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Characterisation of the Amaranth Genetic Resources in the Czech Gene Bank

Dagmar Janovská[1], Petra Hlásná Čepková[2] and Mária Džunková[3]
[1]*Department of Gene Bank, Crop Research Institute*
[2]*Department of Crop Sciences and Agroforestry in Tropics and Subtropics*
Institute of Tropics and Subtropics, Czech University of Life Sciences Prague
[3]*Department of Genomics and Health,*
Centre for Public Health Research (CSISP), Valencia
[1,2]*Czech Republic*
[3]*Spain*

1. Introduction

The human species depends on plants. These constitute the basis for food, supply most of our needs (including clothes and shelter) and are used in industry for manufacturing fuels, medicines, fibres, rubber and other products. However, the number of plants that humans use for food is minimal, compared to the number of species existing in nature. Only 30 crops, the most outstanding of which are rice, wheat and maize, provide 95% of the calories needed in the human diet (Jaramillo & Baena, 2002). However, agricultural biodiversity is in sharp decline due to the effects of modernisation, such as concentration on a few competitive species and changes in diets. Since the beginning of agriculture, the world's farmers have developed roughly 10 000 plant species for use in food and fodder production. Today, only 150 crops feed most of the world's population, and just 12 crops provide 80% of dietary energy from plants, with rice, wheat, maize and potato providing 60%. It is estimated that about three quarters of the genetic diversity found in agricultural crops have been lost over the past century, and this genetic erosion continues (EC, 2007).

Humans need to add to their diet those crops of high yield and quality that can adapt to environmental conditions and resist pests and diseases. Advantage must be taken of native and exotic species, with nutritional or industrial potential, or new varieties must be developed. Improving crops, however, requires reserves of genetic materials whose conservation, management and use have barely begun to receive the attention that they deserve. Humans take advantage of plant genetic resources in as much as they are useful to us, which means that we must understand them, and know how to manage, maintain and use them rationally (Jaramillo & Baena, 2002). Information on genetic diversity and relationships within and among crop species and their wild relatives is essential for the efficient utilization of plant genetic resource collections for the efficient explanation of taxonomic relationships (Chan & Sun, 1997; Drzewiecki et al., 2003).

Amaranthus L. is a genus from *Amaranthaceae* family probably originated in America. This genus contains approximately 70 species of worldwide distribution including pigweeds,

waterhemps, and grain amaranths (Sauer, 1967). The origin of various species of cultivated amaranths is not easy to trace because wild ancestors are pantropical cosmopolitan weeds (Espitia-Rangel, 1994). For human consumption there are cultivated grain amaranths – *A.caudatus, A. cruentus* and *A. hypochondriacus* and vegetable amaranths – mainly *A.dubius, A.tricolor* and *A.cruentus.* Grain amaranths are crop species of New World origin; *A. caudatus* from Andean Peru and Ecuador, *A. cruentus* and *A. hypochondriacus* from Mexico and Central America (Sauer, 1950; Drzewiecki, 2001). Nowadays, the grain amaranths are cultivated from the temperate to tropical zone and the vegetable amaranths mainly in the South Africa and South Asia (Jarošova et al., 1997).

Amaranths are very promising crops. The main reasons could be content of protein, fat and active substances. The content of seed protein is in the range 13 – 18% with very good balanced amino acids. The lysine content is relatively high in the comparison with common cereals. The content of crude proteins in leaves is from 27 to 49% in d.m. what is more than in the leaves in the spinach (Segura-Nieto, 1994). Amaranths have comparable or higher amounts of essential amino acids as whole egg protein (Drzewiecki et al., 2003). The fat content is in the range 0.8-8.0%. The linoleic acid is the predominant fatty acid, with lesser amount of oleic and palmitic acids. The oil also contains squalene, precursor of cholesterol, which is used in the cosmetics and as a penetrant and lubricant (Becker, 1994). Many compounds and extracts from amaranths possessed anti-diabetic, anti-hyperlipidemic, spermatogenic and anti-cholesterolemic effects (Sangameswaran & Jayakar, 2008; Girija et al., 2011), antioxidant and antimicrobial activity (Alvarez-Jubete et al., 2010; Tironi&Anon, 2010). Many consumers purchase amaranth because they want a wheat- and gluten-free product, like the nutritional profile of amaranth, or enjoy "exotic" foods in their diet (Brenner et al., 2000). Amaranth can be used also as a feed for pigs, hens, etc. (Pisarikova et al., 2005). From the cultivation point of view, amaranth is interesting for its heat and drought resistance and very low susceptibility to diseases and pests (Barba de la Rosa, 2009). Considering its agronomic importance, attention should be given to the cultivation, conservation, and sustainable utilization of this promising crop (Ray & Roy, 2009).

Unfortunately, amaranths are also very harmful weeds spread in all over the world. Weedy *Amaranthus* species (pigweeds) have been and continue to be a major problem in agronomic production. The weed amaranth *A. retroflexus* is considered one of the world's worst weeds. A major contributor to the noxious nature of these weedy species is their ability to efficiently adapt to the changes in agricultural management practices that are specifically designed to control and prevent colonization. For example, numerous populations of pigweeds have evolved herbicide resistance (Drzewiecki, 2001; Rayburn et al., 2005).

In the Czech Republic the cultivation of amaranth was introduced in the early 1990s (Michalova 1999; Moudry et al. 1999) and the collection of amaranth genetic resources was established in 1993 in the Czech Gene Bank. Due to the very positive effects on the human health, we try to find out genotypes suitable for the Czech conditions with utilization in the Czech cuisine. On the Czech market, there is very popular food made from amaranth flour such as chips, cookies, and breakfast cereals, etc. However, all amaranth seeds are imported into the Czech Republic from other countries. The demand for vegetable amaranth is also increasing. Presently, in the Gene Bank, there are stored 103 evaluated accessions. In the working collection (in the different stages of evaluations), there are more than 30 accessions. Seed samples of amaranth are obtained from other gene banks, universities, private subjects

or from collecting missions from all over the world. It corresponds with international agreements and with The Czech National Programme on Conservation and Utilization of Plant Genetic Resources and Agro-biodiversity. For maintenance and utilization of plant genetic resources of amaranths, it is very important to know them from all sides. Genetic resources studies are oriented on evaluation of the most important biological characters, with respect to the effective utilization of genetic resources in breeding and agricultural practice. Good characterization and evaluation of genetic resources under conditions similar to those of their origin can provide breeders and users with valuable information on effective utilization of genetic resources for the breeding programmes and utilization. Characterization of genetic resources is focused mainly on morphological characters. The evaluation consists of data on plant growth and development, characteristics of plant stand, analysis of yield elements, etc. (Dotlačil et al., 2001). First steps of evaluations after seed samples receiving, are field evaluations. The phenological and morphological evaluation such as length of vegetation, plant height, length of inflorescence, colour of inflorescence, type of inflorescence, etc., is performed during vegetation. The length of vegetation is very important for amaranth cultivation in the Czech Republic, because many of the amaranths genotypes are sensitive to day-length. They remain in the vegetative period for a long time and create seeds after day-shortening (NRC, 1984). In the Czech Republic, they flower in the second half of September. Because the early frost, they cannot mature their seeds.

