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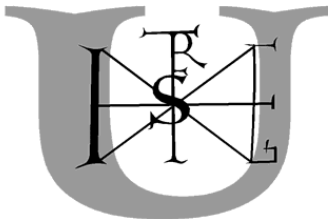

Domestication and molecular evolution of the watermelon

(*Citrullus lanatus*)

PhD Thesis

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Gödöllő
2013

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Contents

1. INTRODUCTION AND AIMS OF THE STUDY

The aDNA samples extracted from remains of plants and animals supply unique materials for the analysis of post-mortem DNA degradation (Brown, 1999; Threadgold and Brown, 2003), domestication and microevolution (Salamini *et al*., 2002; Gugerli *et al*., 2005; Janick and Paris, 2006). Analysis of aDNAs also provides crucial data concerning crop domestication events that have occurred during previous centuries (Gyulai *et al*., 2001, 2006; Bacsó *et al*. 2004; Bisztray *et al*., 2004; Vaughan *et al*., 2007; Schlumbaum *et al*., 2008). Sequences of intact aDNA fragments (Szabó *et al*., 2005; Lágler *et al*., 2005; Gyulai *et al*., 2006), and complete genomes (and mitomes) (Cooper *et al*., 2001; Pääbo *et al*., 2004) of the extinct organisms were also reconstructed by tools of archaeogenetics.

For safe aDNA analysis the most important step is to eliminate both the exogenously and endogenously infected seeds, because bacterial or fungal DNA-remains can contaminate the plant DNA being studied. The surface sterilization seeds and incubation for a month in tissue culture provided optimal aseptic source for aDNA extraction (Tóth *et al*., 2008). Possible DNA cross contamination from the laboratory investigations was also excluded by this aseptic treatment.

Fossilized samples of *Bangiomorpha pubescens* (a red alga) from Canada proved that chloroplasts had developed more than 1.2 billion years ago (Butterfield, 2000). Fossilization coupled with charcoalification leaved floral morphology of ancient *Nymphaeales* perfectly preserved at a site in Sayreville (NJ, USA) from the earliest Upper Cretaceous time (Turonian, ca. 90 million years b.p.) (Crepet *et al*., 2004). Fossils of basal angiosperms (*Archaefructus sp*) were also discovered from lower early Cretaceous period in China (Zhou *et al*., 2003). Extinct angiosperm species (e.g. *Pinus tuzsoni* Greguss; syn. *Pinuxylon tarnocziense* Tuzson) were identified from 20 million year old (Lower Miocene) site at Ipolytarnóc (Hungary) (Andreánszky, 1966; Greguss, 1972; Erdei *et al*., 2007; Hably, 2006; Süss, 2007).

The aims of the study presented were to analyse of aDNA fragments and sequences (ITS, SSR, cpDNA, and *lcy*B gene) of 800-, 600- and 170-year-old *Citrullus* specimens together with a comparison to modern cultivars (1 to 44) with the final aim of molecular and morphological reconstruction of ancient *Citrullus* genotypes.

2. BACKGROUND AND LITERATURE REVIEW

2.1 *Citrullus* species

The monotypic genus *Citrullus* of family *Cucurbitaceae* is comprised of only four diploid (2n = 4x = 22; 4.25 - 4.54 x 108 bp; 0.42 pg DNA) species, including the annual watermelon (*Citrullus lanatus*), the perennial colocynth (syn.: bitter apple) (*Citrullus colocynthis*), and two wild species, growing in Kalahari Desert (Africa): the perennial, monoecious *Citrullus ecirrhosus* with bitter-tasting fruit; and the recently identified, annual *Citrullus rehmii* (De Winter, 1990) with pink and olive green spotted rind, mandarin sized non-edible fruits (Robinson and Decker-Walters, 1997; Sarafis, 1999; Dane and Lang, 2004).

Species watermelon (*C. lanatus*) comprises two subspecies of domesticated watermelon (*C. lanatus lanatus*) with its green fleshed, wild form growing in Namib desert (Sarafis, 1999), and its wild ancestral citron melon (syn.: African tsamma) with also white flesh (*C. lanatus citroides*) (Nakai, 1916; Kanda, 1951; Hanelt, 2001). Domesticated watermelon includes diverse varieties, cultivars, feral forms, mutants (e.g. egusi melon: *C. lanatus mucospermum*; Gusmini *et al*., 2004) and new crossed hybrids (e.g. the first seedless triploid hybrid watermelon developed by Kihara 1951; and the first commercial 'Allsweet'-type hybrid cv. 'Sangria' developed by Tom V. Williams, Syngenta Seeds, 1985) (Maynard *et al*., 2007)

The primary gene centre for watermelon is in South-West Africa, the domestication might have occurred in Northern Africa implied by excavations of six thousand (Barakat, 1990) and five thousand (Wasylikowa and van der Veen, 2004) year-old seed remains. Colocynth grown as medicinal plant, citron as fodder crop, and the domesticated watermelon as fresh fruit production have a history of production in the World.

2.2 Ancient records of *Citrullus*

The most ancient image of watermelon from Pharaohs tomb is known form 3,100 – 2,100 B.C. (Old Kingdom) (Manniche, 1989; Janick *et al*., 2007). Hieroglypf of watermelon is known from 1,550 B.C. (Warid, 1995). The first figures of colocynth (*C. colocynthis*) (known in Arabic as handel) were carved into the cedar wood in Solomon's temple (960 – 586 B.C.), which is the only poisonous (medicinal) plant displayed in the temple (I. Kings 6:18a, Bible) prior to a notes of Exodus from the time 480 year earlier recalling watermelon eaten in Egypt

(1,440 B.C.) (IV. Num. Moses 11:5, Bible). The first painting of colocynth remained in excellent color form in the ancient Dioscorides codex (Dioscorides 1st CENT., and 512 A.D.)

The Greeks and Romans traveling to Egypt must to have known of watermelons probably without discriminating it from colocynth and citron melon (Cox and van der Veen, 2009). Pliny II. wrote about a 'wild' (probably the current colocynth) and two types of 'cultivated' colocynth (probably the current watermelon) one with pale green, and the other with grass green rind, as it has been written: „…Another kind of wild gourd is called Colocynthis. The fruit is smaller than the cultivated one, and full of seeds. The pale variety is more useful than the grass-green one…" (Pliny 23-79 A.D.; Gilmore, 1919; Blake, 1981).

Six hundred years later, when the Iberian Peninsula was conquered by the Berbers (Moors) led by Tarik Ibn Ziyad in 711 A.D., new watermelon types might enter Europe as recorded in the ancient record of Book of Agricuture (Al-Awwam, 1158). In this book two cultivated forms were compared, a black seed type (with dark-green rind which turns black when it ripens) and a red seed type (with green rind which turns to yellow when it ripens) (Blake, 1981).

By 800 A.D. watermelons became popular in India and by 1,100 A.D. in China. The first records of the name of watermelon in Hungarian 'görög dinnye' means 'melon from Greece' is known from 1395 (Finály, 1892). However, the first record on the name melon ('dinna') is known from the 1000's recorded in an ancient certificate (Szamota and Zolnai, 1902-1906) without discriminating cucumbers from melons and watermelons.

Watermelon might have also been introduced to Europe through Crusades (Fischer, 1929) led by either Richard I. the Lionheart (the 3rd Crusades, 1187-1192), or Endre II. the Hungarian King of Árpád Dynasty who led the 5th Crusades (1217-1221). Watermelon spread through Europe quickly and became very popular and commonly cultivated fruit of the Renaissance Europe, with the second color illustration on the frescos in the Villa Farnesina, Rome, Italy, 1517 A.D. painted by Giovanni Martini da Udina (Janick and Paris, 2006). Watermelon reached the New World after Columbus' second voyage in 1493 and dispersed quickly among American natives (Blake, 1981). One of the most ancient forms of small, round fruit with thin, green rind, red flesh and black seeds has survived up to the recent times (Gilmore, 1919).

