Enrichment of Chickpea Genetic Resources Collection Monitored by Microsatellites

Klaudia JOMOVÁ[1], Michaela BENKOVÁ[2] and Ján KRAIC[1,2]

[1]Constantine the Philosopher University, Nitra, Slovakia; [2]Research Institute of Plant Production, Piešťany, Slovakia

Abstract: A set of 49 chickpea accessions maintained in the collection of the Genebank of the Slovak Republic was analyzed using microsatellite markers. The level of genetic diversity and the effectivity of new germplasm acquisition for the collection enrichment were evaluated. Five primer pairs used generated 50 different amplified alleles. Four of them, TA2, TA5, TR1, and TR7, containing a long TAA-tandem repeat, were polymorphic with 11–13 alleles per locus. A single fragment was obtained from all the accessions with the primer pair CATPER flanking a small microsatellite repetition in the mRNA sequence of chickpea cationic peroxidase. The genetic diversity was expressed as a diversity index (DI) and polymorphic information content (PIC) with values in the range from 0.885 to 0.904 and from 0.972 to 0.991, respectively. Recently acquired new germplasm contributed 13 new alleles to the entire collection, i.e. almost 70% of microsatellite diversity originated from the recently acquired accessions. Remarkable is also, that one accession carries a unique allel, not present in the remaining collection. Microsatellite analysis revealed the importance of collecting missions for the enrichment of collections of genetic resources.

Keywords: chickpea; *Cicer arietinum* L.; microsatellite; germplasm; acquisition

The chickpea (*Cicer arietinum* L.) is a legume crop influencing the food supply chain especially in warmer and drier regions, e.g. in countries of the South and West Asia, North and East Africa, South Europe, Australia, and America. This crop is not a dominant legume species in Slovakian agriculture where other grain legumes such as bean and pea predominate. Nevertheless, demand for chickpea is growing here at the present time also due to the interesting nutritive value of seeds and changing climate creating favourable conditions for chickpea cultivation. Moreover, chickpea seeds are considered as an important source of minerals and vitamins necessary for human and animal nutrition and provide a protein-rich supplement to the diet of vegetarians.

The chickpea genetic resources collection maintained in the Genebank of the Slovak Republic, Piešťany, comprises predominantly foreign accessions (71%), in addition to a limited number of Slovak landraces and registered cultivars (only 7%). From the aspect of effective management of the plant genetic resources it is beneficial if the acquisition of any new accessions into the established collection is associated also with proteome and genome based analysis using molecular markers besides morphological and agronomical analyses. This type of characterization has lately been common in plant "genebanking" (Karp *et al.* 1997; Tanksley & McCouch 1997; Cooke & Reeves 2003). The requirements for chickpea accession characterization at the molecular marker level also result from the threat of duplications maintenance, genetic erosion due to the loss of domestic accessions and the sensitivity of plants to environmental stresses as well. It is extremely important to study

the genetic composition of existing genetic resources (lines, cultivars, landraces, wild relatives, progenitors) because they provide a chance to find new and useful genes or alleles.

The choice of a molecular technique depends on plant species and content of expected genetic variation in the framework of the relevant collection. Cultivated *Cicer arietinum* L. is a self-pollinated plant species with low intra- and inter-population variation (Crawford 1990). This was also demonstrated in several studies performed with isoenzymes (Labdi *et al.* 1996; Ahmad *et al.* 2004), seed storage proteins (Ahmad & Slinkard 1992), and RFLPs (Udupa *et al*. 1993, Singh *et al*. 2008). PCR-based techniques and revealing of microsatellite sequences in plant genomes helped to overcome problems with low polymorphism detected also in cultivated chickpea. The sequence-tagged microsatellite sites (STMS), expressed sequence-tags (EST), and amplified fragments of length polymorphism (AFLP) markers for chickpea are accessible at the present time (Hüttel *et al*. 1999; Winter *et al*. 1999; Udupa *et al*. 1999; Buhariwalla *et al*. 2005; Nguyen *et al*. 2004, Cingilli *et al*. 2005). The conservativeness of microsatellite flanking regions in the genus *Cicer* L. gives the possibility to use already available microsatellite chickpea markers at the polymorphism detection in other chickpea accessions (Choumane *et al*. 2000; Sethy *et al*. 2006). The preferred markers for these pur-