For genetic improvement of *Amaranthus*, germplasm collections will play a key role as well. However, only limited information is available on intra- and inter-specific genetic diversity and relationships within *Amaranthus* germplasm collections (Chan & Sun, 1997). In spite of the fact that it has been the object of many studies, the genus *Amaranthus* is still poorly understood, being widely considered as a "difficult" genus. Currently, the taxonomic problems are far from being clarified especially because of the widespread nomenclatural disorder caused chiefly by repeated misapplication of names (Costea et al., 2001) which is shown in Table 1. Due to variation of morphological characters, accurate classification of amaranth genetic resources is not always possible (Transue et al., 1994).

For preliminary identification of *Amaranthus* species, the useful tool can be the number, thickness, orientation and density of branches in inflorescences. The flowers are arranged in small and very contracted cymes, which are agglomerated, axillary and additionally arranged in racemose or spiciform terminal, large and complex synflorescences. Although extremely variable, there is usually a tendency towards a morphological "type" (Costea et al., 2001).

The colour of the seeds is commonly dark-brown to blackish, or whitish-yellowish, sometimes with reddish nuances at the species cultivated as cereals. Many cultivars of *A. caudatus* have pink cotyledons visible through the seed coat. The colour may be uniform or not, in the last case usually with the marginal zone paler. Weedy species and species used as a vegetable have mostly black or dark seeds (Costea et al., 2001; Jarošová et al., 1997; Das, 2011).

Many species of the genus are greatly affected by environmental factors (nutritional elements, water availability, light conditions, injurious factors, etc. exhibiting a great morphological variability with little taxonomic significance (Costea et al., 2001). All the above mentioned characteristics are useful for the taxonomy of the genus but difficult to use

for the current identification of taxa (Costea et al., 2001). Also it is dependent on the cultivation in the field conditions. In the case of a gene bank, when seed samples are received, it is necessary to sow them in the field conditions for the morphological and phenological evaluations. But in the case of weedy species, it would be better to know, if the samples are not harmful weeds. We need to exclude weeds from our collection.

Many different methods of identification have been used for evaluation of amaranth diversity. RAPD analysis was successful in the investigation of the relationships of four *A. hypochondriacus* varieties (Barba de la Rosa et al., 2009). AFLP markers were successfully used to determine species what demonstrated taxonomic ambiguity at the basic morphologic level (Costea et al., 2006). Other methods such as ITS, ISSR and isozyme profile were used to get exhaustive view of interrelationship and relative closeness among amaranth species (Das, 2011; Xu & Sun, 2001). Also other methods such as electrophoresis profiles of proteins have been successfully used to clarify the taxonomy of many families. There was published, that electrophoresis can also be used to characterize the seed protein profiles of species and cultivars, compare cultivars of different geographical origin, and provide taxonomically useful descriptors that are substantially free from environmental influence. This method is rapid, relatively cheap, largely unaffected by the growth environment and eliminate to grow plant to maturity (Juan et al., 2007; Jugran et al., 2010). Drzewiecki (2001) used SDS PAGE of urea-soluble proteins of amaranth seeds for distinguishing both – species and their cultivars. Samples of seven species were divided into three groups by protein patterns according to similarity. According to solubility, Osborne (1907) divided proteins into four classes: albumins soluble in water, globulins soluble in high salt concentration, prolamins soluble in aqueous alcohol and glutelins soluble in acid or alkaline solutions (Segura-Nieto et al., 1994). The division into four protein fractions brings the possibility to see the differences among seed samples more clearly. The first general characterization of the protein fraction spectra of amaranth species was performed by Gorinstein et al. (1991) and Drzewiecki et al. (2003). Finally, Dzunkova et al. (2011) set up the methodology for clear identification of the amaranth species using glutelin protein fraction. The washing off water, salt- and alcohol- soluble proteins in protein fraction separation process makes polymorphic peaks of amaranth glutelins to be distinguished very easily.

SDS PAGE has been the traditional method for analysing glutenin subunit composition of wheat, but the procedure is slow, laborious and non-quantitative. The chip microfluidic technology, based on capillary electrophoresis, provides new opportunities in analysis of wheat HMW-GSs. This procedure is rapid, simple to operate, enabling automatic and immediate quantitative interpretation. Other advantages over traditional gel electrophoresis are lower sample and reagent volume requirements and a reduced exposure to hazardous chemicals (Bradova & Matejova, 2008).

In this work, we focused on evaluation for precise determination of amaranth genetic resources in the Czech Gene Bank. One of our aims was to separate amaranth species according to protein patterns and to verify our hypothesis of different protein fraction pattern based on species and variety. We compared spectra of storage proteins and their fractions of wild weedy and cultivated species of amaranths and verified the suitability of this method for species identification in our collection.

Latin name	Synonyms
Amaranthus caudatus L.[1]	*Amaranthus caudatus* subsp. *caudatus* *Amaranthus caudatus* subsp. *mantegazzianus*
Amaranthus caudatus subsp. *caudatus*[1]	= *Amaranthus alopecurus* Hochst. ex A. Br. & Bouche = *Amaranthus abyssinicus* hort. ex L.H. Bailey. = *Amaranthus caudatus* subsp. *saueri* Jehlik = *Amaranthus caudatus* L. = *Amaranthus maximus* Mill. = *Chenopodium millmi* J.T. del Granado = *Amaranthus caudatus* var. *alopecurus* Moq.
Amaranthus caudatus subsp. *mantegazzianus*[1]	= *Amaranthus edulis* Spegazz. = *Amaranthus mantegazzianus* Passer.
Amaranthus cruentus L. [1]	= *Amaranthus caudatus*auct. = *Amaranthus paniculatus* L. = *Amaranthus hybridus* var. *cruentus* = *Amaranthus sanguineus* L. = *Amaranthus hybridus* '*paniculatus*'. = *Amaranthus speciosus*
Amaranthus retroflexus L. [1]	= *Amaranthus patulus*auct. = *Amaranthus delilei* Richter & Loret
Amaranthus hypochondriacus L. [1]	= *Amaranthus chlorostachys* var. *erythrostachys* (Moq.) Aell. = *Amaranthus leucospermus* S. Wats. = *Amaranthus leucocarpus* S. Wats. = *Amaranthus hybridus* convar. *erythrostachys* (Moq.) Thell. ex Asch. & Graebn. = *Amaranthus hybridus* subsp. *hypochondriacus* (L.) Thell. = *Amaranthus flavus* L. = *Amaranthus frumentacea* Buch.-Ham. = *Amaranthus chlorostachys* var. *leucocarpus* (S. Wats.) Aell. = *Amaranthus anardana* Buch.-Ham.
Amaranthus cannabinus (L.) J.D.Sauer[2]	*Acnida cannabina* L.
Amaranthus deflexus L. [2]	
Amaranthus tuberculatus (Moq.) J.D.Sauer[2]	*Acnida tuberculata* Moq.