2.3 Herbarium samples

One of the oldest watermelon herbarium sample is available from G Bauhin's (1560-1624) collections (personal communication, Mark Spencer, The Natural History Museum, London, UK), who named it *Anguira citrullus* about a hundred year time earlier than Linnaeus. No watermelon herbarium sample remained from C Linnaeus (1753) collections, who named watermelon as *Cucurbita citrullus*, and clocynth as *Cucumis colocynthis* (personal communication, Arne Anderberg, The Linnean Herbarium, Swedish Museum of Natural History, Stockholm, S).

2.4 Ancient Seed remains

The oldest plant remains with proven human activity have revealed only cereal seeds as wild barley (*H. spontaneum*) and wild emmer (*Triticum dicoccoides*) from 19,000 b.p. at Ohalo II., river Jordan (Nadel *et al*., 2004, 2006; Piperno *et al*., 2004). The 17,310±310 b.p. site in Korea (Chungbuk National University, South Korea) revealed the first ancient rice (Oryza sativa) seed remains with extractable amount of aDNA (Suh *et al*., 2000).

The first Cucurbit seeds were excavated from the Spirit Cave (Hoabinh, Thailand) including cucumber type *Cucumis* seeds at least 9,180 ± 360 b.p. as analyzed by C14 of bamboo charcoal (Gorman, 1969).

The oldest, 6,000-year old *Citrullus* (watermelon, *C. l. lanatus*) seeds were excavated in Helwan (Egypt, Africa), at a site 4.000 B.C. (Barakat, 1990). About 5,000-year old seeds were excavated in Uan Muhuggiag, Lybia, Africa from a site 3.000 B.C. (Wasylikowa and van der Veen, 2004). Large quantity of watermelon seeds were deposited in the Pharaoh's tombs of Pyramids as in Thebes (New Kingdom: 1,550-1,070 B.C.; stored in Agricultural Museum, Dokki, Giza, Egypt) (Warid, 1995) and in the pyramid of Tutankhamum ca. 1,330 B.C. (Hepper, 1990; Kroll, 2000; Vartavan and Amorós, 1997). Watermelon seed remains were also excavated from 1,550 B.C. in an old temple near Semna, Nubia (van Zeist, 1983).

Ancient watermelon seeds of the study presented were excavated at sites from 13th CENT. (Debrecen, Hungary), and 15th CENT. (Budapest, Hungary) (Gyulai *et al*., 2006), and collected form a herbal seed collection from 19th CENT. (Pannonhalma) (Vörös, 1971).

2.5 Archaeogenetics

The aDNA samples extracted from remains of plants and animals supply unique materials for the analysis of post-mortem DNA degradation (Brown, 1999; Threadgold and Brown, 2003), domestication and microevolution (Gugerli *et al*., 2005; Gyulai *et al*., 2006; Janick and Paris, 2006). Analysis of aDNAs also provides crucial data concerning crop domestication events that have occurred during previous centuries (Gyulai F *et al*., 1992; Bacsó *et al*., 2004; Bisztray *et al*., 2004; Vaughan *et al*., 2007; Schlumbaum *et al*., 2008). Sequences of intact aDNA fragments (Szabó *et al*., 2005; Lágler *et al*., 2005), and complete genomes (mitomes) (Cooper *et al*., 2001; Pääbo *et al*., 2004) of the extinct organisms were also reconstructed by tools of archaeogenetics.

The aDNA (ancient DNA) samples recovered from excavated remains of plants and animals supply unique materials for tracking domestication (Gugerli *et al*., 2005), microevolution (Gyulai *et al*., 2006), migration (Dane and Liu, 2006) and the analysis of post-mortem DNA degradation (Brown, 1999; Threadgold and Brown, 2003). A numbers of amplifications of intact sequences of aDNA samples (Szabó *et al*., 2005; Lágler *et al*., 2005) and complete genomes (Cooper *et al*., 2001; Pääbo *et al*., 2004) of the extinct organisms have also been reported.

3. MATERIALS AND METHODS

3.1 Seed samples:

800-year-old seed remains of watermelon (*Citrullus l. lanatus*) were excavated at a site from the 13th CENT. (Debrecen, Hungary). In total, 95,133 seed of 206 plant species were identified. Of them 251 watermelon seeds were determined. The 600 year-old seeds were excavated at a site from the 15th CENT. (8th well, Mansion Teleki, King's Palace of Árpád Dinasty, Buda Hill, Budapest, Hungary) (54,415 watermelon seeds in total) (Gyulai *et al*., 2006). Sediment samples were processed by seed sorting and identified in the laboratory according to Schermann (1966). The 19th CENT. (ca. 1836) seeds were collected from the oldest botanical seed collection of Hungary (Pannonhalma) (Vörös, 1971). The collection is recently exhibited at the Hungarian Agricultural Museum, Budapest (Hungary) (Hartyányi and Nováky, 1975). For comparative analysis, forty-four modern *Citrullus* species and varieties were included.

3.2 Elimination of contaminations:

Ancient seeds were surface sterilized by washing with regular detergent (for 3 min) and rinsed three times with distilled water (for 3 min) followed by soaking in ethanol (70% v/v for 1 min) and a bleaching agent (8% NaOCl w/v, for 1 min) with a final rinses with sterile distilled water according to general aseptic culture technique (Gyulai *et al*., 1992). Aseptic seeds were incubated for seven days in tissue culture media to eliminate seeds contaminated either by bacteria or fungi (Gyulai *et al*., 2006). Exogenously and endogenously contaminated seeds infected by fungi and bacteria were eliminated from further analyses. Seeds of the modern varieties were also surface sterilized.

3.3 aDNA extraction:

Aseptic seeds were ground in an aseptic mortar with liquid nitrogen followed by the DNA extraction with CTAB (cethyltrimethylammonium bromide) protocol according to Biss *et al*.,(2003), Yang, (1997) and Cooper and Poinar, (2000). The aDNA isolation was carried out in a laminar air flow cabinets of archaeobotanical lab of the St Stephanus University, Gödöllő. Seed DNA of modern cultivars (0.1 g) was also extracted in CTAB buffer, followed by an RNase-A treatment (Sigma, R-4875) for 30 min at 37°C in each case. The quality and quantity of extracted DNA were measured (2 μl) by a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Delaware, USA – BioScience, Budapest,

Hungary). DNA samples were adjusted to concentration of 30 ng/µl with ddH2O and subjected to PCR amplification (Gyulai *et al*., 2006).

3.4 ALF- SSR analysis:

For nSSR (nuclear microsatellite) analysis twelve primer-pairs were used (Table 1). After amplification, aliquots (2 µl) of PCR products were separated by ALF (automatic laser fluorometer; ALFexpress II DNA Analyser; Amersham Bioscience, Uppsala, Sweden - AP, Budapest, Hungary) according to Röder *et al*., (1998), Huang *et al*., (2002) and Gyulai *et al*., (2006).

