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GENOMIC CHARACTERIZATION OF THE CHILI PEPPERS (*CAPSICUM SOLANACEAE*) GERMPLASM BY CLASSICAL AND MOLECULAR CYTOGENETICS

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Abstract

Within the framework of the IAEA coordinated research project entitled 'Physical mapping technologies for the identification and characterization of mutated genes contributing to crop quality' we carried out genomic characterization of wild and cultivated samples of chilli peppers (genus *Capsicum*) by classical chromosome staining methods (AgNOR and fluorescent chromosome banding) and fluorescent in situ hybridization (FISH). For the first approach, fluorochromes with affinity for specific chromosome regions were used, i.e. chromomycin A3 (CMA) and diamidino-phenyl-indole (DAPI) which have preference for GC-rich and AT-rich regions, respectively. In addition, Ag-staining to detect active nucleolus organizing regions was applied. The heterochromatin could be characterized in respect to type, amount and distribution in the different accessions examined. On the other hand, the number and position of active NORs could be determined. Using FISH, different DNA probes were used in order to map specific sequences in the chromosomes, i.e. 45S and 5S rDNA, telomeric sequences and cloned restriction fragments of repetitive nature. As an example of the work done, we present the results obtained on a sample of *Capsicum annuum* var. *annum* (cultivar NMCA 10272), the most broadly exploited cultivar of chilli pepper. The results allowed us to characterize the *Capsicum* species and accessions and the possible evolutionary pathways for chilli peppers was deduced based on the available cytogenetic data. It is worth mentioning that the research work done under this CRP is part of work being done within an exsting network of chilli pepper research of this important plant group utilized by man and among one of the first cultivated plants in the history of humanity.

1. INTRODUCTION

Solanaceae is an economically important family because it includes several plants of interest for food, drug, stimulant and ornamental uses. On the other hand, some species are toxic or become aggressive weeds. Although the family is cosmopolitan, it has the major concentration of genera and species in South America [1]. In this sense, one highly important group is the New World genus *Capsicum* L. which comprises around 30 species. Five of them, *C. baccatum* L., *C. pubescens* Ruiz et Pav. and the members of the *C. annuum* L. complex (*C. annuum*, *C. frutescens* L. and *C. chinense* Jacq.), were domesticated by American natives and have been exploited world-wide since Columbus because of their fruits. The fruits, which have high nutritional content, specially in vitamins, and are constituents of the human diet, either the pungent forms as spice ('ajíes', 'chillies', 'hot peppers') or the sweet ones as vegetables ('sweet pepper'). Moreover, the genus has medical and ornamental applications [cf. 1-4].

One of the main disadvantages arising from cultivating plants is the resulting susceptibility to a multitude of diseases and pests due to the narrow genetic base from which most of the commercial cultivars are developed. Thus, plant breeders are forced to search for resistance in the existing gene pool and in the wild relatives of the crop species [5,6]. For success in breeding programmes in general, it is essential to achieve basic information on the genetic diversity of the available germplasm, and also on the genomic affinity between the possible donors of valuable alleles and the crop to be improved. Therefore, germplasm characterization is an important aspect for the conservation and utilization of plant genetic resources. In this respect, one of the main aspects to consider is the cytogenetic analysis.

The cytogenetics of *Capsicum* has received early attention for breeding purpose with reference to the domesticated taxa and their possible wild ancestors [3,7]. The success of interspecific crosses,

obviously related to the genomic homology of the parental species, could allow the genetic improvement of cultivars by introducing valuable alleles such as those responsible for pathogen resistance from the wild entities [5,6]. In this sense, a broad gene mapping and genome characterization programme in the genus by classical and molecular cytogenetics will be useful for hybridization and biotechnological approaches, including transformation.

Within the frame of the IAEA coordinated project entitled 'Physical mapping technologies for the identification and characterization of mutated genes contributing to crop quality' we carried out a genomic characterization of wild and cultivated samples of chilli peppers [8-12]. This work comprised a broad programme of classical and molecular studies on somatic chromosomes, i.e., chromosome banding (fluorescent and AgNOR staining) and DNA sequence (ribosomal RNA genes, i.e. 5S and 45S rDNA, telomeric sequences and restriction fragments of repetitive nature) mapping by fluorescence in situ hybridization (FISH), in order to evaluate the inter- and intraspecific cytogenetic variability. We expected to characterize species, varieties and cultivars, with the ultimate aim of enhancing our knowledge of the genome organization and evolution in *Capsicum*, with reference to the origin of the crop species, and attempted to obtain useful information for breeding purposes.