[1]according to Mansfeld'sEncyclopedia of Agricultural and Horticultural Crops (Hanelt& IPGCPR, 2001)
[2]according to IPNI (2011)

Table 1. Synonyms of selected amaranth species

2. Materials and methods

2.1 Plant material

For the evaluation there were used 46 amaranth genotypes from Crop Research Institute in Prague, Czech Republic (CRI) and from USDA, ARS, NCRPIS Iowa State University. In these samples, there were 6 accessions of wild weed and 40 of the cultivated species. The acronyms used for the wild species were as follows: De - *A. deflexus*, Au - *A. australis*, Wr - *A. wrightii*, Tu - *A. tuberculatus*, Cn - *A. cannabinus*, Re - *A. retroflexus*. The cultivated samples were evaluated in the field conditions in 2008 and 2009 according to the list of descriptors for amaranths created for purposes of the Czech Gene Bank. The morphological and phenological characters are evaluated according to List of Descriptors for amaranth created in the Czech Gene Bank. Following traits were evaluated in the field conditions:

- number of days from emergence to inflorescence observation,
- number of days from emergence to flowering,
- number of days from emergence to maturity.

The first two traits were assessed when 50% of plants were in this stage. The numbers of day from emergence to inflorescence observation and the numbers of days from emergence to flowering are important characters due to fact, that certain amaranth genotypes are sensitive to day-length. Maturity was estimated when 75% of the grains were mature. Plant height was measured from the soil surface to the top of the main stem in cm. Length of inflorescence was measured from the downmost branch to the top of inflorescence of the main stem in cm. Weight of thousand seeds (WTS) was weight of thousand seeds in g.

2.2 Total seed protein content and protein fractions content determination

The measurements of total seed protein content and protein fraction content were performed by the Kjeldahl method (Czech state norm 56 0512-12) in Kjeltec automatic analyzer (Kjeltec 2300, Foss Tecator, Sweden) with the protein-nitrogen coefficient set to 6.025. Protein fractions (albumins, globulins, prolamins and total glutelins) were extracted according to the protocol developed for the wheat protein fraction separation by Dvoracek (2006) with some modifications. For the determination of protein fractions content was used 0.5 g of milled amaranth seeds. The protein fractions were extracted by adding 5 ml of solvent (distilled water for albumins, 0.5 M NaCl for globulins, cold 60% ethanol for prolamins), vortexing and centrifuging by 10 000 × g for 15 minutes (Universal 32R HettichCentrifugen, Germany). This procedure was repeated twice and the supernatants from each extraction were saved and poured together. In the case of prolamins, after first addition of solvent, tubes were vortexed and chilled to 4°C for 4 hours; after that the procedure was performed exactly as for albumins and globulins. The protein content of whole seed was also measured by milling 1g of amaranth seeds. For the boiling in the automatic digestion system (2015lift, 2020 digestor, Foss Tecator, Denmark) were used 10 ml from the obtained 15 ml of each fraction extract. Into the each 250 ml tube one catalyser tablet, 3.5 g of K_2SO_4 and $CuSO_4$ mixture and 10 ml of H_2SO_4 were added. In one tube was a blank sample. Tubes were let to boil to the temperature of 420°C for about 1 h 40 min. After cooling for about 10 min, 75 ml of distilled water was added. The content of glutelin and the residual nitrogen fraction was calculated as the difference between the content of the total seed protein and three measured fractions.

2.3 Electrophoresis of the proteins

2.3.1 Extraction of the total seed storage proteins

Five different approaches to the extraction were tested for the development of the best extraction approach:

1. single seed extracted in 18 µl of the extraction solution,
2. bulk of 10 seeds extracted in 50 µl of the extraction solution,
3. bulk of 10 seeds extracted in 100 µl of the extraction solution,
4. bulk of 100 seeds extracted in 200 µl of the extraction solution,
5. bulk of 100 seeds extracted in 400 µl of the extraction solution.

Seed samples were crushed separately and mixed with extraction solution (consisted of 0.0625 M Tris-HCl pH 8.8, 5% (w/v) 2- mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromphenol blue) by vortexing (MS2 Minishaker, IKA, Germany) several times in 1.5 ml tubes. Tubes were allowed to stand at 4 °C for three hours. After this extraction time, the tubes were centrifuged at 12 000 x g for 15 min (Universal 32R HettichCentrifugen, Germany). After the replacement of the samples to the new tubes, the samples were heated in a boiling water bath for 2 min.

Ten seeds from each variety were selected randomly, crushed and put into 2 ml micro tube. The protein fractions were extracted by adding 100 µl of solvent (distilled water for albumins, 0.5 M NaCl for globulins, cold 60% ethanol for prolamins), vortexing and centrifuging by 10 000 × g for 15 minutes (Universal 32R HettichCentrifugen, Germany). This procedure was repeated twice but the supernatants of the second and third wash were always discarded. In the case of prolamins, after first addition of solvent, tubes were vortexed and chilled to 4°C for 4 hours; after that the procedure was performed as in the case of albumins and globulins. Tubes containing protein fractions extract and the seed pellets (glutelins) were freezed to -25°C. After the supernatant in the tubes became solid, the top of the tubes was perforated by a needle to form small holes what serve to prevent the loss of the sample by lyofilisation. The lyofilisation was performed by freeze dryer (Christ, Germany) during 24 h at -58°C and 0.018 mBar. The lyophilized solid samples were mixed with 100 µl extraction solution (consisted of 0.0625 M Tris-HCl pH 8.8, 5% (w/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromphenol blue) by vortexing several times in 1.5 ml tubes. Tubes were allowed to stand at 4 °C for three hours. After this extraction time, the tubes were centrifuged at 12000 x g for 15 min. The supernatants were put into new tubes and heated in boiling water for 2 min.

2.3.2 Protein separation by SDS PAGE

The amaranth protein extracts were separated in conditions of discontinuous electrophoresis (SDS-PAGE) according to Laemmli (1970) 4% stacking gel of pH 6.8, 10% separation gel of pH 8.8 on the polyacrylamide gels of the size 180 x 160 x 0.75 mm.

On the gel was loaded:

- 15 µl of the single seed sample,
- 20 µl of the 10 seed bulk, 100 seed bulk and all the protein fraction samples,
- 7 µl of the protein marker: SigmaMarker Wide Range (MW 6,500-200,000).