Table 1. Primer sequences applied for nuclear SSR analysis

#	Loci	Primer-pair sequences	References
1.	*Cm*TC 51	attggggtttctttgaggtga ccatgtctaaaaactcatgtgg	Katzir *et al*., 1996
2.	*Cm*TC 168	*atcattggatgtgggattctc acagatggatgaaaccttagg	Katzir *et al*., 1996
3.	*CM*ACC 146	caaccaccgactactaagtc cgaccaaacccatccgataa	Katzir *et al*., 1996
4.	*Bngl* 339	ccaaccgtatcagcatcagc gcagagctctcatcgtcttctt	Smith *et al*., 1997
5.	*Bngl* 118-2	gccttccagccgcaaccct cactgcatgcaaaggcaaccaac	Smith *et al*., 1997
6.	*Bngl* 161	gctttcgtcatacacacacattca atggagcatgagcttgcatattt	Smith *et al*., 1997
7.	*Phi* 121	aggaaaatggagccggtgaacca ttggtctggaccaagcacatacac	Smith *et al*., 1997
8.	*Phi* 118-2	atcggatcggctgccgtcaaa agacacgacggtgtgtccatc	Smith *et al*., 1997
9.	*Cl* 1-06	caccctcctccagttgtcattcg aaggtcagcaaagcggcatagg	Jarret *et al*., 1997
10.	*Cl* 1-20	cgcgcgtgaggaccctata aaccgcctcaatcaattgc	Jarret *et al*., 1997
11.	*Cl* 2-23	gaggcggaggagttgagag acaaaacaacgaaacccatagc	Jarret *et al*., 1997
12.	*Cl* 2-140	ctttttcttctgatttgactgg actgtttatcccgacttcacta	Jarret *et al*., 1997

PAGE gel (24 %) using a short thermoplate with 40 samples capacity, was run by 850 V, 50 mA, 50 W at 50°C for 120 min, prior to UV-linkage for 15 min under ReproSet. One primer of each primer pair was labelled by Cy5 fluorescent dye at the 5'-end (Sigma, St. Louis, MO). For sequencing, the left aliquots of PCR product (15 µl) were run and cut out from agarose (1.6 %) gel, purified in a spin column according to the manufacturer's protocol (Sigma, St. Louis, MO).

LycB gene (*lycopene β-cyclase*) gene were probed by primer pairs designed by 'Promer-3' program based on the sequences of (NCBI EF183522, and EF183521) Bang *et al*., (2007) (Table 2).

Table 2. Primer sequences applied for *lcy*B gene analysis

#	Loci	Primer pair sequences	Reference
1.	*LCY*B 314	cctgttcttctggagttctt gaaaaagtgagtggtgtgagga	Bang *et al*., 2007
2.	*LCY*B 1134	aatgatggtgtgaccattcaag cttacaatccaggctaccagg	

The cpDNA were probed at two loci trnaVAL-rps12 (AJ970307; Al Jabani *et al*., 1994) and ycf9-orf62 (AY522531, ay522537 and AY522539; Dane and Liu, 2007) (Table 3).

Table 3. Primer sequences applied for cpDNA

#	Loci	primer-pair sequences	References
1.	*clp*12	agttcgagcctgattatccc gatgaacgctggcggcatgc	Al-Janabi *et al*., 1994
2.	*ycf* 9	aattagagggaggggtctcttgc ataataggctagctctgcactgatg	Dane *et al*., 2004

For ITS analysis primers complementary to the evolutionary conserved spacer regions of the nuclear ribosomal (rDNA) gene cluster of ITS1-5.8S-ITS2 (internal transcribed spacer) were used. Primer pairs were designed by 'Promer-3' program based on the sequences of AJ488232 (Hsiao *et al*., 1995) (ITSL and ITS4): ITS L: 5'-cgcgtttacaaacaaattgtcc-3'; ITS 4/1: 5'-acactacggtggttgatccg -3'; ITS4/2: 5'-gtccccccaaaggatgacgc-3'

PCR amplification: Hot Start PCR (Erlich *et al*., 1991) was combined with Touchdown PCR (Don *et al*., 1991) using AmpliTaq GoldTM Polymerase. Reactions were carried out in a total volume of 25 µl containing genomic DNA of 30-50 ng, 1 x PCR buffer (2.5 mM MgCl2), dNTPs (200 µM each), 20 pmol of each primer and 1.0 U of Taq polymerase. Touchdown PCR was performed by decreasing the annealing temperature by 1.0 ^{o}C / per cycle with each of the initial 12 cycles (PE 9700, Applied Biosystems), followed by a 'touchdown' annealing temperature for the remaining 25 cycles at 56 ^{o}C for 30 s with a final cycle of 72 ^{o}C for 10 min (transgene detection) and hold at 4 ^{o}C. The regular PCR cycles developed for prokariotic cpDNA (Demesure *et al*., 1995; Dane *et al*., 2007) were performed as follows: initial denaturing step at 94 ^{o}C for 5 min, followed by 35 cycles of 94 ^{o}C / 1 min - 55 ^{o}C / 1 min - 65 ^{o}C / 2 min, followed by a final extension step at 65 ^{o}C for 10 min and hold at 4 ^{o}C. A negative control which contained all the necessary PCR components except template DNA was

included in the PCR runs. A minimum of three independent DNA preparations of each sample was used. Amplifications were assayed by agarose (1.8 %, SeaKem LE, FMC) gel electrophoresis (Owl system), stained with ethidium bromide (0.5 ng/µl) running at 80 V in 1 X TBE buffer. Each successful reaction with scorable bands was repeated at least twice. Transilluminated gels were analyzed by the ChemilImager v 5.5 computer program (Alpha Innotech Corporation - Bio-Science Kft, Budapest, Hungary). A negative control which contained all the necessary PCR components except template DNA was included in the PCR runs. Fragments were purified in a spin column (Sigma 5-6501) according to the manufacturer's protocol and subjected for sequencing.

Sequencing: PCR fragments were isolated from the agarose gel with a spin column (Sigma, 56501) and subjected to automated fluorescent DNA sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Hungary). Multiple Sequence Alignments (MSA). MSAs were carried out by either BioEdit Sequence Alignment Editor (NCSU, USA) (Hall 1999), CLUSTALW (Thompson *et al*., 1994) software programs or the on-line MULTALIN computer program (http:// npsa_pbil.ibcp.fr/cgibin/npsa_automat.pl?page=/ NPSA/npsa_multalinan.html). BLAST (Basic Local Alignment Search Tool) analysis (Altschul *et al*., 1997) was carried out by a computer program of NCBI (National Center for Biotechnology Information).

Distance trees based on DNA sequences were edited by either MEGA4 (Tamura *et al*., 2007) program. For MEGA4 the following steps were applied: Bootstrap Test of Phylogeny (1000); Neighbor-Joining; Gaps (Complete deletions); Substitution model (Nucleotide Maximum Composite Likelihood) according to Tamura *et al*., (2007). Diagrams were edited by Microsoft Office Excel program (9625 West 76th Street, Eden Prairie, MN 55344, USA). Cluster analysis was carried out by either or MEGA4 (Tamura *et al.,* 2007) or SPSS-11 program package using the Jaccard Similarity Index (Jaccard, 1908) (Average Linkage, within group) based on the presence versus absence of SSR alleles and nucleotides of DNA.

Data analysis: PIC value (polymorphism index content) of each SSR was calculated using the formula of Anderson *et al.,* (1993): PIC = 1 - ∑n-ipi2, where pi is the frequency of the ith allele. Cluster analysis was carried out by the SPSS-11 program package using the Jaccard Similarity Index (Jaccard, 1908) (Average Linkage, within group) based on the presence versus absence of SSR alleles.

4. RESULTS AND DISCUSSION

4.1 Morphological evidences

Watermelon seeds excavated at both medieval sites appeared to be extremely well preserved (Fig. 1) due to anaerobic conditions at site Debrecen (13th CENT.), and in the slime of a deep well in Budapest (15th CENT.) (Gyulai *et al.*, 2006). The herbarium sample seeds form the 19th CENT. were stored under precise conditions in glass containers (Vörös, 1971).