Table 1. Analyzed chickpea (*Cicer arietinum* L.) accessions

No.	Accession code/name	Origin	No.	Accession code/name	Origin
1	Flip 84-22	Syria	26	VSP 21	India
2	Flip 84-60 C	Syria	27	VSP 22	India
3	Flip 84-81 C	Syria	28	VSP 23	India
4	Flip 84-82 C	Syria	29	VSP 24	India
5	Flip 84-96 C	Syria	30	VSP 26	India
6	Flip 84-148 C	Syria	31	VSP 27	India
7	Flip 84-149 C	Syria	32	VSP 28	India
8	Flip 84-155 C	Syria	33	ILC 613	Syria
9	Flip 84-159 C	Syria	34	ILC 620	Syria
10	Flip 85-42 C	Syria	35	187 (Sitno 97)	Slovakia
11	Flip 85-64 C	Syria	36	135 (Sitno 97)	Slovakia
12	Flip 85-88 C	Syria	37	Krajová z Kráľovej	Slovakia
13	Flip 85-126 C	Syria	38	Baku	Azerbaijan
14	VSP 1	India	39	Maškovský Bagovec	Slovakia
15	VSP 3	India	40	Slovák	Slovakia
16	VSP 4	India	41	018 Považie 96	Slovakia
17	VSP 5	India	42	Flip 85-17 C	Syria
18	VSP 6	India	43	ICCC 8	Syria
19	VSP 7	India	44	Flip 85-112	Syria
20	VSP 11	India	45	VSP 29	India
21	VSP 12	India	46	Flip 84-68 C	Syria
22	VSP 13	India	47	Flip 146 C	Syria
23	VSP 14	India	48	Flip 84-161 C	Syria
24	VSP 18	India	49	VSP 17	India
25	VSP 19	India			

poses are single-locus co-dominant microsatellite markers whose usefulness in chickpea has already been reported (Udupa *et al*. 1999; Cingilli *et al*. 2005). These markers helped to overcome limited polymorphism within cultivated chickpea. The STMS chickpea marker map is accessible with several hundreds of STMS markers (Hűttel *et al*. 1999; Winter *et al*. 1999; Tekeoglu *et al*. 2002) and the chickpea marker map including different molecular markers has been developed (Winter *et al*. 2000; Radhika *et al.* 2007).

The objectives of this study were to analyse the level of diversity at microsatellite loci within the monitored set of chickpea genetic resources, and to use this tool for the monitoring of diversity enrichment in a chickpea collection by new germplasm acquisitions.

MATERIAL AND METHODS

Forty-nine chickpea accessions obtained from the Genebank of the Slovak Republic at Piešťany were used for the study including one Slovak registered cultivar and six landraces collected across Slovakia (Table 1). Forty-two of them (21 originated from Syria and 21 from India) were added to the collection as accessions enriching the already existing national collection. Their way to the national collection was through germplasm exchange. During operations and adoption some of the accessions were relabelled. In additional, one genotype originated from Azerbaijan.

DNA was isolated from young leaves according to Dellaporta *et al*. (1993). Five pairs of primers flanking microsatellite sequences were used for amplifications (Table 2). Four of them (TA2, TA5, TR1, TR7) were described by Winter *et al*. (1999) as one of the earliest available chickpea microsatellite sequences. Moreover, these markers were effective in diversity study in a composite chickpea collection (Upadhyaya *et al*. 2008). The sequence of the fifth primer pair CATPER (5-3: ttctagagctattactttgct, tggtttgtaaggtcaatgat) was retrieved from the EMBL nucleotide sequence database (http://www.ncbi.nlm.nih.gov) and derived from a part of the mRNA sequence of chickpea cationic peroxidase.

