fEst^{100} was identified at this locus. In some cases, there appear slightly coloured bands located at the anode side. These bands were accepted as an artifact. The results thus interpreted showed the monomer nature of the quaternary structure of proteins in *P. vulgaris*. Fluorimetric esterases were not successfully established in all samples and were therefore excluded from the analyses for assessment of polymorphic loci. Through numerous studies conducted by Weeden & Marx (1984); Aebersold et al. (1987); Weeden & Wendel (1989); Murphy et al. (1990); Zoro (1999), where the isozyme phenotype of the heterozygous individuals is characterized by three bands, it is assumed that genes in *P. lunatus* encode dimeric proteins.

6.2.5 Glutamate dehydrogenase (GDH)

In the studied *P. vulgaris* genotypes a single *Gdh* monomorphic locus was observed, with allele GDH^{100}, which is subject to interpretation for the purposes of our study (results not

shown in figure). In addition to the interpretable band, in the enzymograms there was one more accompanying band (trace). In studies conducted on *P. lunatus* by Zoro (1994), two zones of activity (monomorphic for this species were observed, each of which was encoded by a single gene controlling the isozymes of GDH. By extending the time of germination, activity of a second gene locus can be expected to be expressed.

6.2.6 Isocitrate dehydrogenase (IDH)

In the common bean genotypes analyzed, three zones of activity of IDH were identified, two of which are poorly stained (Fig. 1f). The cathodic zone has the lowest enzyme activity. There were no variations in any of the zones. Based on the results obtained, the quaternary structure of the IDH isozyme cannot be expressed. In other studies, these isozymes have been identified as dimeric proteins (Gurie & Ledig, 1978; Kiang & Gorman, 1985; Weeden & Lamb, 1987; Simonsen & Heneen, 1995). Due to poor visualization of two of the zones in the statistical analyses, only the locus encoding the most highly mobile band of IDH-1 was used.

6.2.7 Malate dehydrogenase (MDH)

Zymograms of MDH are shown in Fig. 1g. Many bands were observed in this enzyme system, but only five of them were typical of homozygous individuals. Some of these five bands were poorly stained. Three loci control the expression of these bands. The three bands comigrated in the intermediate zone. One of these bands, the closest to the anodic one, was considered to be the product of *Mdh*-2, and the other two were products of other loci or artifacts. *Mdh*-1 was represented by two alleles, but in our case by one homozygous allozyme phenotype. Experiments using extraction buffer containing ascorbic acid have revealed that the isozymes encoded by *Mdh*-1 *Mdh*-2 were located in the cytoplasm, while those of *Mdh*-3 were mitochondrial. In fact, the samples extracted using a standard buffer represented all isozymes, while in extraction using a solution with a very low pH only the activity of *Mdh*-3 was manifested. The poorly stained uninterpretable bands might be products of the activity of isozymes localized in other cellular compartments such as peroxisome and chloroplast, in which MDH activity was observed (Gietl, 1992). This hypothesis was verified by the fact that isozymes localized in different cellular compartments were usually encoded by different genes and differed in their electrophoretic mobility, the kinetics of the reaction they catalyze and the nature of their inhibitor (Newton, 1983). Due to the complexity of the MDH zymograms, the dimeric nature of this enzyme system was not clearly established in some species of the genus *Phaseolus*, such as *P. lunatus* (Zoro, 1999). In other species their dimeric nature was clarified (Goodman et al., 1980; Weeden & Wendel, 1989; Murphy et al., 1990). The fact that no band of interaction between the products of the *Mdh*-1 *Mdh*-2 was observed pointed out that some heterodimers were inactive (Zoro, 1999). To obtain information about the genetic determinism of the MDH isozymes, it is necessary to carry out crosses between the alternative homozygotes.