In this report, the cytogenetic characterization of a sample of the most important and widespread pepper crop, *C. annuum* var. *annuum*, a species possibly domesticated in southern highland Mexico [3,4], is presented as an example of the work done under the CRP.

2. MATERIALS AND METHODS

2.1. Plant material

The chilli pepper germplasm bank at the Multidisciplinary Institute of Plant Biology (IMBIV), National University of Cordoba, Argentina, comprises around 400 accessions, including cultivated and wild relatives from different localities. The studied sample presented in this paper is *Capsicum annuum* var. *annuum* cultivar NMCA 10272 from Mexico, was provided by Paul W. Bosland, New Mexico State University, USA, and cultivated at the greenhouse of the IMBIV.

2.2. Chromosome preparations

Root tips (5-10mm long) obtained following seed germination were pre-treated with a saturated solution of p-dichlorobenzene for 2h in the dark at room temperature, then, fixed in freshly prepared absolute ethanol/glacial acetic acid (3:1) and stored at -20ºC until preparation. Somatic chromosome spreads were prepared according to Schwarzacher *et al.* [13]. Root apices were macerated in an enzyme solution [1% (w/v) cellulase plus 10% (v/v) pectinase] at 37ºC for 40 min, and then squashed in 45% acetic acid. After removal of the coverslip with CO_2, slides were air dried, aged for 1-2days at room temperature, and then kept at -20ºC until use.

2.3. Chromosome banding methods

Silver impregnation to detect active nucleolar organizing regions (NORs) was performed according to the Ag-I procedure by Bloom and Goodpasture [14] with the modifications of Kodama *et al.* [15]. Fluorescent chromosome banding to reveal amount, distribution, and type of constitutive heterochromatin was done according to the triple staining method of Schweizer and Ambros [16] with the fluorochromes chromomycin A3 (GC-specific binding), distamycin A and 4'-6-diamidino-2-phenylindole (AT-specific binding) (CMA/DA/DAPI).

2.4. Fluorescent in situ hybridization (FISH)

The 45S rDNA and 5S rDNA repeated sequences were localized using the following DNA probes: R2, a 6.5-kb fragment of the 18S-5.8S-26S (45S) rDNA repeat unit from *Arabidopsis thaliana*, including internal transcribed spacers ITS1 and ITS2 and a short 5' segment of the intergenic region (IGR) [17], and pXV1, a 349 bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris*, including the

adjacent intergenic spacer [18]. The first probe was labelled with biotin-11-dUTP (Sigma, USA) and the second one with digoxigenin-11-dUTP (Roche, Germany), both by nick translation. The telomeric sequences were localized using an *Arabidopsis*-like telomeric probe amplified by PCR with the oligomer primers (5'-TTTAGGG-3')s and (5'-CCCTAAA-3')s according to Ijdo *et al.* [19] labelled with biotin-11-dUTP (Sigma). Labelling of the probes, pre-treatment of the preparations, chromosome and probe denaturation, conditions for the in situ hybridization, post-hybridization washing, blocking, indirect detection by fluorochrome conjugated antibodies (Dako, Denmark), i.e. anti-biotin conjugated to tetramethyl-rhodamine isothiocyanate (TRITC) and anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC), and chromosome counterstaining with DAPI were performed according to Moscone *et al.* [20,21].

2.5. Microscopy and image acquisition

After the application of the different methods, metaphase chromosomes were observed and photographed, depending on the procedure, with transmitted light or epifluorescence in a Leica DMLB microscope equipped with the appropriate filter sets, a Leica DC250 digital camera, and the IM 1000 Leica image management system. Particularly, for epifluorescence red, green, and blue images were captured in black and white using appropriate filters for TRITC, CMA, FITC, and DAPI excitation, respectively. Digital images were pseudocoloured and combined using the IM 1000 Leica software package (Leica, Switzerland), and then, imported into Adobe Photoshop 7.0 for final processing.