PCR analyses were performed in the PTC-200 thermal cycler (MJ Research, USA) in a total volume of 20 µl, containing 25 ng of template DNA, 1 × PCR buffer, 1.5 mmol/dm^3 $MgCl_2$, 0.1 mmol/dm^3 of each primer, 0.1 mmol/dm^3 of dNTP mixture, and 0.8 U of *Taq*-DNA polymerase (Life Technologies, USA). An initial denaturation step at 94°C for 2 min followed by 35 cycles of 20 s at 94°C, 50 s at 55°C, and 50 s at 60°C. Final extended period was at 60°C for 5 min. Four microlitres of reaction mix were loaded and separated in 6% denatured polyacrylamide gel in a DNA sequencing unit (Scie-Plas TVS 1400, UK). DNA bands were visualised by silver staining (Bassam *et al*. 1991). The fragment sizes were determined using the molecular weight standards with 25 base pairs (bp) and 50 bp molecular ladders (Life Technologies, USA). Frequencies of incidence of all polymorphic alleles were calculated and used for determination of statistical parameters: diversity index (Weir 1997), probability of identity (Paetkau *et al*. 1995) and polymorphic information content (Weber 1990).

RESULTS AND DISCUSSION

Altogether fifty different, reproducible microsatellites have been amplified within the studied chickpea collection. No length variation was detected at the microsatellite locus CATPER where only one allele (211 bp) was analyzed. No variation

Table 2. Statistical data from the analyzed SSR loci of 49 chickpeas

SSR marker	Core motif	DI	PI	PIC
TR 1	$(TAA)_{31}$	0.900	0.024	0.976
TA 2	$(TAA)_{16}TGA(TAA)_{19}$	0.904	0.009	0.991
TA 5	$(TAA)_{29}$	0.885	0.028	0.972
TR 7	$(TAA)_{25}$	0.891	0.025	0.976
CATPER	$(ATT)_6$	–	–	–

DI – diversity index; PI – probability of identity; PIC – polymorphic information content

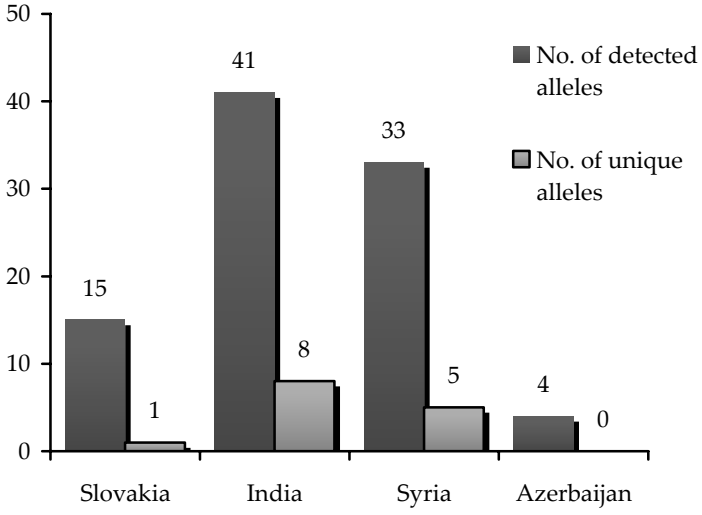

Figure 1. The number of detected alleles and the number of unique alleles in geographically based sub-collections within all 49 identified microsatellite alleles

at this locus in comparison with the other four can be related to the origin of this microsatellite in DNA coding sequence even though EST markers were used for the analysis of chickpea diversity (Buhariwalla *et al*. 2005). The remaining four microsatellite loci were polymorphic. We previously published data on the number of alleles per locus (11–13), size of alleles, and genetic similarity within this set of chickpeas (Jomová *et al*. 2005). Calculated values of diversity index (DI) ranged from 0.885 to 0.904, polymorphic information content (PIC) from 0.972 to 0.991 (Table 2), indicating high variation at the $(TAA)_x$ containing microsatellite loci in chickpea. This confirms observations of Winter *et al*. (1999) on abundance and polymorphism of TAA motif even between closely related chickpea species and Upadhyaya *et al*. (2008) during the study of the chickpea composite collection. We detected the highest values of DI and PIC at the TA2 locus similarly like in the study of Udupa *et al*. (1999). Low indices of probability (PI) values for desired microsatellite loci indicate a high potential of these markers for the discrimination of chickpea accessions within collections.