6.2.8 Malate dehydrogenase (NADP+) (MDHP)

This enzyme also called malic enzyme (ME), malate NADP+ dependent enzyme represents a single interpretable band (Fig. 1h), a product of the activity of one gene locus. No polymorphism was observed. Weeden (1984) found correlation between Me and RbcS-

Rubisco (ribulose biphosphate carboxylase) loci, which manifested themselves to be interconnected (r>30 cM). Therefore, it was one of them that we picked for our investigation.

ME catalyzes the metabolism of malic acid to pyruvate. ME activity was found in all plant organs, and it increases during fruit development in many plants, where it is an important determinant of flavour. In tomato, four malate dehydrogenase genes (*Mdh*-1, 2, 3, 4) are known, several of which have been placed on the genetic map. In mapping populations generated between different species, the number and location of *Me* genes has not been reported.

Linkage analysis using Mapmarker indicated the *Me*-1 locus was located on chromosome 5, between the RFLP markers TG379 and TG23 (Chetelat, 1999).

The exceptionally wide cross (F_1 hybrid from *Solanum lycopersicum cv. VF36* x *S. lycopersicoides LA2951*) has proven a rich source of isozyme variation, allowing the determination of map locations of several previously unmapped genes, including *Mdh*-1, *Dia*-1, -2, -3, and –4 (Chetelat et al., 1997).

6.2.9 Superoxide dismutase (SOD)

The enzyme was visualized simultaneously with dihydrolipoamide dehydrogenase (DDH) in Fig. 2a. On the same gels, the activity of DDH was visualized as dark bands. SOD-1 was represented by two white bands '123' and '100'. It was assumed that each zone was determined by one gene: *Sod*-1 and *Sod*-2, which determined the SOD isozymes. The second locus was difficult to interpret. All these bands were monomorphic.

To assess the genetic diversity it is necessary to express the polymorphic loci and alleles for each locus. In self-pollinating species such as *P. vulgaris*, their number is small. This fact restricts the use of allozymes as molecular markers. In this case, the studied material, which is otherwise multifarious in terms of morphological and economic characters – yield, resistance to diseases and pests, is monomorphic in terms of the electrophoretic profile of the above studied enzymes. These enzymes cannot be used to assess the heterogeneity of the studied population of common bean species grown in the region of Plovdiv. Despite the monomorphism of the enzyme systems in the research material, it could be used in the breeding process. Of particular interest is the search for an economically important character, difficult to identify or determine, whose gene(s) and the enzyme encoding gene(s) are inherited together. The preference of a particular character in the breeding process can lead to "fixing" a population (variety, accession) toward the enzyme. When in such a correlation is established, the isoenzyme may serve as a marker.

The low variability detected throuth isozymes suggests the use of DNA-markers to characterize the genetic variability of the green common bean. Enzyme electrophoresis does not show variability to its full extent. Only about 30% of the substitutions at a genetic level result in substitution of amino acid with altered clear load of the protein (Pasteur et al., 1987). Many triplets encode one and the same amino acid. Normally, replacing one amino acid with another bearing the same charge would not impact the clear charge of the protein. Therefore, the identical electrophoretic mobility does not necessarily mean one and the same sequence of DNA. In all cases, however, the quantity of the unmarked variability would be proportionate to the quantity of the marked one (Crawford, 1990).