The electrophoresis was performed on 90 mA (45 mA / gel) and let to run for about 4 hours. The gels were stained with a solution of 0.1% (w/v) Coomasie Brilliant Blue (CBB) R250, 50% (w/v) methanol, 10% acetic acid, 0.02% (w/v) bromphenol blue salt for 1 day and destained with a solution of 25% (w/v) denatured alcohol and 3.5% (w/v) acetic acid, what lasted also 1 day. Gels were preserved in solution: 45% (w/v) denatured alcohol, 3% (w/v) glycerol for 2 hours, then dried and stored into cellophane sheets. The whole procedure including the test of the different extraction concentrations, the protein fraction separation procedure and the electrophoresis was repeated for the control of the correct experiment performance.

2.3.3 Chip electrophoresis

All the extracted protein fraction samples were analyzed by chip capillary electrophoresis using commercial Experion Pro260 Analysis Kit for 10 Chips and the Experion automated electrophoresis system (Bio-Rad Laboratories, USA) for protein quantification according to the manufacturer's instructions. Experion automated electrophoresis station performs automatically all the steps of the gel-based electrophoresis (samples separation, staining, destaining, imaging, band detections, and data analysis).

2.4 Statistical analysis

For the statistical evaluation of morphological traits, analysis of variance (ANOVA) and the Tukey HSD test were used (software -Statistica 7.0 CZ). In the case of protein fraction proportion in accessions with different seed colour, the basic statistics of R statistics 2.10.0 software were used for calculation of mean x, standard deviation sx and p-values (adjusted by Holm correction, two sided Welch Two Sample t-test used).

The SDS-PAGE spectra of total seed storage proteins and protein fractions were compared and confronted with the spectra of the chip capillary electrophoresis. The bands in the spectra were analyzed regarding the positions of the bands and also the relative intensity of the bands. The intensity of the bands was analyzed individually for each sample considering the intensity of the internal markers of the chip electrophoresis and the general intensity of all the bands in the sample. The intensity of the bands was expressed as the relative protein concentration measured by chip capillary electrophoresis what was the multiplication of numbers 0, 1, 2, 3 used in our statistics (0– no band, 1- light band, 2 – medium intensity band, 3 – dark band). The spectra expressed as the numerical values were analyzed by R statistics 2.10.0 software. The relationships between accessions were expressed by Pearson correlation using single linkage. The hierarchical clustering dendrogram was cut at the level of correlation 0.99 to show the well defined clusters.

3. Results and Discussion

3.1 Morphological and phenological evaluation

Mean data of morphological and phenological evaluations of amaranth are shown in table 2 and 3. From our long-term observations, genotypes with number of days from emergence to flowering higher than 100 days likely does not mature before early frost in autumn. The vegetation period in evaluated collection ranged from 92±0.00 to 163.00±0.00 days. Also height of plants in maturity and length of inflorescence is a very useful character. Both are

	From emergence to inflorescence observation (days)	From emergence to flowering (days)	From emergence to maturity (days)
Genotype	Mean±SD	Mean±SD	Mean±SD
6	56.00±1.41^{abc}	72.00±7.07^{bcdef}	107.00±16.97^{ab}
11	51.50±7.78^{abc}	67.00±4.24^{abcdef}	120.50±7.78^{ab}
12	47.50±4.50^{abc}	64.00±11.37^{abcdef}	101.00±16.97^{ab}
21	46.50±3.54^{abc}	64.00±8.49^{abcdef}	108.50±24.75^{ab}
23	51.00±2.83^{abc}	64.00±9.90^{abcdef}	109.00±25.46^{ab}
24	46.00±0.00^{abc}	64.00±9.90^{abcdef}	102.00±25.46^{ab}
35	51.50±2.12^{abc}	64.50±7.78^{abcdef}	100.50±26.16^{ab}
43	64.00±0.00^{abc}	86.00±0.00^{ef}	122.00±0.00^{ab}
44	62.00±5.66^{abc}	105.00±0.00^{abcdef}	131.00±36.77^{ab}
45	44.00±0.00^{abc}	63.00±0.00^{abcdef}	145.00±0.00^{ab}
51	49.00±4.24^{abc}	67.00±5.66^{abcdef}	107.00±26.87^{ab}
62	45.00±1.41^{abc}	64.50±9.19^{abcdef}	126.00±0.00^{ab}
70	52.00±1.41^{abc}	68.50±3.54^{abcdef}	112.50±19.09^{ab}
71	45.50±4.50^{abc}	65.00±4.24^{abcdef}	111.50±17.68^{ab}
72	50.00±7.07^{abc}	68.00±2.83^{abcdef}	100.00±26.87^{ab}
73	41.00±0.00^{ab}	56.50±0.71^{abcd}	113.50±0.71^{ab}
75	52.00±2.83^{abc}	75.00±0.00^{cdef}	104.50±16.26^{ab}
76	47.50±0.71^{abc}	61.00±4.24^{abcdef}	114.50±3.54^{ab}
80	44.00±0.00^{abc}	69.00±0.00^{bcdef}	124.00±0.00^{ab}
92	75.00±7.07^{abc}	98.00±0.00^{abcdef}	163.00±0.00^{b}
95	34.50±19.09^{ab}	51.00±7.07^{abcd}	111.00±1.41^{ab}
96	36.50±12.02^{ab}	60.50±10.61^{abcdef}	106.00±16.97^{ab}
98	47.50±12.02^{abc}	78.00±0.00^{def}	121.00±0.00^{ab}
99	45.00±0.00^{abc}	63.00±0.00^{abcdef}	111.00±0.00^{ab}
101	42.50±0.71^{abc}	55.50±3.54^{abcd}	110.00±1.41^{ab}
104	43.50±7.78^{abc}	54.00±4.24^{abcd}	114.50±20.51^{ab}
107	45.00±0.00^{abc}	65.00±0.00^{abcdef}	111.00±0.00^{ab}
109	48.00±0.00^{abc}	88.00±0.00^{f}	101.50±14.85^{ab}
110	49.50±2.12^{abc}	64.50±4.50^{abcdef}	118.00±11.31^{ab}
111	52.50±2.12^{abc}	63.00±1.41^{abcdef}	116.50±9.19^{ab}
112	55.50±7.78^{abc}	70.50±10.61^{bcdef}	120.00±14.14^{ab}
120	51.50±0.71^{abc}	70.00±0.00^{bcdef}	116.00±22.63^{ab}
121	31.50±23.33^{ab}	57.00±5.66^{abcd}	92.00±0.00^{a}
123	41.50±14.85^{ab}	53.00±15.56^{abcd}	114.00±25.46^{ab}
124	47.50±3.54^{abc}	60.00±2.83^{abcde}	115.00±11.31^{ab}
125	49.5±0.71^{abc}	58.00±0.00^{abcd}	105.50±7.78^{ab}
132	35.00±0.00^{ab}	47.00±1.41^{ab}	97.00±15.56^{ab}
134	35.00±2.83^{ab}	48.50±0.71^{abc}	97.50±16.26^{ab}
136	27.50±19.09^{a}	41.00±16.97^{a}	103.00±22.62^{ab}
143	41.50±3.54^{ab}	60.00±4.24^{abcde}	98.50±17.68^{ab}
Year			
2008	45.44±11.67^{a}	66.21±13.60^{a}	115.68±15.42^{a}
2009	48.17±8.78^{a}	63.83±13.06^{a}	108.27±19.29^{b}

SD-standard deviation
Analysis of variance (ANOVA) and the Tukey HSD test were used for statistical evaluation (software - Statistica 7.0 CZ).
Different letters in the same row are statistically significant at $p > 0.05$.