However, the main topic of our study was to exploit microsatellite polymorphism as a tool for the evaluation of enrichment within the national collection of chickpea genetic resources. This collection contained only forty-five accessions before the inclusion of a large set of new germplasms originating from Syria and India received in the nineties of the last century. The six Slovak chickpeas were used as representatives of the basic collection in the evaluation of enrichment, which possessed altogether fifteen, i.e. 30.6% of all forty-nine detected microsatellite alleles. Reversely, chickpeas from India (83.7%) and Syria (67.3%) possessed the highest number of alleles (Figure 1), supporting that just this germplasm is favourable for development and enhancement of the native collection. This common concept for genetic diversity extension, i.e. extensive germplasm collecting based on the eco-geographical principle, was suggested by Abbo *et al*. (2003). From the aspect of maintenance of rare and unique alleles there arise at least two other interesting questions. How does the acquisition of a new germplasm contribute to enrichment of the existing national gene pool? Can "domestic" germplasm also possess some unique alleles? Thirty-five out of 49 alleles in our study were common for chickpeas regardless of their geographic origin. The Indian and Syrian chickpeas obtained by germplasm exchange contributed altogether 13 new, unique alleles (Figure 1) to the entire collection. Remarkable is also that a small subgroup of Slovakian chickpeas possesses one unique allele which could be born during the breeding process as a consequence of the transition of alleles from parental genotypes or by mutation process. It confirms an important role of the national genebank, i.e. collecting and protection of the original germplasm on the one hand and hunting and acquisition of new gene resources for further exploitation on the other hand. Better understanding of the new germplasm acquisition and comprehensiveness of domestic germplasm are demonstrated in Figure 2a–d. The new germplasm brought unique or very low frequent alleles at all four loci and highly enriched the genetic diversity of the primary collection especially at the TA2 locus, where twelve new