6.3 Polymorphic enzymes

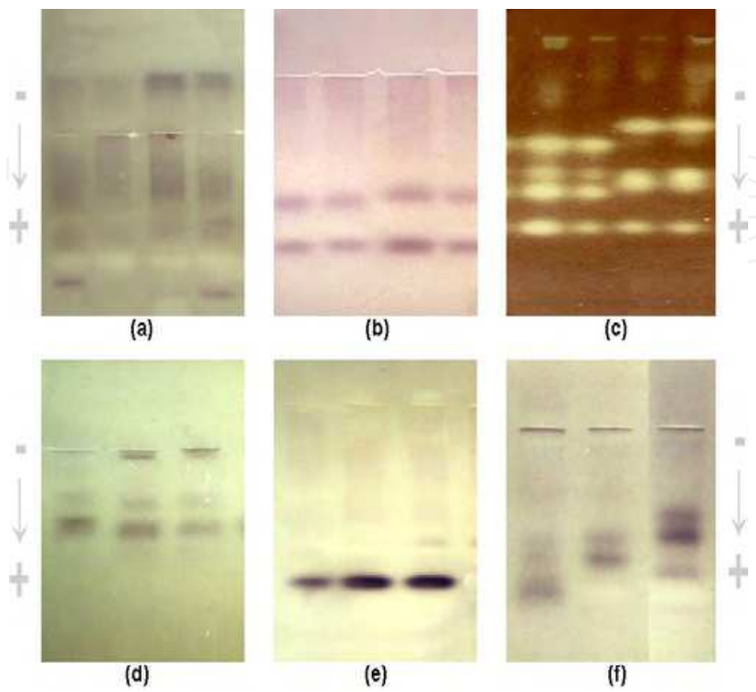

Fig. 2. Zymogrammes of the polymorphic enzyme systems in French bean genotypes grown in the region of Plovdiv:

a. Aconitate hydratase (ACOH). Profiles 1, 2: ACO-$1^{100/100}$; ACO-$2^{100/100}$; Profiles 3, 4: ACO-$1^{100/100}$; ACO-$2^{107/107}$.
b. Superoxide dismutase (SOD) of common bean genotypes. Profiles 1, 2, 3: SOD-$1^{100/100}$; SOD-$2^{123/123}$. Dihydrolipoamide dehydrogenase (DDH). Profiles 1, 2, 3: DDH-$1^{100/100}$; DDH-$2^{100/100}$. Profile 4: DDH-$1^{90/90}$; DDH-$2^{100/100}$.
c. Glucose-6-phosphate isomerase (GPI). Profiles 1, 2: GPI-$1^{100/100}$; GPI-$2^{100/100}$; GPI-$3^{100/100}$; Profiles 3, 4: GPI-$1^{100/100}$; GPI-$2^{96/100}$; GPI-$3^{96/96}$.
d. Glucose-6-phosphate dehygrogenase (G6PDH). Profile 1: G6PDH-$1^{94/94}$; G6PDH-$2^{94/94}$; G6PDH-$3^{94/94}$; Profile 2: G6PDH-$1^{100/100}$; G6PDH-$2^{100/100}$; G6PDH-$3^{100/100}$; Profile 3: G6PDH-$1^{100/100}$; G6PDH-$2^{100/100}$; G6PDH-$3^{94/94}$.
e. Phosphoglucomutase (PGM). Profile 1: PGM-$1^{100/100}$; PGM-$2^{100/100}$; Profile 2: PGM-$1^{100/100}$; PGM-$2^{100/100}$; Profile 3: PGM-$1^{100/100}$; PGM-$2^{67/67}$.
f. Shikimate dehydrogenase (SKDH). Profile 1: SKDH-$1^{100/100}$; SKDH-$2^{133/133}$; Profile 2: SKDH-$1^{133/133}$; SKDH-$2^{196/196}$; Profile 3: SKDH-$1^{100/133}$, SKDH-$2^{133/196}$.

6.3.1 Aconitate hydratase (ACOH)

The zymograms of ACOH are characterized by two zones of activity, regarded as products of two loci (*Aco*-1 and *Aco*-2) (Fig. 2b). The product of locus *Aco*-1 is monomorphic, while in locus *Aco*-2 polymorphism was observed between the populations - two alleles producing the allozymes ACO-2^{100} and ACO-2^{107}. No heterozygous individuals were found in the

populations studied. In the study of the species *P. lunatus* (Zoro, 1999) three different allozymes have been identified, products of three alleles at locus *Aco*-2, and in the heterozygous individuals two bands. This observation indicates the monomeric nature of this enzyme system. Similar results were seen in species of the genus *Sorghum* (Morden et al., 1988) and in *Cicer arietinum* (Kazan et al., 1993). These authors not only observed the two loci, in addition they have proved that the activity of *Aco*-1 is limited in the cytoplasmic fraction, while the expression of *Aco*-2 is associated with the mitochondrial extracts. If the research continues at the level of cellular fractionation, that could clarify the results, because most often it is cytoplasmic enzymes that are polymorphic (Weeden & Gottlieb, 1980).