Table 2. Phenological evaluation of amaranths

	Inflorescence length (cm)	Plant height (cm)	WTS (g)	Colour of seed
Genotype	Mean±SD	Mean±SD	**Mean±SD**	
6	54.00±1.41^{de}	137.50±3.54^{bcd}	0.75±0.01^{cdefghijk}	pale
11	56.50±0.71^{de}	137.50±45.96^{bcd}	0.69±0.08^{bcdefghij}	pale
12	29.50±0.71^{bc}	117.50±3.54^{abcd}	0.68±0.04^{bcdefghij}	pale
21	34.00±1.41^{bcd}	90.00±7.07^{abc}	0.58±0.03^{abcdefg}	pink
23	44.50±0.71^{cde}	127.50±3.54^{bcd}	0.76±0.08^{cdefghijk}	black
24	66.00±1.41^{e}	152.50±3.54^{cd}	0.75±0.07^{cdefghijk}	black
35	30.50±0.71^{bc}	167.50±3.54^{d}	0.90±0.00^{ijk}	pale
43	36.00±0.00^{bcd}	150.00±0.00^{cd}	0.74±0.04^{cdefghijk}	pale
44	24.50±0.71^{a}	147.50±3.54^{cd}	0.74±0.00^{cdefghijk}	pale
45	29.00±0.00^{bc}	100.00±0.00^{abc}	0.88±0.00^{hijk}	pale
51	29.00±0.00^{bc}	142.50±3.54^{bcd}	0.85±0.00^{hijk}	pale
62	37.50±0.71^{bcd}	137.50±3.54^{bcd}	0.66±0.06^{abcdefghi}	black
70	45.50±0.71^{cde}	102.50±3.54^{abcd}	0.78±0.05^{efghijk}	pale
71	52.50±0.71^{cde}	132.50±3.54^{bcd}	0.86±0.15^{hijk}	pale
72	46.50±0.71^{cde}	122.50±3.54^{bcd}	0.93±0.10^{jk}	pale
73	41.50±0.71^{cde}	130.00±8.49^{bcd}	0.71±0.01^{cdefghijk}	pale
75	60.00±0.00^{e}	132.50±3.54^{bcd}	0.91±0.10^{ijk}	pale
76	36.50±3.54^{bcd}	109.00±4.24^{abcd}	0.84±0.08^{ghijk}	pale
80	47.00±0.00^{cde}	125.00±0.00^{bcd}	0.50±0.00^{abcd}	black
92	51.00±1.41^{de}	142.50±3.54^{bcd}	0.84±0.00^{ghijk}	pale
95	35.50±0.71^{bcd}	92.50±3.54^{abc}	0.63±0.11^{abcdefgh}	black
96	34.00±1.41^{bcd}	92.60±3.54^{abc}	0.40±0.04^{a}	black
98	43.50±0.71^{cde}	152.50±3.54^{cd}	0.96±0.00^{k}	pale
99	44.00±0.00^{cde}	110.00±0.00^{abcd}	0.70±0.00^{bcdefghijk}	black
101	38.00±0.00^{bcd}	127.50±36.06^{bcd}	0.5±0.02^{abc}	pink
104	42.50±0.71^{cde}	92.50±3.54^{abc}	0.63±0.04^{abcdefgh}	black
107	22.00±0.00^{a}	53.00±0.00^{a}	0.52±0.00^{abcde}	black
109	34.50±0.71^{bcd}	77.50±3.54^{ab}	0.44±0.04^{ab}	black
110	49.50±0.71^{cde}	155.00±7.07^{cd}	0.70±0.00^{bcdefghijk}	black
111	52.00±1.41^{de}	137.50±3.54^{bcd}	0.52±0.11^{abcde}	black
112	53.00±2.83^{de}	127.50±3.54^{bcd}	0.84±0.01^{ghijk}	pale
120	51.50±2.12^{de}	95.00±42.43^{abc}	0.73±0.03^{cdefghijk}	pale
121	36.50±2.12^{bcd}	115.00±21.21^{abcd}	0.54±0.17^{abcdef}	pale
123	47.00±0.00^{cde}	102.50±3.54^{abcd}	0.80±0.00^{fghijk}	pale
124	54.50±0.71^{de}	137.50±3.54^{bcd}	0.82±0.06^{ghijk}	pale
125	51.50±0.71^{de}	127.50±3.54^{bcd}	0.75±0.14^{cdefghijk}	pale
132	46.50±4.50^{cde}	114.50±23.33^{abcd}	0.77±0.01^{efghijk}	pale
134	44.50±3.54^{cde}	119.50±17.68^{bcd}	0.81±0.08^{fghijk}	pale
136	45.50±3.54^{cde}	92.50±38.89^{abc}	0.77±0.01^{defghijk}	pale
143	39.50±2.12^{bcd}	107.50±38.89^{abcd}	0.79±0.02^{efghijk}	pale
Year				
2008	42.71±9.90^{a}	122.85±26.21a	0.72±0.15^{a}	
2009	43.22±9.87^{a}	119.02±26.36^{a}	0.73±0.14^{a}	

Different letters in the same row are statistically significant at $p > 0.05$.
SD-standard deviation
Analysis of variance (ANOVA) and the Tukey HSD test were used for statistical evaluation (software - Statistica 7.0 CZ).

Table 3. Morphological evaluation of amaranth

important for mechanized harvest by combine harvester. Lower plants with mean inflorescence are better for grain production and mechanized harvest. From our collection it is for example accession '120' with 95.00±42.43 cm height and 51.50±2.12 cm length of inflorescence. Taller genotypes are useful to develop varieties for feed utilization (Wu eta l., 2000). On the other hand, plant height could be influenced by increasing of number of plant per m^2 (Jarošová et al., 1997). The value of weight of thousand seeds (WTS) is shown in table 3. In the relation with seed colour is clear, that the biggest WTS was observed in pale seeded samples. The seed size of the genera ranges from 0.37 to 1.21 g per 1000 seed weight according to Espitia-Rangel (1994). He noted that the low value corresponding to wild and weedy species and the high values to cultivated grain species. In our experiments the WTS ranged from 0.39 to 0.96 g.