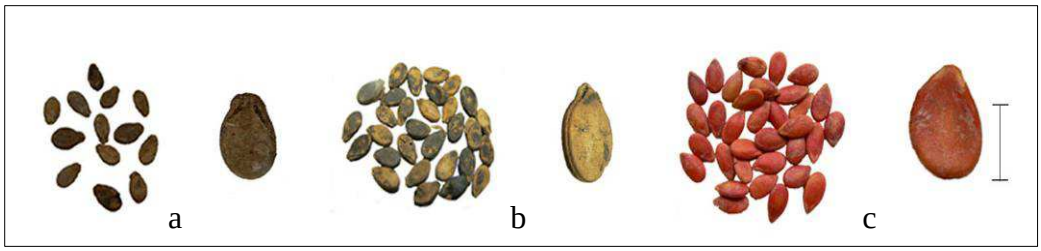

Figure 1. Seed morphology (groups and individuals) of ancient seeds (*Citrullus l. lanatus*) from the 13th CENT. (Debrecen, Hungary); 15th CENT. (Budapest, Hungary) and 18th CENT. (*Citrullus l. citroides*) (Pannonhalma, Hungary) (scale bar 1cm)

4.2 Microsatellite evidences

Microsatellites (SSR) of nuclear DNA (nDNA) analysis revealed a sum of 701 fragments of 23 SSR alleles at 12 SSR loci among the medieval (13th and 15th CENT.), 19th CENT. and modern *Citrullus* specimens. Molecular dendrogram (fig. 2) based on nSSR fragment diversity loci revealed that 13th CENT. sample (# 45) showed close genetic similarity to modern watermelon cv. 'Kecskeméti vh' (#36) and the 15th CENT. sample (Budapest) showed close similarity to cv. 'Csárdaszállás' (# 14). As expected from seed morphology, the 19th CENT. *Citrullus* showed close molecular similarity to modern citron melons (*C. l. citroides*) (# 4-6) with white flesh color.

Allelic diversity of microsatellites were reliably detected in aDNAs of 300 – 1,100-year old seagrass (*Posidonia oceanica*) (Raniello and Procaccini, 2002). SSRs were used to morphologically reconstruct 600-year old melon (*Cucumis staivus*) (Szabó *et al.*, 2005) and millet (*Panicum miliaceum*) (Lágler *et al.*, 2005; Gyulai *et al.*, 2006). SSR analysis was also applied to herbarium samples of common reed (*Phargmites australis*) of about 100-year-old to track plant invasion in North America (Saltonstall, 2003).

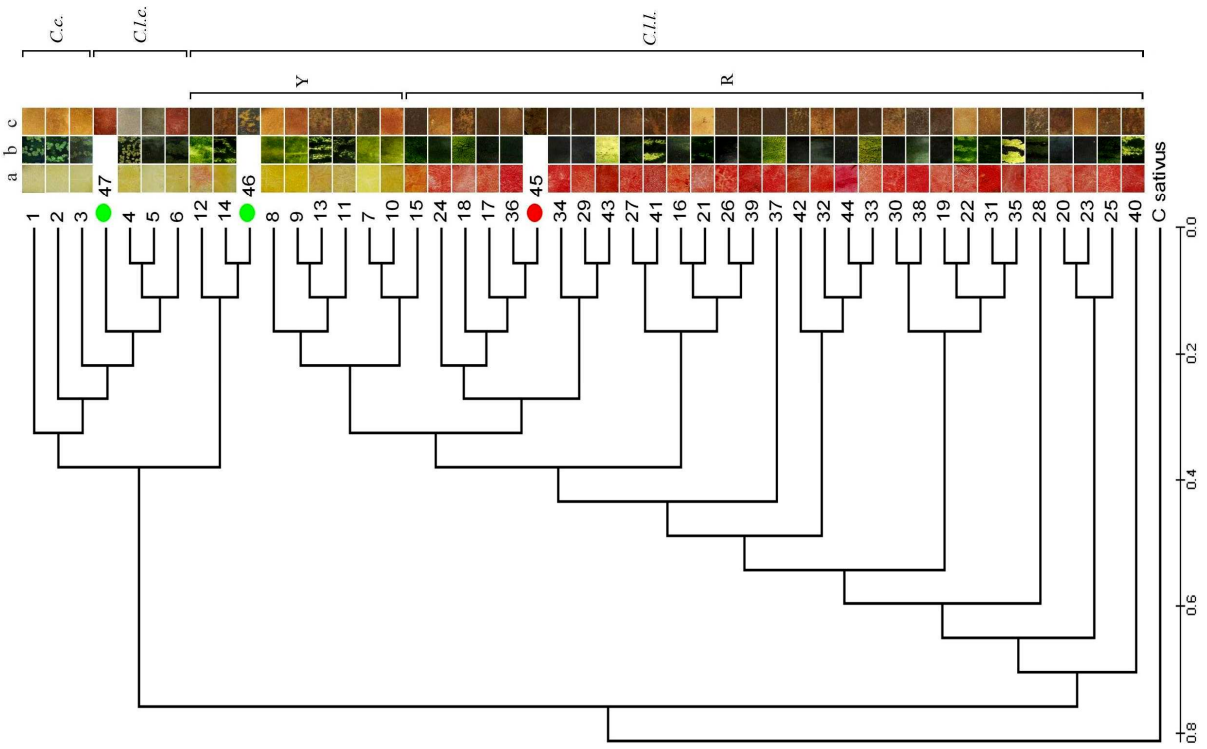

Figure 2. Molecular (SSR) dendrogram (SPSS 16) of current varieties of colocynth (*Citrullus colocynthis*, # 1-3), citron melon (*Citrullus lanatus citroides*, # 4-6) and watermelon (*Citrullus lanatus lanatus*, # 7-44) compared to archaeological and herbarium samples (# 45-47)

4.3 cpDNA and ITS analysis.

Chloroplast genome specific primers provides highly sensitive methods for analyzing cpDNA in the total DNA samples without using the former tedious ultracentrifuge separations (Al-Jabani *et al.*, 1994; Demesure *et al.*, 1995 ; Dane *et al.*, 2007). The level of SNP polymorphism of cpDNA in plant species varies from nil, as in pearl millet (*Pennisetum glaucum*) (Gepts and Clegg, 1989), compared to low level in soybean (*Glycine soya*) (Xu *et al.*, 2002), chestnut (*Castanea sativa*) (Fineschi *et al.*, 2000) and pear (*Pyrus ssp.)* (Katayama and Uematsu 2003), with high polymorphisms in wild beet (*Beta vulgaris ssp maritima*) (Forcioli *et al.*, 1998) and several tree species such as Prunus (Mohanty *et al.*, 2001) and olive (*Olea europaea*; Besnard *et al.*, 2002). *Citrullus* species were found to also have high SNP polymorphism with 6 SNP (1.73 %) along the tRNA-VAL – rps12 sequence (346 nt) (fig. 3).