Studies of the genetic diversity of species of the genus *Phaseolus* often involve examination of diaphorase (DIAP) as an enzyme representing polymorphism. According to Nodari et al. (1992), who have published the related mapping, the alleles encoding DIAP isozymes (diaphorase) and ACO-2 are in the fifth group of linked genes. Therefore, our study focused only on the enzyme ACO.

According to literature data, in common bean the DIAP enzyme system has a quaternary structure (Sprecher, 1988). In 1990, Murphy et al. concluded that the structure of the enzyme can be monomeric or dimeric depending on the species observed.

6.3.2 Dihydrolipoamide dehydrogenase (DDH)

In the conditions of our study, two zones of enzyme activity are reproducible (DDH-1 and DDH-2), encoded by two loci (*Ddh*-1 and *Ddh*-2). Zone DDH-2 was observed as monomorphic. There is a concomitant band. Polymorphism was found in locus *Ddh*-1. Fig. 2a shows the two alleles of locus *Ddh*-1: DDH-$1^{100/100}$ and DDH-$1^{90/90}$.

From the heterozygous phenotype DDH-$1^{90/100}$, established in single individuals in two populations, the monomeric nature of the enzyme was accepted as likely, barring the assumption that there may be a band of interaction that is not manifested in the gels. At the level of this study, without any crossings or survey of the segregation of the character, it is not possible to make a definitive conclusion about the monomeric enzyme nature.

6.3.3 Glucose-6-phosphate isomerase (GPI)

For GPI four zones of activity were observed: a, b, c and d (Fig. 2c). The zymogram is characterized by two cytosolic loci, named *Gpi*-1 and *Gpi*-2, respectively, and one plastid locus *Gpi*-3, which migrates more slowly. These three groups identified in our study were detected in studies of various plant organisms carried by Weeden & Wendel (1989), in apple conducted by Weeden & Lamb (1987), in many *Leguminosae*, such as in *P. lunatus* (Maquet, 1995; Zoro, 1999). In our case, no interaction was detected between cytosolic loci, as in the studies of *P. lunatus*, where the two zones comigrated. The most anodic band was monomorphic in the populations studied here. In contrast, in *P. lunatus* (Maquet, 1995; Zoro, 1999) the same band was polymorphic and comigrates with the next electrophoretic mobility band. In the populations of this study, polymorphism was found in the second band, for which the following genotypes (allelic states) have been established: *Gpi*-$2^{100/100}$ and a small number of cases *Gpi*-$2^{99/100}$ (the latter are not shown in Fig. 2c). The null allele described by Zoro (1999) (absence of allele for locus *Gpi*-2) was not established in this study. There was a correlation between the variation of the second zone and the variation in the

fourth one, where two alleles, '100' and '96', were observed. The subunits of *Gpi*-3, the authors believed, they formed electrophoretically active heterodimers with the cytosolic loci. This contradicted the observations in Lima bean, where there was interaction established between *Gpi*-1 and *Gpi*-2 and the band in the intermediate position was taken to represent inter-gene heterodimer between these two genes. Observations on natural hybrids in the studied material of *P. lunatus* gave reason to Maquet (1995) and Zoro (1999) to assume that the quaternary structure of this enzyme was dimeric. Heterozygous individual was not found in this study, having allozyme phenotype characterized by three bands. One such observation would confirm the hypothesis about the dimeric structure of this enzyme system. However, in other taxa it has been demonstrated that the GPI isozymes have a dimeric structure (Guries & Ledig, 1978; Harry, 1986; Weeden & Lamb, 1987).