3.2 Protein content and content of protein fractions

The results of the protein content analysis showed that the highest protein content (17.32 ± 0.82%) had *A. cruentus*accessions followed by *A. caudatus* (17.24±0.65%) and *A. hypochondriacus* (16.89±0.80%). It corresponds with other published data. Segura-Nieto et al. (1994) published, that the range of protein content is following: *A. cruentus* 13.2 – 18.2%, *A. hypochondriacus* 17.9% and *A. caudatus* 17.6 – 18.4%. The range of the total protein content into our collection (12.43 – 17.33%) was similar to the results of other authors investigating various amaranth genotypes (Barba de la Rosa et al., 2009). The amaranth albumins, globulins and prolamins formed 9.2 – 14.65%, 9.78 – 13.81% and 1.76 – 3.3% of total seed protein, respectively (Table 4). The glutelins with the residual nitrogen were the most abundant. It was in accordance with the results of Bressani & Garcia-Vela (1990) and Bejosano & Corke (1999a). The very low content of prolamins (1.76 – 3.3%) confirmed the results of several authors (Gorinstein et al., 1991a; Bejosano & Corke, 1999a; Petr et al., 2003). However, another group of authors reported several times more prolamins (Correa et al., 1986; Zheleznov et al., 1997; Vasco-Mendez & Paredes-Lopez, 1995). The differences between the results of these two groups of authors might be due to the different extraction methods (Fidantsi & Doxastakis, 2001). Significant differences between black, pale and pink coloured seeds in the content of albumins were detected. Content of albumins of the black seeded group (9.64 ± 0.40%) was significantly lower (p-value 4.10^{-3}) than of the pale seeded group (13.21 ± 1.45%) and also lower than of the pink seeded group (11.39 ± 0.00; p-value 2.10^{-2}). Bresani & Garcia-Vela (1990) did not observed any differences in the protein fractions distribution among species or cultivars of the same species, independent of the fractionation sequence used. However, our results showed that the black seeded varieties had the lowest albumin content. No significant differences in other protein fractions were detected.

	Seed colour			
	black	**pale**	**pink**	**range**
WTS (g)	0.60 ± 0.12	0.79 ± 0.09	0.54 ± 0.04	0.39 - 0.96
Protein content in %	15.69 ± 0.60	16.69 ± 0.78	16.04 ± 0.00	12.43 – 17.33
Albumins	9.64 ± 0.40	13.21± 1.45	11.39 ± 0.00	9.2 – 14.65
Globulins	10.92 ± 0.78 1	11.76 ± 1.72	10.75 ± 0.00	9.78 – 13.81
Prolamins	2.37 ± 0.82	2.68 ± 0.44	2.00 ± 0.00	1.76 – 3.3
Glutelins + residual nitrogen	77.07 ± 0.33	72.35 ± 2.67	75.86 ± 0.00	69.13 – 77.44

Table 4. Total seed protein content and protein fraction content (in % of DW) of investigated accessions with respect the seed colour.

3.3 Methodical approach to protein extraction

According to our results, the chip capillary electrophoresis could replace the standard SDS-PAGE procedure, because it produced comparable results and what is more it could be performed routinely also in small laboratories thanks to its rapid performance. On the other hand, the chip capillary electrophoresis showed wider range of proteins spectra (up to 260 kDa).

The test of different concentrations was used for selection of the best extraction approach for chip and SDS-PAGE electrophoresis. By the chip capillary electrophoresis, the bulked samples of 100 seeds in 400 µl of extraction buffer were also tested. The chip capillary electrophoresis showed the high sensitivity and therefore the high concentration of the protein in the main bands resulted in their illegility. The protocol of chip electrophoresis does not provide many possibilities to chase the loaded amount of the sample. The satisfactory results of the chip electrophoresis brought the use of the single seeds.

For the SDS PAGE there were used single seed samples, bulked samples of 10 seeds extracted in 50 and 100 µl and bulked samples of 100 seeds extracted in 200 and 400 µl of extraction solution were used. The protein patterns of the samples extracted from the single seeds did not show the intensity required for the analysis of all the bands in the spectra (Figure 1). On the other hand, samples obtained by extraction of 10 seeds in 50 µl and 100 seeds extracted in 200 µl of extraction solution did not show clearly separated bands, what resulted in their illegility. In comparison with the spectra of the less concentrated samples (single seeds, 10 seeds in 100 µl of extraction solution), the main bands of the more concentrated samples were thick and joined together. The bands, which were in the less concentrated samples less intensive, were expressed so intensively that formed dark background what resulted in the impossibility of identification of the individual bands in the protein spectra. The protein spectra of the samples obtained by the extraction of 100 seeds in 400 µl were also over expressed, but the less intensive bands did not form the background, so the mayor bands were more easily identified, but several mayor bands joined together.

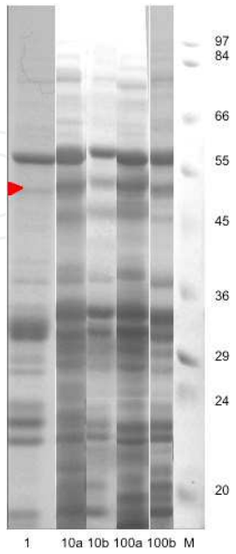
As the best approach for the total seed storage protein extraction for classical SDS-PAGE we selected bulked samples of 10 seed extracted in 100 µl of extraction solution. The bulked samples of 10 seeds extracted in 100 µl to be the most suitable tools, because of their clear expression of protein patterns and moreover they can be used when samples with higher number of seeds are not available. This selected approach differed from methodology selected by Drzewiecki (2001) who used 50 µl or by Gorinstein et al. (2005) who used 62.5 µl of extraction solution for 10 seeds bulked samples. The need for using more extraction solution in our study might be to consequence of higher protein extraction as a result of the proper seed crushing performed in our study which was not mentioned in the methodology description of other authors (Drzewiecki, 2001; Gorinstein et al., 2005).

When using total seed storage protein spectra for accessions identification by chip electrophoresis the single seed samples with several repetitions showed up as the best approach. These results were with accordance with Bradova & Matejova (2008) that compared whole seed storage proteins of wheat.

3.4 Polymorphism of the glutelins

The electrophoresis of the glutelin fraction is widely used for crop varieties identification. There were published several articles about wheat (Matejova&Bradova, 2008; Dutta et al.,

2011), rice (Gorinstein et al., 2003), barley (Smith & Simpson, 1983), lupine (Vaz et al., 2004) etc. varieties identification based on glutelin patterns. Similarly amaranth glutelins showed polymorphism not only in position of bands but also in their intensity.



1 - a single seed extracted in 18 µl,
10a - bulk of 10 seeds extracted in 50 µl,
10b - bulk of 10 seeds extracted in 100 µl,
100b - bulk of 100 seeds extracted in 400 µl of the extraction solution.
M - wide range protein marker (bands in kDa).