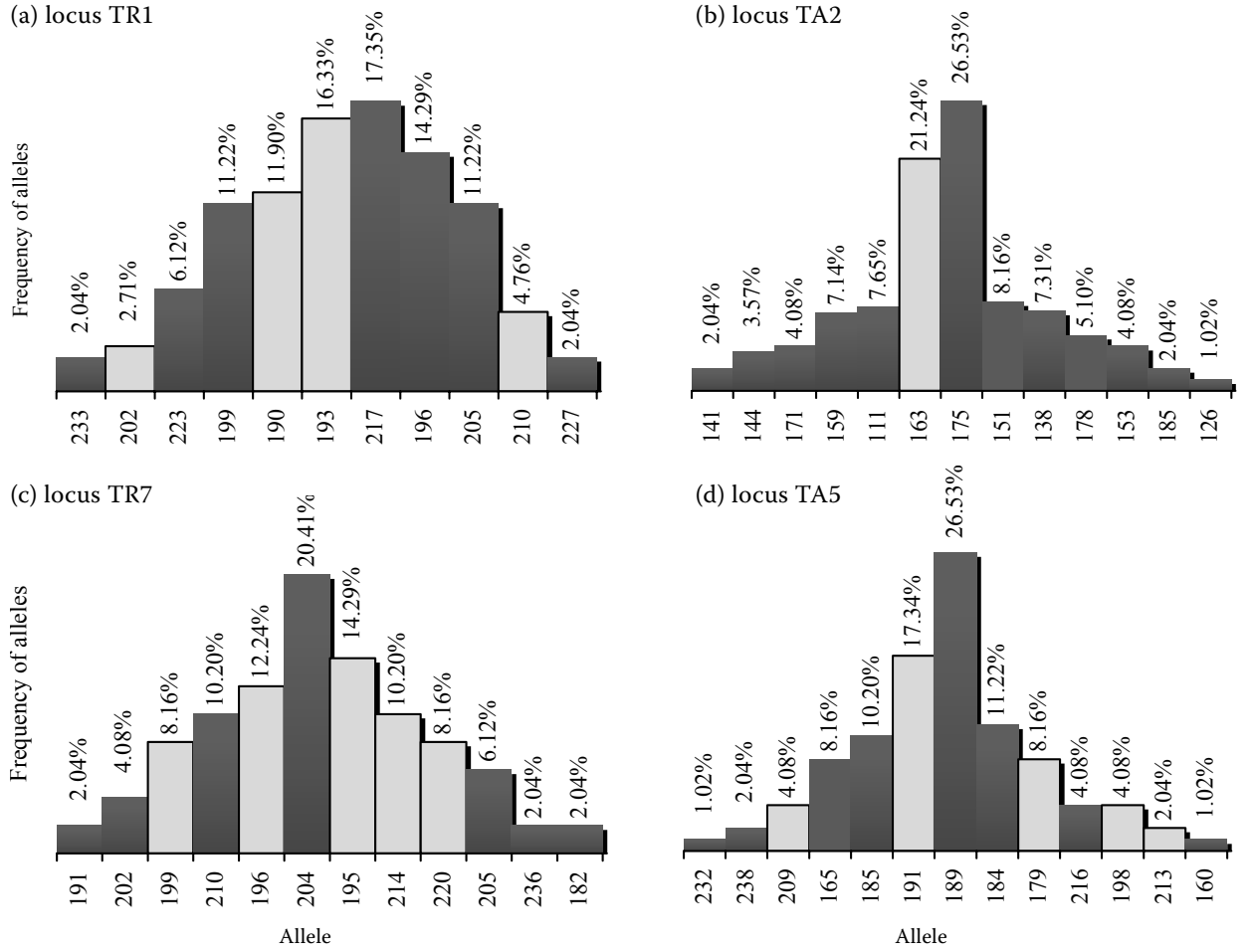

Figure 2. Frequency of alleles at four microsatellite loci (grey columns represent Slovak accessions, black columns accessions from other countries)

alleles were gained and the domestic germplasm was very narrow (Figure 2b). Distribution of allele frequency was similar at all four loci. One or two alleles usually dominated over others and alleles with low frequency (bellow 5%) and unique alleles were also detected. Our results support the idea that microsatellites can report the status of genetic diversity in the maintained national chickpea collection but could be applied broadly to the monitoring of collection development.

Another common problem in genetic resources collecting is the redundancy of identical samples, i.e. duplicates. In our previous study (Jomová *et al*. 2005) we observed that three pairs from the identical set of chickpeas were undistinguishable. It was based on this hypothesis: if no marker differentiating two genotypes is available, the genotypes have the same genetic constitution. These are regarded as being identical, and can be considered as duplicates. However, the number of markers used in our study was too low for the unambiguous confirmation of duplicates. Virk *et al*. (1995) estimated that an adequate number of polymorphic markers to detect at least one difference between the suspected pairs of rice duplicates at 99% probability was twenty-six. Even more molecular markers were used by Shan *et al*. (2007) for the verification of chickpea duplicates and they suggested an AFLP technique for the identification of duplicates.

The improvement of collections of crop genetic resources in genebanks is based on the acquisition of germplasm by different approaches mainly including collecting missions, exchange of material, and adoption from public or commercial breeding programmes. Methods of directed construction of collections based on consistent selection of new and available germplasm by curators are used. The process of selection is usually based on the evaluation of morphological and agronomical traits,

phytopathological characteristics, and breeding value. Optimal approaches should combine both commercial and diversity aspects, which meets the demands of plant breeders as well as conservationists (van Treuren *et al*. 2008). These authors used and suggested microsatellite markers for marker-assisted optimization of strategy for the acquisition of modern lettuce cultivars (germplasm) into a genebank collection. Rao (2004) reviewed the exploitation of different biotechnological tools, including molecular markers, in plant genetic resources management. Results of our study support their applicability; moreover, microsatellites can provide effective molecular tools for the study of new germplasm acquisition activities.