6.3.4 Glucose-6-phosphate dehydrogenase (G6PDH)

There are two polymorphic zones of activity on gels stained for G6PDH, one of which is well manifested and can be interpreted (Fig. 2d). The electrophoretic profile of this enzyme system is influenced by the power of germination, therefore it is not easily interpreted in the conditions of this study (sprouted seeds are tested). The electrophoretic variants of the level of each zone of activity segregate independently of those in the other zone. The result suggests that two loci control the expression of the G6PDH isozymes: *G6pdh*-1 and *G6pdh*-2. Both alleles producing the allozyme phenotypes $G6PDH^{94}$ $G6PDH^{100}$ and are identified in locus *G6pdh*-1. At the level of this locus heterozygous phenotypes were observed, characterized by two bands, '100' and '94' (the figure shows only one allozyme). These results indicate monomeric quaternary structure of the isozymes in representatives of *P. vulgaris*. According to Murphy et al. (1990), many quaternary structures have been suggested for the G6PDH isoenzymes.

6.3.5 Phosphoglucomutase (PGM)

Figure 2e shows the two zones of activity identified on the gels. They are regarded as products of two loci: *Pgm*-1 and *Pgm*-2. The zone closer to the anode is monomorphic in the studied populations. In the slower migrating zone two alleles, '100' and '67', are identified. Heterozygous individuals are not established. In *P. lunatus* the heterozygotes are characterized by two bands, thus confirming the expected phenotype of monomeric enzymes. The monomeric structure of the PGM isozymes has been repeatedly confirmed (Harry, 1986; Shore & Barrett, 1987; Weeden & Wendel, 1989).

6.3.6 Shikimate dehydrogenase (SKDH)

In the analysis of samples of all the gels, two comigrating zones of activity were observed (Fig. 2f). Both areas of activity can be controlled by a single locus, one of them being an artifact. However, they can also be products of expression of two genetically related genes, *Skdh*-1 and *Skdh*-2. Three allelic variants, producing allozymic phenotypes are identified: $SKDH^{100}$, $SKDH^{133}$ and $SKDH^{196}$. The following genotypes were observed: in homozygous individuals - $SKDH^{100/100}$, $SKDH^{196/196}$ and $SKDH^{133/133}$, in heterozygous individuals – $SKDH^{100/133}$, $SKDH^{133/196}$ and $SKDH^{100/196}$. Such profiles are identical to those of dimeric enzymes. Therefore, it is assumed that this enzyme system is controlled by one locus (*Skdh*) or two genetically linked loci (*Skdh*-1 and *Skdh*-2) having codominant alleles. Similar results were seen

in *P. lunatus* (Zoro, 1999), but in this species only two allozymic forms of the enzyme were observed. For the representatives of *P. lunatus* a monomeric quaternary structure of the enzyme was established. Because of the fact that the anodic band in the homozygotes $SKDH^{100/100}$ or $SKDH^{133/133}$ opposes the cathodic band of the homozygotes $SKDH^{196/196}$ or another respective one, in *P. lunatus* the heterozygous phenotype is characterized by three bands (Zoro, 1999). Weeden & Gottlieb (1980a) observed two forms of SKDH in *Clarkia williamsonii*: one cytoplasmic and one chloroplastic form. But the nature of genetic relationship, existing between the loci expressed by both forms of SKDH, has not been studied until now.

The results obtained for SKDH can be interpreted as follows: *P. vulgaris* has two genes - *Skdh*-1 and *Skdh*-2. These two genes produce polypeptide subunits that interact to form active tetrameric isozymes. The subunits combine randomly to form inter-gene homodimers and three inter-gene heterotetramers that in homozygous plants manifest five bands. The fact that the fifth line is of very weak intensity does not support such interpretation of the results and makes it highly unlikely. The crossover between alternative homozygotes of loci 1 and 2, and the analysis of the segregation of heterozygotes could contribute more information to the genetic determinism of the SKDH isozymes.