2.6. Karyotype analysis

Five to ten metaphase plates per sample were used to make the karyotype characterization. The arm ratio [r=q (long arm length) / p (short arm length)] was used to classify the chromosomes according to Levan *et al.* [22] in m (metacentric, r=1.00-1.69), sm (submetacentric, r=1.70-2.99) and st (subtelocentric, r = 3.00 – 6.99). In Table 1 and Figs. 1-3 the chromosomes were numbered according to Moscone *et al*. [10,11]. The procedure for measurements of chromosomes and fluorescent heterochromatic bands, in order to build the respective idiograms, is described in Moscone *et al.* [11]. Karyotype variants below the species level were considered as 'cytotypes'.

TABLE 1. MEASUREMENTS OF THE CHROMOSOME COMPLEMENT IN *C. ANNUUM* VAR. *ANNUUM* CV. NMCA 10272

Pair	Length		r (q/p)	Type	Position of fhcb	Length of fhcb	
	Absolute (µm) X (sd)	Relative (% of HKL)	X (sd)			Absolute (µm) X (sd)	Relative (% of HKL)
1	7.10 (0.66)	10.40	1.06 (0.04)	m	q	0.09 (0.04)	0.14
2	6.19 (0.80)	9.07	1.18 (0.12)	m			
3	5.76 (0.71)	8.44	1.15 (0.08)	m			
4	5.74 (0.71)	8.41	1.17 (0.10)	m	p	0.05 (0.01)	0.07
5	5.71 (0.72)	8.37	1.15 (0.09)	m			
6	6.00 (0.49)	8.79	1.09 (0.05)	m	p^(77.35)	0.20 (0.06)	0.29
					q^(61.23)	0.05 (0.01)	0.07
7	5.53 (0.45)	8.10	1.13 (0.08)	m			
8	5.41 (0.68)	7.93	1.33 (0.14)	m	p^(88.12)	0.05 (0.03)	0.07
9	5.29 (0.54)	7.75	1.20 (0.10)	m	p	0.08 (0.02)	0.12
10	5.17 (0.44)	7.58	1.14 (0.07)	m			
11	5.48 (0.31)	8.04	1.85 (0.29)	sm-NOR	p	0.70 (0.01)	1.03
12	4.86 (0.62)	7.13	3.08 (0.31)	st-NOR	p	0.43 (0.08)	0.63

Abbreviations: *cv*, cultivar; *X*, mean value; *sd*, standard deviation; *HKL*, haploid karyotype length; *r*, braquial index; *q*, long arm; *p*, short arm; *m*, metacentric; *sm*, submetacentric; *st*, subtelocentric chromosome; *NOR*, nucleolus organizing region; *fhcb*, fluorescent heterochromatic band.
^ intercalary band (in brackets is indicated the distance to the centromere).

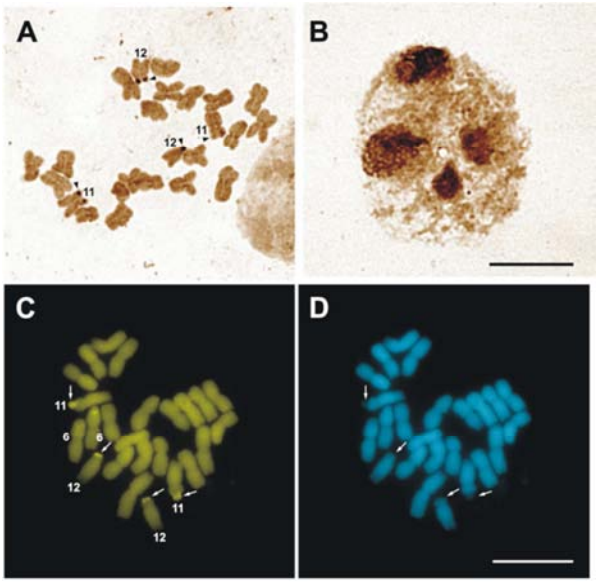

Fig. 1. Chromosome banding in Capsicum annuum var annuum cv. NMCA 10272 (2n = 24). A-B, Ag-NOR banding showing two chromosome pairs (Nos. 11 and 12) with active nucleolus organizing regions in metaphase pointed with arrowheads (A), and four Ag-stained nucleoli in an interphase nucleus (B). C-D, Fluorescent banding pattern displaying CMA+ (C) and DAPI- (D) heterochromatic bands; arrows indicate bands related to NORs. Scale bars represent 10 µm.