Acknowledgement. This study was supported by the Ministry of Agriculture of the Slovak Republic, Projects No. 27-11 and No. 2003 SP 27/028 OD 01/028 OD 01.

References

Abbo S., Berger J., Turner N.C. (2003): Evolution of cultivated chickpea: four bottlenecks limit diversity and constrain adaptation. Functional Plant Biology, **30**: 1081–1087.

Ahmad F., Slinkard A.E. (1992): Genetic relationships in the genus *Cicer* L. as revealed by polyacrylamide gel electrophoresis of seed storage proteins. Theoretical and Applied Genetics, **84**: 688–692.

Ahmad F., Gaur P.M., Slinkard A.E. (2004): Isozyme polymorphism and phylogenetic interpretations in the genus *Cicer* L. Theoretical and Applied Genetics, **83**: 620–627.

Bassam B.J., Caetano-Anolles G., Gresshoff P.M. (1991): Fast and sensitive silver staining of DNA in polyacrylamide gels. Analytical Biochemistry, **196**: 80–83.

Buhariwalla H.K., Jayashree B., Eshwar K., Crouch J.H. (2005): Development of ESTs from chickpea roots and their use in diversity analysis of the *Cicer* genus. BMC Plant Biology, **5**: 16.

Cingilli H., Altinkut A., Akcin A. (2005): The use of microsatellite markers in the annual and perennial *Cicer* species growing in Turkey. Biology, **60**: 93–98.

Choumane W., Winter P., Weigand F., Kahl G. (2000): Conservation and variability of sequence-tagged microsatellite sites (STMSs) from chickpea (*Cicer aerietinum* L.) within the genus *Cicer*. Theoretical and Applied Genetics, **101**: 269–278.

Cooke R.J., Reeves J.C. (2003): Plant genetic resources and molecular markers: variety registration in a new era. Plant Genetic Resources: Characterization and Utilization, **2–3**: 81–87.

Crawford D.J. (1990): Plant Molecular Systematics: Macromolecular Approaches. John Wiley & Sons Canada, Ltd., New York.

Dellaporta S.L., Wood J., Hicks J.B. (1993): A plant DNA minipreparation: Version II. Plant Molecular Biology Reporter, **4**: 19–21.

Hüttel B., Winter P., Weising K., Choumane W., Weigand F., Kahl G. (1999): Sequence-tagged microsatellite site markers for chickpea. Genome, **42**: 210–217.

Jomová K., Benková M., Žáková M., Gregová E., Kraic J. (2005): Clustering of chickpea (*Cicer arietinum* L.) accessions. Genetic Resources and Crop Evolution, **52**: 1039–1048.

Karp A., Kresovich S., Bhat K.V., Ayad W.G., Hodgkin T. (1997): Molecular tools in plant genetic resources conservation: a guide to the technologies. IPGRI Technical Bulletin No. 2, International Plant Genetic Resources Institute, Rome.

Labdi M., Robertson L.D., Singh K.B., Charrier A. (1996): Genetic diversity and phylogenetic relationships among the annual *Cicer* species as revealed by isozyme polymorphism. Euphytica, **88**: 181–188.

Nguyen T.T., Taylor P.W.J., Redden R.J., Ford R. (2004): Genetic diversity estimates in *Cicer* using AFLP analysis. Plant Breeding, **123**: 173–179.

Paetkau D., Calvert W., Stirling I., Strobeck C. (1995): Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology, **4**: 347–354.

Radhika P., Gowda S.J.M., Kadoo N.Y., Mhase L.B., Jamadagni B.M., Sainani M.N., Chandra S., Gupta V.S. (2007): Development of an integrated intraspecific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations. Theoretical and Applied Genetics, **115**: 209–216.

Rao N.K. (2004): Plant genetic resources: Advancing conservation and use through biotechnology. African Journal of Biotechnology, **3**: 136–145.

Sethy N.K., Shokeen B., Edwards K.J., Bhatia S. (2006): Development of microsatellite markers and analysis of intraspecific genetic variability in chickpea (*Cicer arietinum* L.). Theoretical and Applied Genetics, **112**: 1416–1428.