The enzyme systems studied were chosen to be products of unconnected genes, often representing different areas of the genome in the genus *Phaseolus*. Moreover, through the adopted research methods of the enzyme systems above described, the polymorphism of *Phaseolus vulgaris* is covered more completely as the loci encoding them are located on chromosomes other than those on which are the phaseolin-encoding loci.

The monomorphic and polymorphic isozymes established in this study, and respective alleles, are shown in Table 3.

Designation of the loci and alleles studied, direction of migration of the isoenzymes and polymorphism of the enzyme systems found in the study of our genotypes and established for representatives of the species according to data in literature are shown in Table 3. For comparison, literature data for *P. lunatus* was used about studies by Maquet (1995) and Zoro (1999). From the 24 analyzed enzyme loci and the screening of the 14 genotypes and accessions, eleven expressed polymorphisms and a total of 43 alleles were observed, most loci having a common allele. The proportion of polymorphic loci varied from 0% (i.e., Acc.6787) to 13.04% (i.e., BL614), with a mean of 6.15%. Accession 6787 is completely fixed in all loci analysed – zero genetic diversity. In the other genotypes studied there is an average of 1.43 polymorphic loci. The distribution of genotypes at loci showed a significant deficiency of heterozigots. "Pressure" exerted by the breeding is connected probably with polymorphic loci. Only 26% of 15 enzyme systems were polymorphic, which explains the origin of their very narrow size region, as well as the nature of the enzymes studied (all with the exception of CAP, participate in vital functions of plant organisms where the mutations are rarely observed). The sample of 45 to 51, average 49.50 individuals covers available alleles in the population, although in homozygous form.

For the cultivated *P. vulgaris* genotypes studied, data from isozyme electrophoresis indicated genetic variability mainly within the individuals of the accessions and the genotypes, i.e. at the interpopulation level. The values of allelic richness, heterozygosity and interpopulation gene flow were low contrary to the expected one. We recommend preserving the small number of the observed rare alleles at interpopulation level as a resource of maintenance of genetic

diversity that could be an appropriate management method. The observed substantial level of genetic uniformity threatens the studied genotypes. As a result of the obtained data, we also recommend a reintroduction of genotypes having rare alleles at regular time intervals to avoid the endangerment of a genetic drift. The genetic knowledge of the germplasm resources in the target species might directly result in better conservation and management control of the implications for the plant breeding.

Enzyme	Locus number	Locus designation	Migration direction	Alleles observed	Polymorphism / Number of alleles (%) observed in:	
					P. vulgaris	*P. lunatus*
ACOH	2	*Aco*-1 *Aco*-2	A	100 100, 107	Yes / 3	Yes / 4
ADH	2	*Adh*-1 *Adh*-2	A	100 100	No / 2	Yes / 4
CAP	1	*Cap*	A	100	No / 1	Yes / 4
DDH	2	*Ddh*-1 *Ddh* -2	A	100, 90 100	Yes / 3	-
END	1	*End*	A	100	No / 1	Yes / 4
EST	3	c*Est*-1 c*Est*-2 f*Est*-1	A C A	100 100 100	No / 3	Yes / 6
GDH	1	*Gdh*	A	100	No / 1	No / 2
G6PDH	2	*G6pdh*-1 *G6pdh*-2	A	100, 94 100, 94	Yes / 4	Yes / 5
GPI	3	*Gpi*-1 *Gpi*-2 *Gpi*-3	A	100 100 100, 96	Yes / 4	Yes / 5
IDH	3	*Idh*-1 *Idh*-2 *Idh*-3	A	100 100 100	No / 3	Yes / 5
MDH	3	*Mdh*-1 *Mdh* -2 *Mdh* -3	A	100 100, 84 100	Yes / 4	Yes / 6
MDHP	1	*Mdhp*	A	100	No / 1	-
PGM	2	*Pgm*-1 *Pgm*-2	A	100 100, 67	Yes / 3	Yes / 5
SKDH	3	*Skdh*-1 *Skdh*-2 *Skdh*-3	A	100, 196 100, 133 100, 133, 196	Yes / 7	Yes / 3
SOD	2	*Sod*-1 *Sod*-2	A	100, 123 100	No / 3	No / 2
N = 15	N = 31	N = 31		N = 43	P = 26%	P = 10.32 %