Fig. 1. SDS – PAGE spectra of total seed storage proteins of sample obtained by different extraction approaches.

In the cluster dendrogram (Figure2), there were clearly separated the grain and the wild monoecious and the wild dioecious accessions. All investigated amaranth species had in common three major bands of the MW 21 – 23 kDa, but remarkable differences in the rest of the spectra were the reason for the segregation into three main clusters. The glutelin spectra of the grain amaranth varieties were very similar to the total seed storage protein patterns, but the main polymorphic bands were better distinguished because of the washing off the first three fractions during fraction separation procedure which probably formed the "background" of the spectra. The principal polymorphism was detected in following band positions 38, 39, 54, 58, 60, 64 and 65 kDa with three intensity levels (1-3). The amaranth glutelins showed up as the most abundant protein fraction by SDS-PAGE analysis also in the study of Bejosano&Corke (1999). The division of the grain amaranth glutelins into three major groups reported also Gorinstein et al. (2004) and Barba de la Rosa et al. (2009).

Figure 2 indicated three well defined clusters: grain species, monoecious wild species and dioecious wild species. The grain species *A. cruentus*, *A. hypochondriacus*, *A. caudatus* closely matched together with one sample *A. mantegazzianus.* There were clearly segregated clusters with the wild monoecious species (*A.wrightii*, *A. delfexus* and *A. retroflexus*) and the wild dioecous species (*A. australis*, *A. cannabinus* and *A. tuberculatus*).

A. caudatus group presentedwas two accessions '21' and '101' characterized by the dark band 60 kDa and the light band 39 kDa in their glutelin spectra. The *A. cruentus* cluster was clearly separated in the dendrogram of hierarchical distancing by the presence of the dark band of 58 kDa and of the light band in the position of 39 kDa. *A. hypochondriacus* accessions were characterized by the lack of any band in the position 58 kDa and by the presence of the dark band 54 kDa and the light band 38 kDa. The typical band (in the position 54 kDa) used for *A. hypochondriacus* recognition was qualified as characteristic for *A. hypochondriacus* by several authors (Drzewiecki, 2001; Marcone, 2002; Gorinstein et al., 2005), but its position was determined differently: as 55 kDa (Marcone, 2002) or 52 kDa (Drzewiecki, 2001) or in the case of protein fractions as 55 kDa, too (Thanapornpoonpong et al., 2008). The characteristic presence of the band 58 kDa in *A. cruentus* spectra and of the band 54 kDa in *A. hypochondriacus* spectra was confirmed by the results of Thanapornpoonpong et al. (2008).

Some of the accessions possessed extra light band of 65 kDa and were aggregated close to the *A. hypochondriacus* cluster. Their similarity to the other *A. hypochondriacus* varieties was expressed by very high correlation 0.987.

The dark band of 54 kDa, the dark band of 64 kDa and the light band in the position 65 kDa showed up in the glutelin spectra of the accession '134'. The accession '80' had the same glutelin spectra, but its band of 54 kDa was of medium intensity. These two varieties might be the hybrids of *A. hypochondriacus* and other unknown species which could have dark band of 64 kDa and light band of 39 kDa or they might be *A. hypochondriacus* varieties with some special properties that were not considered in our study. The accessions '132' with the dark band of 60 kDa typical for *A. caudatus* accessions was also present in the spectra and therefore the correlation between these accessions and the *A. caudatus* accessions was as high as 0.911. These accessions also showed the light band of 38 kDa and the medium intensity band of 54 kDa (typical marker for *A. hypochondriacus* spectra).

The dioecious wild species *A. australis*, *A. cannabinus* and *A. tuberculatus* formed a totally distinct cluster. They possessed several major dark bands of lower molecular weight 32 - 50 kDa. From this group, *A. cannabinus* and *A. australis* were the most similar, their correlation was 0.675. The monoecious wild species (*A. wrightii*, *A. deflexus* and *A. retroflexus*) and the dioecious wild species had in common one light band in the position of 65 kDa. The major dark bands of the monoecious wild species were of MW 29 - 66 kDa. The spectra of the monoecious wild species had some similarities with the spectra of the grain species. The grain species spectra were characterized by the two bands of MW 31 and 33 kDa while in the spectra of *A. retroflexus* these bands were just "shifted up" to MW 32 and 34 kDa. Protein fractions spectra of the wild species had not been published yet by other researchers. The results indicated the high correlation of the spectra of *A. retroflexus* and *A. wrightii* what confirmed the similarity observed by the first morphological descriptions made by Watson (1877).

Accessions possessing several bands of different intensities in the polymorphic area were qualified as the hybrid accessions. The accession '99' had in its spectra several bands in the polymorphic area: the dark band of 54 kDa, light band of 58 kDa, medium intensity band in the position of 60 kDa and the light band of 65 kDa. Its similarity with *A. hypochondriacus* was expressed as correlation 0.901 and to the accession '95'. The accession '95' differed from the accession '99' just in the intensity of the bands of 58 kDa and 60 kDa (correlation 0.971). Varieties '62' and '110' were designated as hybrid varieties. They had the both bands of 54 kDa (marker for *A. cruentus*) and 58 kDa (marker for *A. hypochondriacus*) of medium

intensity. Moreover, they possessed the light band of 39 kDa. The presence of the light band 39 kDa (typical marker for *A. cruentus*) was the reason for their higher correlation with *A. cruentus* group (0.920) than with A. *hypochondriacus* group (0.892). The variety '111' was exceptional. Moreover, it had higher correlation with *A. hypochondriacus* varieties (0.960).