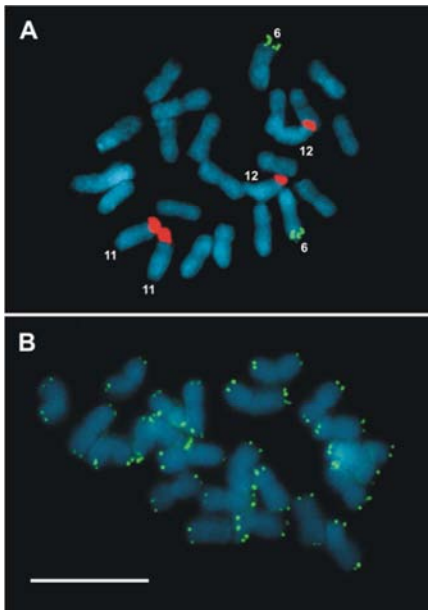

Fig. 2. Fluorescent in situ hybridisation (FISH) in Capsicum annuum var annuum cv. NMCA 10272 (2n = 24). A, Mapping of rRNA genes, 45S rDNA loci (two pairs) are displayed in red and 5S rDNA loci (one pair) in green. B, Mapping of telomeric sequences which are displayed in green. Scale bar represents 10 µm.

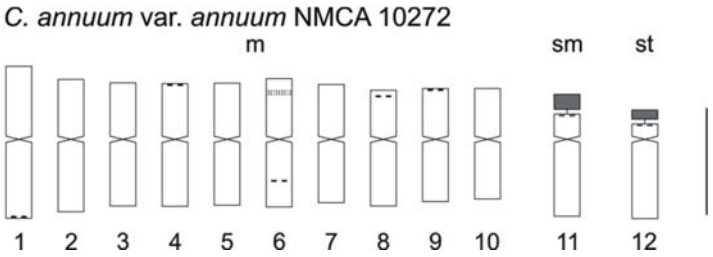

Fig. 3. Idiogram of Capsicum annuum var annuum cv. NMCA 10272 (2n = 24). All heterochromatic bands are CMA+/DAPI- , which include the rDNA loci. 45 S rDNA loci are shown in grey and the 5S rDNA locus in a hatched intercalary region. Scale bar represents 5 µm.

3. RESULTS AND DISCUSSION

Illustrations of chromosome banding, i.e., AgNOR staining, fluorescent CMA/DA/DAPI staining, and FISH experiments for localizing 5S and 18S, 5.8S, 26S (45S) rDNA and telomeric repeated sequences in *C. annuum* var. *annuum* cv. NMCA 10272, and the resulting idiogram are shown in Figs. 1-3. The measurements of the chromosomes from which the idiogram was built are given in Table 1.

The cultivar NMCA 10272 was, selected to show how the cytogenetic methodological approaches applied to chilli peppers is useful for chromosome identification. NMCA 10272 displayed 2n = 24, with a karyotype formula of 10m+1sm+1st chromosome pairs, where pairs nos. 11 (sm) and 12 (st) have active nucleolus organizing regions (NORs) on short arm. The total haploid karyotype length was 68.24µm. As it is typical in the genus, the fluorescent heterochromatic bands are CMA+/DAPI-, which indicates a GC-rich heterochromatin constitution. As usual in this species, the total heterochromatin amount of the cultivar was low (2.42%) and mostly placed in the terminal regions of the chromosomes.

The FISH patterns of the 45S ribosomal RNA gene family concerning number, position and size of loci differ between species, varieties and cultivars of *Capsicum* and resemble, although not completely, the corresponding specific fluorescent banding patterns after the triple staining with chromomycin, distamycin and DAPI, which shows CMA+/DAPI- bands [9,11,23]. This phenomenon, which was also observed in other plant groups (*Brassica* L.) [24], could be explained by possible cross-hybridization of the 45S probe with the CMA+/DAPI- (GC-rich) heterochromatic regions in the genus, as it is known that in most plant species ribosomal DNA is rather GC-rich [25]. It should be considered that the R2 and pTa71 probes used in our studies and by Park *et al.* [26], contain at least part of the intergenic 45S rDNA spacer (IGR), which carries repetitive elements and, thus, could be cross reactive [27]. In *Nicotiana* L., a satellite sequence with a GC-rich subrepeat, which is homologous to the 45S rDNA IGR and occurs outside of rDNA loci in species of section *Tomentosae* Goodsp was been reported. [28].