Table 3. Summarized data of enzyme locus of cotyledon tissues from the studied *P. vulgaris* genotypes, compared to literature data on *P. lunatus*.
Notes: N, number of established loci; A, anodic migration; C, cathodic migration; P, polymorphism; %, percentage of polymorphic loci; Yes – detected; No – non detected polymorphism. PL, number of polymorphic loci; PLP (%), percentage of polymorphic loci

Factors that may cause such a result are most often the impact of a narrow gene flow, the predominantly autogamous reproductive system possessed by the species, the breeding "pressure" by surrounding microenvironment. The analysis of the genotype composition allows to assume that the studied populations were influenced by factors that have fixed genes encoding enzyme systems.

The manifestation of the homogeneity of the selected group of representatives of *P. vulgaris* contributes to the assessed low level of genetic diversity in the working collection. From the analyses carried out in the gene pools bank of wild forms, compared with the cultivated ones, a vast preponderance is evident in the parameters used for assessment of variability, including those calculated on the basis of enzyme systems. A comparison within the same species, common bean, promotes the development of a strategy to increase its genetic diversity. This in turn will allow an increase in the genetic basis for improving the economic performance of the lines of the working collection and solving the breeding issues posed by them.

Despite the importance of the methods *ex situ* for conservation and breeding of the "big crops" (Falk, 1990), there are many problems related with their application. The problems mainly concern the inadequate procedures for selection of material for sampling and the lack of representativeness of the material maintained in genebanks (Altieri et al., 1987; Brown et al., 1997). The methods for collecting field samples have been thoroughly explored in research using models and theory of population genetics (Ewens, 1972; Marshall et al., 1975; Oka, 1975; Crossa, 1989; Yonezawa et al., 1989; Falk, 1991; Crossa et al., 1993).

Enzyme electrophoresis is one of the techniques frequently applied for analyses in studies requiring screening analyses of the genetic diversity of several loci in large numbers of individuals. Further efforts should be devoted to analysing additional accessions and natural populations of this food legume from the region by the introduced non-expensive, easy of implementation and resolution methods. Genetic tests from segregation data and linkage relationships between polymorphic loci should be investigated to check current results.

7. Conclusions

In this paper the previously described procedures were optimised and applied for analysing common bean accessions and genotypes.

Twenty four loci of 15 enzyme systems studied in cotyledons of French bean from the Plovdiv region were determined. A total of 43 alleles were scored at these loci and in 26% of all a polymorphism was detected.

The data obtained for monomorphic and polymorphic isozyme loci contributed to characterize the genetic basis and the genetic variation within *P. vulgaris* accessions and genotypes that could be used in gene bank to maintain as much genetic diversity as possible, in breeding for crosses and new varieties developing.

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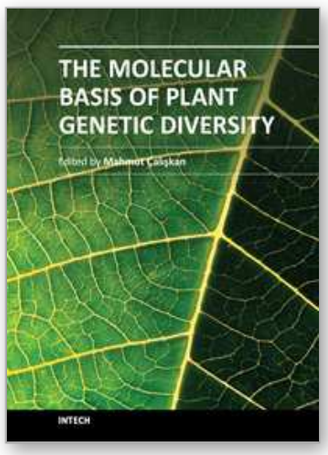

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The Molecular Basis of Plant Genetic Diversity presents chapters revealing the magnitude of genetic variations existing in plant populations. Natural populations contain a considerable genetic variability which provides a genomic flexibility that can be used as a raw material for adaptation to changing environmental conditions. The analysis of genetic diversity provides information about allelic variation at a given locus. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in plants and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

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