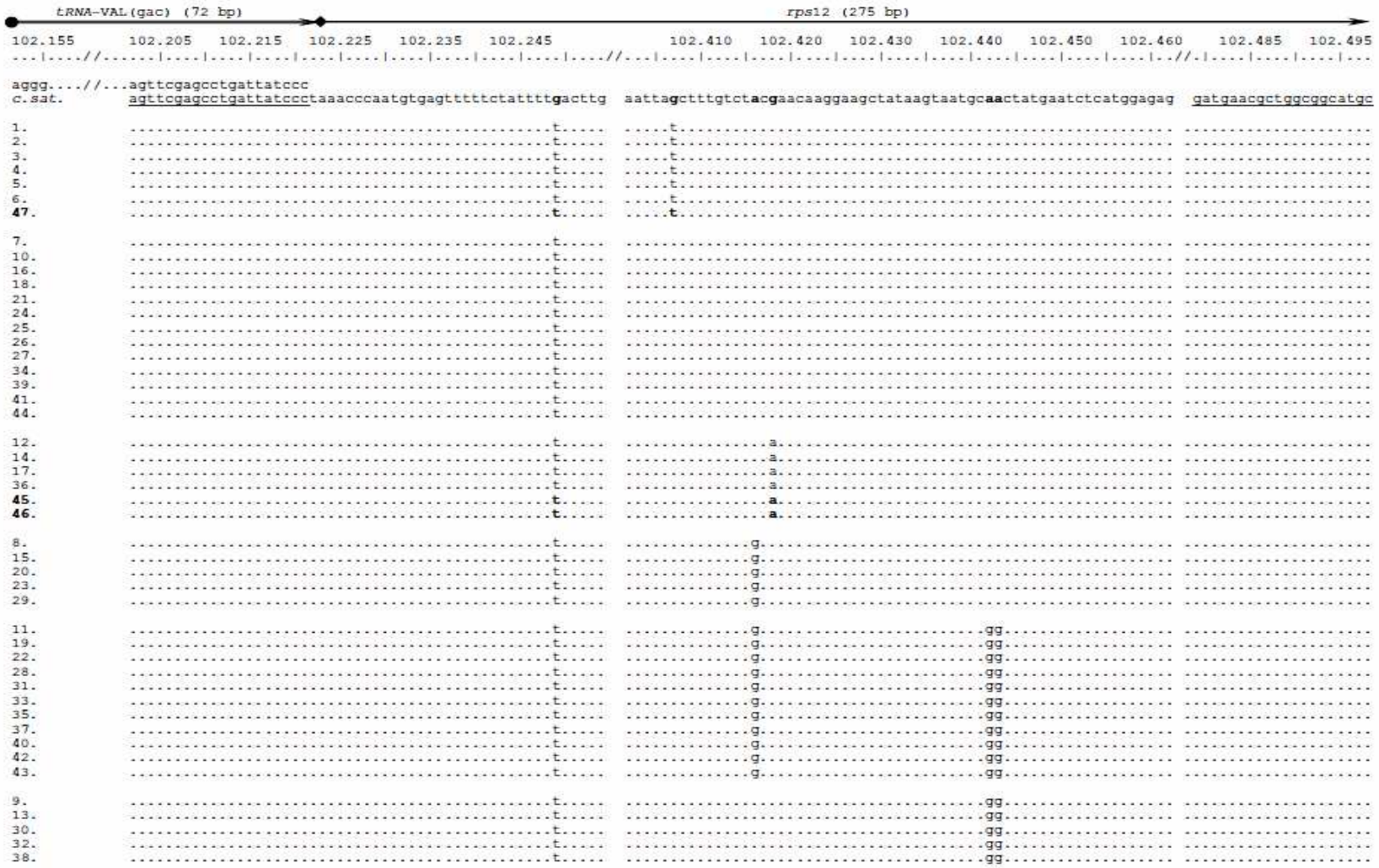

Figure 3. Sequence analysis of cpDNA on *tRNA*-Val - *rps*12 loci of current varieties of colocynth (*Citrullus colocynthis*, # 1-3), citron melon (*Citrullus lanatus citroides*, # 4-6) and watermelon (*Citrullus lanatus lanatus*, # 7-44) compared to archaeological and herbarium samples (# 45-47).

The morphological reconstruction revealed that 13th CENT. watermelon might have been a red flesh type (carrying the homozygote recessive alleles of lycb/ lycb gene) with green rind similar to modern watermelon cv. 'Kecskeméti vh' (#36) (based on SSR similarities). The 15th CENT. watermelon might have been a yellow flesh type (carrying the homozygote dominant alleles of lycB/ lycB gene) with striped rind similar to modern watermelon cv. 'Csárdaszállás' (# 14) (based on SSR similarities). As expected from seed morphology, the 19th CENT. *Citrullus* showed close molecular similarity to modern citron melons (C. l. citroides) (# 4-6) with white flesh color (based on SSR similarities). The ITS-analysis of rDNA supplied a further watermelon-specific marker by separating watermelons (*C. lanatus lanatus*) from citrons (*C. lanatus citroides*) and colocynths (*C. colocynthis*). The rDNA sequences of modern cultivars of colocynth (#1-3), citron melons (#4-6) and the 19th CENT. *Citrullus* showed the same SNP pattern at the ITS1-5.8S-ITS2 locus, however modern citron melon cv. 'De Banat' (#5) was found to carry a watermelon-specific rDNA allele at heterozygote form, which might indicate an evolutionary step from bitter tasted citron towards watermelons. In contrast, modern watermelon cv. 'Túrkeve' (#20) was found to carry a citron-specific rDNA allele at heterozygote form, which indicates that watermelon cv. 'Túrkeve' (#20) is the most ancient watermelon type among the accessions studied (fig. 4).

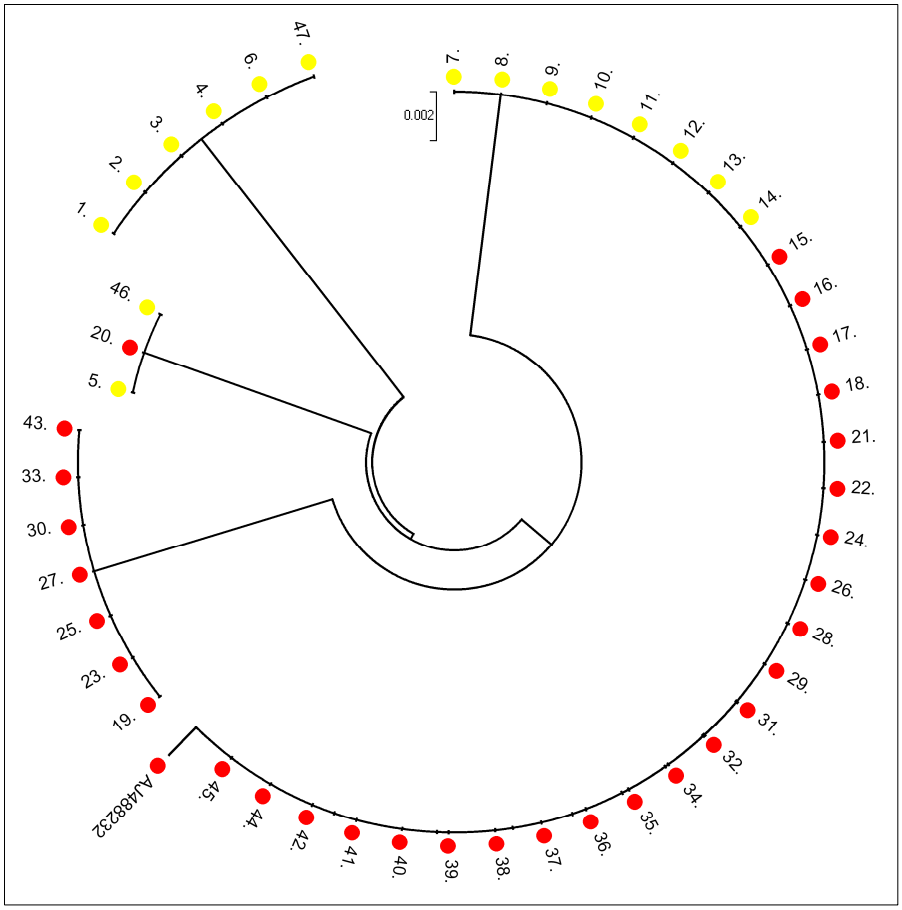

Figure 4. Molecular (ITS1-5.8S-ITS2) dendrogram (MEGA 4) of current varieties of colocynth (*Citrullus colocynthis*, # 1-3), citron melon (*Citrullus lanatus citroides*, # 4-6) and watermelon (*Citrullus lanatus lanatus*, # 7-44) compared to archaeological and herbarium samples (# 45-47).

4.4 Reconstruction of Flesh and Rind types

Flesh color of watermelons varies from white to yellow - canary yellow - salmon yellow and orange mainly due to pigment compositions of xanthophylls. The pink - red - purple colors varies mainly due to pigment compositions of lycopenes. Genes coding for white flesh color (w) were QTL-mapped (quantitative trait loci) on chromosome (syn.: linkage group) 6 (Hashizume *et al.,* 1996). Genes responsible for yellow and red color were mapped on chromosome 2. These gene loci indicate different transition colors between yellow and red (canary yellow, pale yellow) (Hashizume *et al.,* 2003). QTL responsible for red flesh color had another locus on chromosome 8. This locus showed genetic linkage with QTL for high sugar (brix value) content (Hashizume *et al.,* 2003). This result strongly indicate the reason of over numbered red flesh watermelons compared to cultivars with white and yellow flesh colors, as selection for sweeter watermelons during domestication has been coupled with selection for red flesh color at the same time (Hashizume *et al.,* 2003). Further genetic loci for color determination were recently determined, namely Y (red, dominant), yo (orange, recessive), y (salmon yellow, recessive), C (canary yellow, dominant) and c (red, recessice), respectively (reviewed in Bang *et al.,* 2007).