In the diploid complements of the taxa studied during this project, the number of 45S rDNA loci was variable, whilst invariably there was only one pair of 5S sites. Prior to this work, data on rRNA genes localization by FISH have been reported by other authors only in one accession each of the five cultivated *Capsicum* species, including *C. annuum*, *C. baccatum* and *C. pubescens* [26,29]. In *C. annuum* cultivar different from the one analysed in our study, only one pair of 45S rDNA loci was reported by non-fluorescent in situ hybridization methods [30].

Fluorescent chromosome banding patterns, AgNORs number and position and cytological mapping of rRNA gene clusters has revealed chromosome homologies between chilli pepper species [10,11,23] and allowed the speculation of species relationships and the possible evolutionary pathways. Generally, the information from our classical and molecular cytogenetic studies supports previous conclusions on systematic affinities and phylogenetic relationships in *Capsicum*, based on different methodological approaches [1,3,8,12,31]. It should be noted that the cytogenetic results obtained during this project allowed us to speculate a widely accepted evolutionary picture in the genus, where the x = 13 basic number appeared two times, once very early the evolution of the genus, and after that in a more advanced line. In addition, the observed intraspecific variation at cytological level helped in finding the possible original place of domestication for the cultivated pepper species [4], i.e. *C. annuum* var. *annuum* in Mexico and Peru, *C. chinense* and *C. frutescens* in Brazil, *C. baccatum* var. *pendulum* in Bolivia, and *C. pubescens* in Peru. Most of the cytogenetic information generated under this CRP was included in a related global project [32].

In the near future, we will attempt to localize in the chromosomes of the *Capsicum* species an available set of gene-derived markers (resistance gene analogues or RGAs), which are related to disease resistance genes of *Arachis* (Leguminosae). It should be noted that chilli peppers and peanut crops could be affected by similar diseases and pests, such as fungal leaf spots (*Cercospora*) and nematode root-knots (*Meloidogyne*), the resistance searched in the wild relatives [7]. Finally, the construction of integrated molecular linkage maps in members of the *C. annuum* complex [33-35]

open the prospect to attempt comparisons between the physical chromosomes identified cytogenetically and the pure genetic linkage groups, using chromosome-specific BAC clones based on genetically mapped restriction fragment length polymorphism (RFLP) markers [36,37].

4. CONCLUSIONS

The distribution and size variation of fluorescent heterochromatic bands, AgNORs and rDNA gene clusters, allowed us to distinguish wild and domesticated chilli peppers. Fluorescent banding, silver staining and rDNA FISH patterns were useful even with discriminate cultivars from the same species. This is significant, particularly, since most chromosomes in chilli pepper species are of similar in size and difficult to distinguish one from another. Thus, the methods used in study demonstrated to be very valuable for characterizing pepper germplasm.

FISH technique using ribosomal RNA gene (5S and 45S rDNA) probes combined in single experiments, together with fluorescent and AgNOR banding methods [32], brought out useful markers for chromosome identification and for further DNA sequence mapping by FISH, including genes contributing to crop quality in peppers.

Classical and molecular cytogenetics provided sufficient chromosome landmarks to postulate homologies between species. Valuable data to enhance the knowledge of the phylogeny, genome organization and evolution in *Capsicum* were obtained.

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REFERENCES

[1] HUNZIKER, A.T. (2001). Genera Solanacearum. The genera of Solanaceae illustrated, arranged according to a new system. Gantner Verlag, Koenigstein.

[2] International Board for Plant Genetic Resources (IBPGR). (1983). Genetic resources of *Capsicum*, a global plan of action. Food and Agriculture Organization of the United Nations, Rome.

[3] PICKERSGILL, B. (1991). Cytogenetics and evolution of *Capsicum* L. In: Tsuchiya, T.and Gupta, P.K. (eds.), Chromosome engineering in plants: genetics, breeding, evolution. Part B, pp. 139-160. Elsevier, Amsterdam.

[4] ESHBAUGH, W.H. (1993). Peppers: history and exploitation of a serendipitous new crop discovery. In: Janick, J. and Simon, J.E. (eds.), New crops, pp. 132-139. J. Wiley and Sons, New York.

[5] BOUKEMA, I.W. (1980). Allelism of genes controlling resistance to TMV in *Capsicum* L. Euphytica 29:433-439.