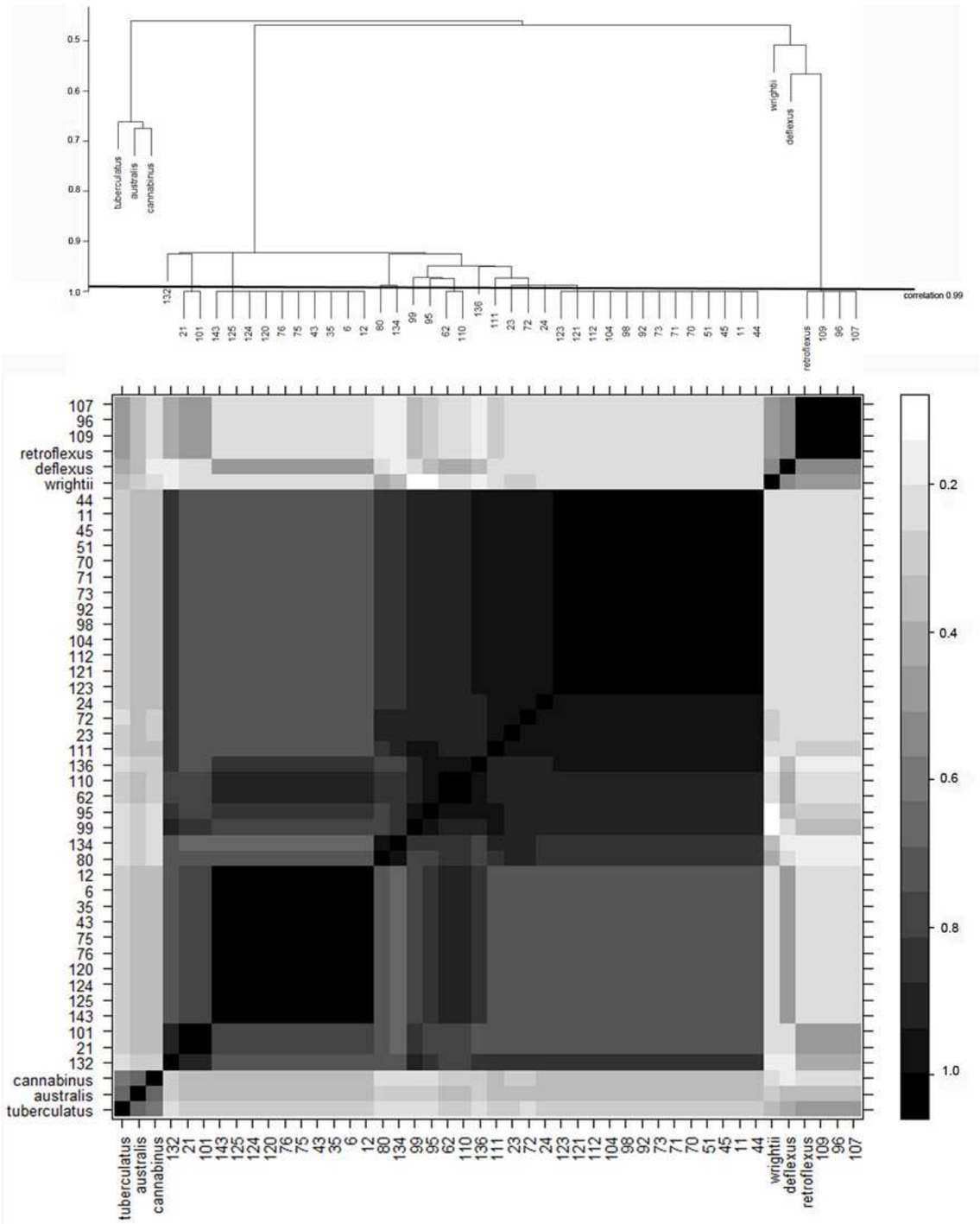


Fig. 2. Relations among amaranth samples expressed by Pearson correlation in dendrogram

4. Conclusion

Amaranth is mostly named as a crop of the future. Due to very good contents of protein, oil and many components with positive effects to humans, it is one of the promising crops. In the Czech Republic, there was interest of amaranth growing in the fields and the consumption of amaranth products is increasing as well. Most of grain raw material is imported to the Czech Republic from other countries, but there is increasing demand of Czech amaranth production. For amaranth cultivation it is necessary to know, what species could be grown. Because amaranth is not native in Europe, we have to receive seeds from other sides. In Czech legislation act about invasive weeds exists. Several amaranth species are included in this Act. In order to avoid cultivation of weedy amaranths, it is necessary to know the characteristics of the cultivated species and do not confuse them. Due to vegetable and weedy amaranth have black seed colour, it is impossible to use this trait as a marker. Amaranth glutelins were the best tool for the amaranth species identification, because they showed high polymorphism not only in position of bands but also in their intensity. The method used here was based on the data concerning the relative intensity and the position of the bands in the glutelin spectra obtained by the chip capillary electrophoresis what resulted in the exact similarity calculation of the protein fraction spectra and thus in the segregation of the cultivated grain species, the monoecious wild species and the dioecious wild species into three separate clusters. Each of the grain amaranth species was characterized by one dark band in the polymorphic region (54 – 65 kDa), while the hybrids possessed more bands of different relative intensity. The study brought several new contributions to the amaranth genetic research and is a very useful tool for species identification before cultivation in the field conditions. Unfortunately, this method is not so sensitive for individual amaranth genotype identification. We work on it in our current tasks.

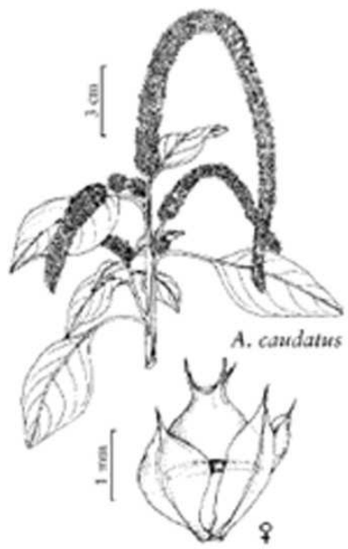


Fig. 3. *A. caudatus* (Standley, 1949)



Fig. 4. *A. hypochondriacus* (NRC, 1984)



Fig. 5. *A. cruentus* (Bojian et al., 2003)



Fig. 6. *A. cannabinus* (Standley, 1949)

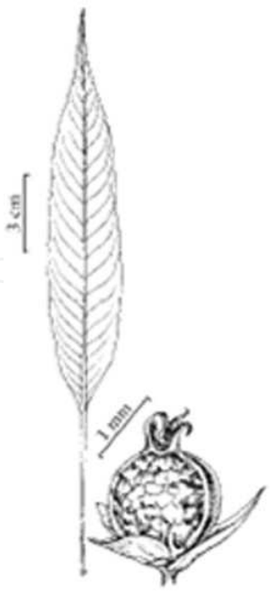


Fig. 7. *A. australis* (Standley, 1949)



Fig. 8. *A. retroflexus* (Standley, 1949)



Fig. 9. *A. tuberculatus* (Standley, 1949)

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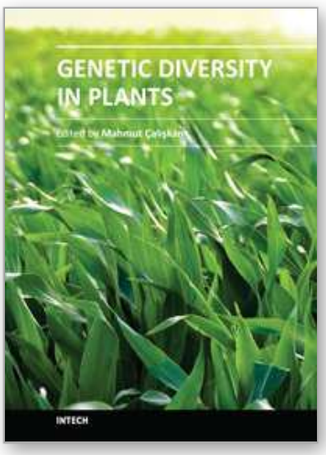
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Genetic diversity is of fundamental importance in the continuity of a species as it provides the necessary adaptation to the prevailing biotic and abiotic environmental conditions, and enables change in the genetic composition to cope with changes in the environment. Genetic Diversity in Plants presents chapters revealing the magnitude of genetic variation existing in plant populations. 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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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Phone: +86-21-62489820
Fax: +86-21-62489821

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