Shan F., Clarke H.J., Yan G., Plummer A., Siddique K.H.M. (2007): Identification of duplicates and fingerprinting of primary and secondary wild annual *Cicer* gene pools using AFLP markers. Genetic Resources and Crop Evolution, **54**: 519–527.

Singh A., Devarumath R.M., Ramarao S., Singh V.P., Raina S.N. (2008): Assessment of genetic diversity, and

phylogenetic relationships based on ribosomal DNA repeat unit length variation and Internal Transcribed Spacer (ITS) sequences in chickpea (*Cicer arietinum*) cultivars and its wild species. Genetic Resources and Crop Evolution, **55**: 65–79.

Tanksley S., McCouch S.R. (1997): Seed banks and molecular maps: unlocking genetic potential from the wild. Science, **277**: 1063–1066.

Tekeoglu M., Rajesh P.N., Muehlbauer F.J. (2002): Integration of sequence tagged microsatellite sites to the chickpea genetic map. Theoretical and Applied Genetics, **105**: 847–854.

Udupa S.M., Sharma A., Sharma R.P., Pai R.A. (1993): Narrow genetic variability in *Cicer arietinum* L. as revealed by RFLP analysis. Journal of Plant Biochemistry and Biotechnology, **2**: 83–86.

Udupa S.S., Robertson L.D., Weigand F., Baum M., Kahl G. (1999): Allelic variation at $(TAA)_n$ microsatellite loci in a world collection of chickpea (*Cicer arietinum* L.) germplasm. Molecular and General Genetics, **261**: 354–363.

Upadhyaya H.D., Dwivedi S.L., Baum M., Varshney R.K., Udupa S.M., Gowda C.L.L., Hoisington D., Singh D. (2008): Genetic structure, diversity, and allelic richness in composite collection and reference set in chickpea (*Cicer arietinum* L.). BMC Plant Biology, **8**: 106.

van Treuren R., van Hintum T.J.L., van de Wiel C.C.M. (2008): Marker-assisted optimization of an expert-based strategy for the acquisition of modern lettuce varieties to improve a genebank collection. Theoretical and Applied Genetics, **55**: 319–330.

Virk P.S., Newbury H.J., Jackson M.T., Ford-Lloyd B.V. (1995): The identification of duplicate accessions within a rice germplasm collection using RAPD analysis. Theoretical and Applied Genetics, **90**: 1049–1055.

Weber J.L. (1990): Informativeness of human (dC-dA)n.(dG-dT)n polymorphisms. Genomics, **7**: 524–530.

Weir B.S. (1997): Genetic Data Analysis II: Methods for Discrete Population Genetic Data. Sinauer Associates, Sunderland.

Winter P., Pfaff T., Udupa S.M., Huttel B., Sharma,P. C., Sahi S., Arreguin-Espinoza R., Weigand F., Muehlbauer F.J., Kahl G. (1999): Characterization and mapping of sequence-tagged microsatellite sites in the chickpea (*Cicer arietinum* L.) genome. Molecular and General Genetics, **262**: 90–101.

Winter P., Benko-Iseppon A.M., Hüttel B., Ratnaparkhe M., Tullu A., Sonnante G., Pfaff T., Tekeoglu M., Santra D., Sant V.J., Rajesh P.N., Kahl G., Muehlbauer F.J. (2000): A linkage map of the chickpea (*Cicer arietinum* L.) genome based on recombinant inbred lines from a *C. arietinum* × *C. reticulatum* cross: localization of resistence genes for fusarium wilt races 4 and 5. Theoretical and Applied Genetics, **101**: 1155–1163.

Received for publication September 8, 2008
Accepted after corrections January 30, 2009

Corresponding author:

Doc. RNDr. Klaudia Jomová, PhD., Univerzita Konštantína Filozofa, Fakulta prírodných vied, Katedra chémie, Trieda Andreja Hlinku 1, 949 74 Nitra, Slovenská republika
tel.: + 421 376 408 656, e-mail: kjomova@ukf.sk