The enzyme LYCB (*lycopene β-cyclase*) encoded by lycb gene play a central role in plant color development by converting lycopene to carotenoids with ring structure. SNP (single nucleotide polymorphism) markers in lycb gene, which discriminates yellow and red flesh watermelons were developed recently (Bang *et al.,* 2007).

The SNPs at the 1182th base pair (bp) were also found to be A=T (Adenine = Thymine) in all CY (canary yellow) watermelons with homozygote dominant (lycB/lycB) genotype. However, all red flesh watermelons had G≡C (Guanine ≡ Cytosine) bp at the 1182th bp indicating a homozygote recessive genotype (lycb/lycb). The heterozygotes (lycB/lycb) also encodes for dominant CY flesh type carrying both alleles (T=A and G ≡ C). No heterozygote was found in the samples studied. The other SNP at the 518th nt (Bang *et al.,* 2007) with the opposite way of substitutions (G ≡ C in canary yellow, and T=A in red) were also found to be identical. Amino acid sequence analysis of LYCB enzyme revealed that this SNP resulted in an aminoacid substitutios from Ph to Val at the 226th amino acid locus, which might be impair the catalytic function of lycB gene (Bang *et al.,* 2007).

The 19th CENT. (#47) and 15th CENT. (#46) samples and all modern colocynths (#1-3), citrons (#4-6), and yellow flesh watermelons (# 7-14) had the homozygote dominant CY-allele at

both SNP loci of lycB gene with G≡C bp (base pair) at the 518th nt, and T=A at 1182th nt, respectively. The 13th CENT. (#45) sample and all red flesh modern watermelons (# 15 - 44) had the recessive homozygote allele (lycb) at both SNP loci with T=A at the 518th nt and G≡C at the 1182th nt, respectively (fig. 5). Red flesh watermelon appeared also in the painting of Still Life with Melons and Carafe of White Wine (1603 A.D.) painted by Caravaggio (Janick, 2004; Janick *et al.*, 2007). No colocynths and citrons were found with red flesh color indicating that the recessive allele of *lyc*b gene developed later during the domestication.

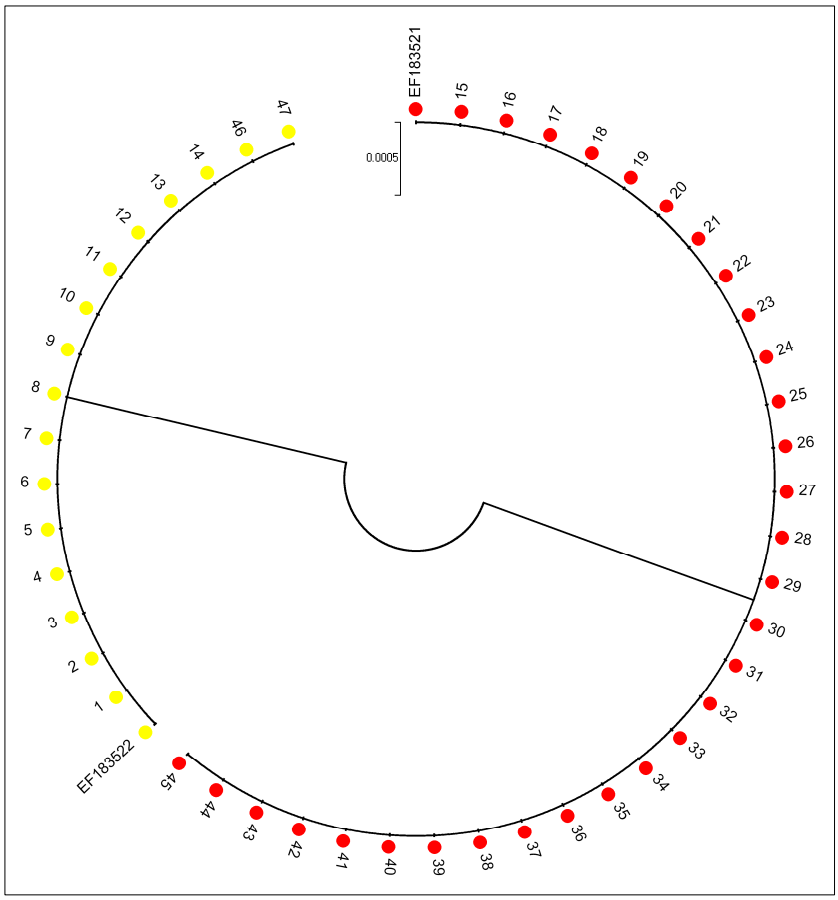

Figure 5. Molecular (*lyc*b) dendrogram (MEGA 4) of current varieties of colocynth (*Citrullus colocynthis*, # 1-3), citron melon (*Citrullus lanatus citroides*, # 4-6) and watermelon (*Citrullus lanatus lanatus*, # 7-44) compared to archaeological and herbarium samples (# 45-47).

Watermelons fruit shapes are round to cylindrical. Unexpectedly, the most ancient, 5000 year old record in Pharaohs tomb (3.100 – 2.100 B.C., Old Kingdom) shows not a round but an elongated watermelon with green strips (Manniche, 1989; Janick *et al.*, 2007). Fruit rind (exocarp) color varies from pale green to dark green, with or without whitish strips, or small whitish spots.

The most ancient European color wall paintings (1517) show watermelons with pale green rinds (Janick *et al.*, 2007) which indicate an ancient rind type, as a QTL locus (gs) responsible for dark-green rind was found to be dominant over the light-green rind (Hashizume *et al.*, 2003).

SUMMARY

Watermelon seeds excavated at both medieval sites analyzed in the study presented appeared to be extremely well preserved due to anaerobic conditions at site Debrecen (13th CENT.), and in the slime of a deep well in Budapest (15th CENT.) covered by water, apparently used as dust holes in the Middle Ages (Gyulai *et al*., 2006). The herbarium sample seeds form the 19th CENT. were stored under precise conditions in glass containers (Vörös, 1971).

Molecular dendrogram of the study presented based on 701 SSR fragments in total identified at eleven nuclear microsatellite (nSSR) loci revealed that middle age samples show close lineages to ancient varieties currently growing in Hungary with red flesh colour. Allelic diversity of microsatellites were reliably detected in aDNAs of 300 – 1,100-year old seagrass (*Posidonia oceanica*) (Raniello and Procaccini, 2002). SSRs were used to morphologically reconstruct 600-year old melon (*Cucumis melo*) (Szabó *et al*., 2005a) and millet (*Panicum miliaceum*) (Lágler *et al*., 2005; Lágler, Gyulai *et al*., 2006). SSR analysis was also applied to herbarium samples of common reed (*Phargmites australis*) of about 100-year-old to track plant invasion in North America (Saltonstall, 2003). Results of seed morphology correlated strongly to molecular results. The 13th -14th CENT. sample (Debrecen) showed similarity to cv. 'Kecskeméti vöröshéjú'; the 15th CENT. sample (Budapest) showed similarity to cv. 'Belyj dlinnij' (# 12). These results also reflect the preferential cultivation of red flesh – and not yellow flesh- watermelon in the Middle Age of Hungary. Red flesh watermelon also appeared in the painting of Still Life with Melons and Carafe of White Wine (1603 A.D.) painted by Caravaggio (Janick, 2004; Janick *et al*., 2007). Molecular data obtained might provide further tools for watermelon breeders. The 170-year-old herbarium sample (Pannonhalma, Hungary) showed close molecular similarity to citron melon (*Citrullus lanatus citroides*) cv.'Újszilvás' which reflects the importance of citron melon as fodder in the Middle-Age Hungary.