[6] BOITEUX, L.S., T. NAGATA, W.P. DUTRA and. M.E.N. FONSECA (1993). Sources of resistance to tomato spotted wilt virus (TSWV) in cultivated and wild species of *Capsicum*. Euphytica 67:89-94.

[7] LIPPERT, L.F., P.G. SMITH and B.O. BERGH (1966). Cytogenetics of the vegetable crops. Garden pepper, *Capsicum* sp. Botanical Review (Lancaster) 32:24-55.

[8] MOSCONE, E.A., M. LAMBROU, A.T. HUNZIKER and F. EHRENDORFER (1993a). Giemsa C-banded karyotypes in *Capsicum* (Solanaceae). Plant Systematics and Evolution 186:213-229.

[9] MOSCONE, E.A., M. LAMBROU, F. EHRENDORFER and A.T. HUNZIKER (1993b). Fluorescent chromosome banding in *Capsicum* (Solanaceae). In: Heubl, G.R. (ed.). Jornadas de Taxonomía Vegetal, pag. 8. Frank, Munich.

[10] MOSCONE, E.A., J. LOIDL, F. EHRENDORFER and A.T. HUNZIKER (1995). Analysis of active nucleolus organizing regions in *Capsicum* (Solanaceae) by silver staining. American Journal of Botany 82:276-287.

[11] MOSCONE, E.A., M. LAMBROU and F. EHRENDORFER (1996a). Fluorescent chromosome banding in the cultivated species of *Capsicum* (Solanaceae). Plant Systematics and Evolution 202:37-63.

[12] MOSCONE, E.A., M. BARANYI, I. EBERT, J. GREILHUBER, F. EHRENDORFER and A.T. HUNZIKER (2003). Analysis of nuclear DNA content in *Capsicum* (Solanaceae) by flow cytometry and Feulgen densitometry. Annals of Botany 92:21-29.

[13] SCHWARZACHER, T., P. AMBROS and D. SCHWEIZER (1980). Application of Giemsa banding to orchid karyotype analysis. Plant Systematics and Evolution 134:293-297.

[14] BLOOM, S.E. and C. GOODPASTURE (1976). An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Human Genetics 34:199-206.

[15] KODAMA, Y., M.C. YOSHIDA and M. SASAKI (1980). An improved silver staining technique for nucleolus organizer regions by using nylon cloth. Japanese Journal of Human Genetics 25:229-233.

[16] SCHWEIZER, D. and AMBROS, P.F. (1994). Chromosome banding: stain combinations for specific regions. p.97-112. In: Gosden, J.R. (ed.), Chromosome Analysis Protocols. Methods in Molecular Biology, Vol. 29. Humana Press, Totowa, USA.

[17] WANZENBÖCK, E.-M., C. SCHÖFER, D. SCHWEIZER and A. BACHMAIR (1997). Ribosomal transcription units integrated via T-DNA transformation associate with the nucleolus and do not require upstream repeat sequences for activity in *Arabidopsis thaliana*. The Plant Journal 11:1007-1016.

[18] SCHMIDT, T., T. SCHWARZACHER and J.S. HESLOP-HARRISON (1994). Physical mapping of rRNA genes by fluorescent in situ hybridization and structural analysis of 5S rRNA genes and intergenic spacer sequences in sugar beet (*Beta vulgaris*). Theoretical and Applied Genetics 88:629-636.

[19] IJDO, J.W., R.A. WELLS, A. BALDINI and S.T. REEDERS (1991). Improved telomere detection using a telomere repeat probe $(TTAGGG)_n$ generated by PCR. Nucleic Acids Research 19:4780.

[20] MOSCONE, E.A., M.A. MATZKE and A.J.M. MATZKE (1996b). The use of combined FISH/GISH in conjunction with DAPI counterstaining to identify chromosomes containing transgene inserts in amphidiploid tobacco. Chromosoma 105:231-236.

[21] MOSCONE, E.A., F. KLEIN, M. LAMBROU, J. FUCHS and D. SCHWEIZER (1999). Quantitative karyotyping and dual-color FISH mapping of 5S and 18S-25S rDNA probes in the cultivated *Phaseolus* species (Leguminosae). Genome 42:1224-1233.

[22] LEVAN, A., K. FREDGA and A.A. SANDBERG (1964). Nomenclature for centromeric position on chromosomes. Hereditas 52:201-220.