Watermelons are divided into several morphological types; based on fruit weight as personal size with to 2.7 kg / 6 lbs, icebox type to 6.8 kg/15 lbs, and picnic type above 6.8 kg/15 lbs. Fruit shapes are round to cylindrical. Unexpectedly, the most ancient, 5000 year old record in Pharaohs tomb (3.100 – 2.100 B.C., Old Kingdom,) shows not round but elongated fruit with green strips (Manniche, 1989; Janick *et al*., 2007). Fruit rind (exocarp) varies from thin to thick and brittle to tough with colors from pale green to dark green, with or without whitish

strips, or small whitish spots. The most ancient European color wall paintings (1517) show watermelons with pale green rind (Janick *et al*., 2007) which indicate an ancient rind type, as a QTL locus (gs) responsible for dark-green rind was found to be dominant over the light-green rind (Hashizume *et al*., 2003).

Flesh color of watermelons varies from white; to yellow - canary yellow - salmon yellow - orange mainly due to pigment compositions of xanthophylls. The pink - red - purple colors mainly due to pigments of lycopenes. Genes coding for white flesh color (w) were QTL-mapped (quantitative trait loci) on chromosome (syn.: linkage group) 6 (Hashizume *et al*., 1996). Genes responsible for yellow and red color were mapped on chromosome 2. These gene loci indicate the transition colors between yellow and red (canary yellow, pale yellow) (Hashizume *et al*., 2003). QTL responsible for red flesh color had another locus on chromosome 8. This locus showed genetic linkage with QTL for high sugar content (Hashizume *et al*., 2003). This result strongly indicate the reason of over numbered red flesh watermelons compared to cultivars with white and yellow flesh colors, as selection for sweeter watermelons during domestication has been coupled with selection for red flesh color at the same time (Hashizume *et al*., 2003). Some further genetic loci for color determination were recently determined by breeding tools (crossings), namely Y (red, dominant), yo (orange, recessive), y (salmon yellow, recessive), C (canary yellow, dominant) and c (red, recessive), respectively (reviewed in Bang *et al*., 2007).

The enzyme LCYB (*lycopene β-cyclase*) encoded by lcyb gene play a central role in plant color development by converting lycopene to carotenoids with ring structure. SNP (single nucleotide polymorphism) markers in lcyb gene (NCBI EF183521) were which discriminated yellow and red flesh watermelons (Bang *et al*., 2007). The 19th CENT. and 15th CENT. samples along with modern colocynts, citrons, and modern (# 7-15) yellow flesh watermelons (*Citrullus lanatus lanatus*) showed CY-type SNPs at both loci 518th (G≡C) and 1182th (T=A) of lcyb gene. The 13th CENT. sample and all red flesh modern watermelons (# 16 - 44) showed the red-type SNPs at both loci 518th (T=A) and 1182th (G≡C) of lcyb gene. No colocynts and citrons were found with red flesh color.

5. LIST OF PUBLICATIONS OF ZOLTAN TÓTH

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1. Gyulai G, Z Szabó, B Wichmann, A Bittsánszky, LWaters Jr, **Z Tóth**, F Dane (2012) Conservation genetics - Heat Map analysis of nuSSRs of aDNA of archaeological watermelons (Cucurbitaceae, Citrullus l. lanatus) compared to current varieties. Genes, Genomes and Genomics 6 (SI1): 86-96.
2. Başli Ag, G Gyulai, **Z Tóth**, A Güner, Z Szabó, Vl Stakhov, L Murenyetz, Sg Yashina, L Heszky, Sv Gubin (2009) Light and Scanning Electron Microscopic Analysis of Silene stenophylla Seeds Excavated from Pleistocene-Age (Kolyma). *Anadolu Univ J Sci and Technol* 10:161-167.
3. Güner A, G Gyulai, **Z Tóth**, Ga Başli, Z Szabó, F Gyulai, L Heszky (2009) Grape (*Vitis vinifera*) seeds from Antiquity and the Middle Ages Excavated in Hungary - LM and SEM analysis. *Anadolu Univ J Sci Technol* 10:205-213.
4. Gyulai G, M Humphreys, R Lágler, Z Szabó, **Z Tóth**, A Bittsánszky, F Gyulai, L Heszky (2006) Seed remains of common millet from the 4th (Mongolia) and 15th (Hungary) centuries; AFLP, SSR, and mtDNA sequence recoveries. *Seed Science Research* 16:179-191. (***IF: 1,892***)
5. Bittsánszky A, G Gyulai, M Humphreys, G Gullner, Zs Csintalan, J Kiss, Z Szabó, R Lágler, **Z Tóth**, H Rennenberg, L Heszky and T Kőmíves (2006) RT-PCR analysis and stress response capacity of transgenic *gsh*I-poplar clones (*Populus* x *canescens*) in response to paraquat exposure. *Z Naturforschung* 61c:699-730. (***IF: 0,756***)
6. Gyulai G**, Z Tóth**, Z Szabó, F Gyulai, R Lágler, L Kocsis, L Heszky (2009) Domestication Events of Grape (*Vitis vinifera)* from Antiquity and the Middle Ages in Hungary. *Hung Agric Resi* 2009/4.
7. **Tóth Z**, G Gyulai, L Horváth, Z Szabó, L Heszky (2007) Watermelon (*Citrullus l. lanatus*) production in Hungary from the Middle Ages. *Hung Agric Res* 2007/4: 14-19.
8. Lágler R, G Gyulai, Z Szabó, **Z Tóth**, A Bittsánszky, L Horváth, J Kiss, F Gyulai, L Heszky (2006) Molecular diversity of common millet (*P. miliaceum*) compared to archaeological samples excavated from the 4th and 15th centuries. *Hung Agric Res* 2006/1:14-19.

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***International* (1)**

9. Gyulai G, **Z Tóth**, A Bittsánszky (2011) Flesh color reconstruction from aDNAs of Citrullus seeds from the 13th, 15th, and 19th cents (Hungary). In. Plant Archaeogenetics. Ed. by G Gyulai. Chapter 7. pp. 69-87. Nova Sci Publisher Inc., New York, USA. ISBN 978-1-61122-644-7.
10. Gyulai G, **Z Tóth**, A Bittsánszky, Z Szabó, G Gullner, J Kiss, T Kőmíves and L Heszky (2008) Gene up-regulation by DNA demethylation in 35S-*gsh*I-transgenic poplars (*Populus* x *canescens*). in: Genetically Modified Plants: New Research Trends. Eds. T Wolf and J Koch, *Nova Science Publisher*, Inc. USA, Chapter 8, pp. 173-191. ISBN 978-1-60456-696-3.