[23] SCALDAFERRO, M.A., J.G. SEIJO, M.C. ACOSTA, G.E. BARBOZA, D.A. DUCASSE and E.A. MOSCONE (2006). Genomic characterization of the germplasm in peppers (*Capsicum – Solanaceae*) by fluorescent in situ hybridization. Plant Science (Bulgaria) 43:291-297.

[24] HASTEROK, R. and J. MALUSZYNSKA 2000. Cytogenetic analysis of diploid *Brassica* species. Acta Biologica Cracoviensia Series Botanica 42/1:145-153.

[25] HEMLEBEN, V., M. GANAL, J. GERSTNER, K. SCHIEBEL and R.A. TORRES (1988). Organization and length heterogeneity of plant ribosomal RNA genes. In: Kahl, G. (ed.), Architecture of eukaryotic genes, pp. 371-383. VHC, Weinheim.

[26] PARK, Y.-K., B.-D. KIM, B.-S. KIM, K.C. ARMSTRONG and N.-S. KIM (1999). Karyotyping of the chromosomes and physical mapping of the 5S rRNA and 18S-26S rRNA gene families in five different species in *Capsicum*. Genes and Genetic Systems 74:149-157.

[27] BROWN, S.E., J.L. STEPHENS, N.L.V. LAPITAN and D.L. KNUDSON (1999). rDNA loci in barley and wheat: probe pTa71 FISH fact or fiction. Plant and Animal Genome VII Conference, San Diego.
http://www.intl-pag.org/pag/7/abstracts/pag7237.html.

[28] LIM, K.Y., K. SKALICKA, B. KOUKALOVA, R.A. VOLKOV, R. MATYASEK, V. HEMLEBEN, A.R. LEITCH and A. KOVARIK (2004). Dynamic changes in the distribution of a satellite homologous to intergenic 26-18S rDNA spacer in the evolution of *Nicotiana*. Genetics 166:1935-1946.

[29] PARK, Y.-K., K.-C. PARK, C.-H. PARK and N.-S. KIM (2000). Chromosomal localization and sequence variation of 5S rRNA gene in five *Capsicum* species. Molecules and Cells 10:18-24.

[30] TANKSLEY, S.D., R. BERNATZKY, N.L. LAPITAN and J.P. PRINCE (1988). Conservation of gene repertoire but not gene order in pepper and tomato. Proceedings of the National Academy of Sciences USA 85:6419-6423.

[31] MCLEOD, M.J., S.I. GUTTMAN, W.H. ESHBAUGH and R.E. RAYLE (1983). An electrophoretic study of evolution in *Capsicum* (Solanaceae). Evolution 37: 562-574.

[32] MOSCONE, E.A., M.A. SCALDAFERRO, M. GRABIELE, N.M. CECCHINI, Y. SÁNCHEZ GARCÍA, R. JARRET, J.R. DAVIÑA, D.A. DUCASSE, G.E. BARBOZA and F. EHRENDOFER (2007). The evolution of chili peppers (*Capsicum – Solanaceae*): a cytogenetic perspective. Acta Horticulturae 745:137-169.

[33] PRINCE, J.P., E. POCHARD and S.D. TANKSLEY (1993). Construction of a molecular linkage map of pepper and a comparison of synteny with tomato. Genome 36:404-417.

[34] LEFEBVRE, V., A. PALLOIX, C. CARANTA and E. POCHARD (1995). Construction of an intraspecific integrated linkage map of pepper using molecular markers and doubled-haploid progenies. Genome 38:112-121.

[35] LIVINGSTONE, K.D., V.K. LACKNEY, J.R. BLAUTH, R. VAN WIJK and M.K. JAHN (1999). Genome mapping in *Capsicum* and the evolution of genome structure in the Solanaceae. Genetics 152:1183-1202.

[36] DONG, F., J. SONG, S.K. NAESS, J.P. HELGESON, C. GEBHARDT and J. JIANG (2000). Development and applications of a set of chromosome-specific cytogenetic DNA markers in potato. Theoretical and Applied Genetics 101:1001-1007.

[37] PEDROSA, A., E.A. MOSCONE, C.E. VALLEJOS, A. BACHMAIR and D. SCHWEIZER (2001). Integrating common bean (*Phaseolus vulgaris* L.) linkage and chromosomal maps. Molecular Genetics of Model Legumes: Impact for Legume Biology and Breeding, pag. 23. Golm, Germany.