Hungarian Scientific Journals (6)

11. **Tóth Z**, Gyulai G, Szabó Z, Heszky L (2008) Sejtmagi mikroszatellita és cpDNS szekveniák diverzitása görögdinnyében (*C. lanatus*). *Agr Vidékfejl Szemle* 2008/3(1): 1-5.
12. **Tóth Z**, Gyulai G, Szabó Z, Horváth L, Gyulai G, Heszky L (2007) Mikroszatellita lokuszok evolúciója a görögdinnyében (*Citrullus lanatus*) a középkor óta; $(CT)_3$ deléció a $(CT)_{26}$ nSSR-ban. *Agrártud Közl* 27: 125-134.
13. Szabó Z, Gyulai G, **Tóth Z**, Heszky L (2007) SNP elemzés az rDNA ITS-5.8S-ITS2 lokuszán a mai és a középkori sárgadinnyében (*Cucumis melo*). *Agrártud Közl* 27: 27: 120-124
14. Bittsánszky A, Gyulai G, **Tóth Z**, Horváth M, Fekete I, Szabó Z, Heltai Gy, Gullner G, Kőmíves T, Heszky L (2008) Molekuláris nyárfanemesítés (*Populus x canescens*) ökoremediációs alkalmazásra. *Agr Vidékfejl Szemle* vol. 3. 2008/2:184-189.
15. Lágler R, Gyulai G, Szabó Z, **Tóth Z**, Heszky L (2007) A köles (*Panicum miliaceum*) SSR- és ISSR szekvencia-stabilitása a 4. és 15. századi régészeti leletektől a mai fajtákig**.** *Agrártud Közl* 27: 10-19.
16. Horváth L, Gyulai G, Szabó Z, Lágler R, **Tóth Z**, Heszky L (2007) Morfológiai diverzitás sárgadinnyében (*Cucumis melo*); egy középkori típus fajtarekonstrukciója. *Agrártud Közl* 27: 84-90.

Peer Reviewed Proceedings
***English* (4)**

17. **Tóth Z**, G Gyulai, Z Szabó, F Gyulai, L Heszky (2008a) New Citrullus haplotypes at the tRNA-Val – rps12 locus of cpDNA.1 Cucurbitaceae 2008, Proceedings of the IXth EUCARPIA meeting on genetics and breeding of Cucurbitaceae (Pitrat M, ed), INRA, Avignon (France), May 21-24th, 2008, pp.335-310.
18. **Tóth Z**, G Gyulai, Z Szabó, A Bittsánszky, L Heszky (2008b) Genotype (nSSR) and haplotype (cpDNA) identification in watermelons (Citrullus l. lanatus). Gen. Meet. EUCARPIA, Valencia, Spain, pp. 253-257.

19. Szabó Z, G Gyulai, **Z Tóth**, A Bittsánszky, L Heszky (2008) Sequence diversity at the loci of nuclear SSRs and ITS1-5.8S-ITS2 of rDNA of 47 melon (Cucumis melo) cultivars and an extinct landrace excavated from the 15th century. General Meeting EUCARPIA, Valencia, Spain, pp. 244-249.
20. Szabó Z, G Gyulai, **Z Tóth**, L Heszky (2008) Morphological and molecular diversity of 47 melon (Cucumis melo) cultivars compared to an extinct landrace excavated from the 15th Century. Cucurbitaceae 2008, Proceedings of the IXth EUCARPIA meeting on genetics and breeding of Cucurbitaceae (Pitrat M, ed), INRA, Avignon (France), May 21-24th, 2008, pp.313-321.

***Hungarian* (4)**

21. **Tóth Z**, Gyulai G, Kenéz Á, Szabó Z, Bittsánszky A, Lágler R, Gyulai F, Horváth L, Heszky L (2009) Molekuláris domesztikáció a *Citrullus* nemzetségben az ITS (ITS1-5.8s-ITS2), NSSR, SNP (*lcy*b) és cpDNS (*ycf*9-*orf*62; *trn*val-*rps*12) lokuszokon. Hagyomány és haladás a növénynemesítésben. XV. Növénynemesítési Tudományos Napok, Budapest, ISBN: 978-963-508-575-0, pp. 507-511.
22. **Tóth Z**, G Gyulai, Z Szabó, L Heszky (2008c) Az nSSR és cpDNS lokuszok evolúciója a görögdinnyében (Citrullus lanatus). 4. Erdei Ferenc Tud Konf., Kecskemét, aug.27-28. (ed Ferenc Á.), II. kötet pp.866-870.
23. Kenéz Á, Gyulai G, **Tóth Z**, Szabó Zoltán, Lágler R, Heszky L, Gyulai F (2009) Római Kori (Keszthely-Fenékpuszta, (5. sz.) Növényleletek Azonosítása: I. Egyszikűek. Hagyomány és haladás a növénynemesítésben. XV. Növénynemesítési Tudományos Napok, Budapest, ISBN: 978-963-508-575-0, pp. 233-237.
24. Bittsánszky A, Gyulai G, Gullner G, **Tóth Z**, Kiss J, Szabó Z, Heszky L, Kőmíves T (2009) Paraquat-toleráns nyárfa *in vitro* szelekciója és molekuláris jellemzése. Hagyomány és haladás a növénynemesítésben. XV. Növénynemesítési Tudományos Napok, Budapest, ISBN: 978-963-508-575-0, pp. 31-35.

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22. **Tóth Z**, Gyulai G, A Başli, R Lágler, A Güner, Z Szabó, A Kis, A Bittsánszky, L Heszky (2007) Morphological and molecular reconstruction of 15th and 18th cent. watermelons (C. lanatus). IWGP14, 14th Int Symp Work Group for Palaeoethnobotany, 17-23 June, 2007, Kraków, Poland.
23. **Tóth Z**, G Gyulai, L Horváth, F Gyulai (2006) Molecular and morphological reconstruction of a medieval melon (Cucumis melo). 36th International Symposium on Archaeometry (ISA 2006), május 2 - 6, Quebec City, Canada.
24. Lágler R, Gyulai G, A Güner, **Z Tóth**, A Kis, Z Szabó, Ga Başli, A Bittsánszky, L Heszky (2007) Morphological and molecular analysis of ancient common millet (P. miliaceum) seeds. IWGP14, 14th Int Symp Work Group for Palaeoethnobotany, 17-23 June, 2007, Kraków, Poland.
25. Stakhov V, Gyulai G, Szabó Z, Kovács L, Murenyetz L, Lagler R, **Tóth Z**, Yashina S, Bittsánszky A, Heszky L, Gubin S (2007) Pleistocene-Age Silene stenophylla seeds excavated in Russia – A Scanning Electron Microscopic Analysis. Botany and Plant biology Joint Congress, Chicago, Illiois, USA, July 7-11. ID: 2131
26. Szabó Z, Gyulai G, Ga Başli, **Z Tóth**, A Güner, R. Lágler, L Kovács, A Kis, A Bittsánszky, L Kocsis, L Heszky, F Gyulai (2007) Analysis of Grape (Vitis vinifera) Seeds from Antiquity and the Middle Ages Excavated in Hungary. 14th Int Symp Work Group for Palaeoethnobotany, 17-23 June, 2007, Kraków, Poland.
27. Szabó Z, Gyulai G, Kovács L, **Tóth Z**, Lagler R, Bittsanszky A, Kocsis L (2007) Ancient DNA analysis and morphology of grape seeds from antiquity and the middle agesexcavated in Hungary. Botany and Plant biology Joint Congress, Chicago, Illiois, USA, July 7-11. ID: 2119

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28. **Tóth Z**, Gyulai G, Szabó Z, Gyulai F, Horváth L, Kiss J, Heszky L (2008) Haplotípusok azonosítása görögdinnyében (Citrullus lanatus). XIVdik Növénynemesítési Tudományos Napok, MTA Budapest, 2008. március 12. p. 14.
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30. **Tóth Z**, Gyulai G, Szabó Z, Mórocz S, Hajósné N M, Lágler R, Kálmán L, Kiss J, Bock I, Bittsánszky A, Koncz S, Bottka S, Heszky L (2006) SSR és cpSSR lókuszok Q-PCR és ALF-SSR elemzése kukorica (Zea mays L.) vonalakban és hibridekben, XII. Növénynemesítési Tudományos Napok, Budapest, p. 175.
31. Horváth L, Gyulai G, Szabó Z, Lágler R, **Tóth Z**, Bittsánszky A, Lehoczky P, Gyulai F, Heszky L (2007) Morfológiai diverzitás a sárgadinnyében (Cucumis melo); egy középkori típus fajtarekonstrukciója. XIII. Növénynemesítési Tudományos Napok, p.79.Budapest.
32. Kis A, Gyulai G, Lágler R, Szabó Z, Gubin Sv, **Tóth Z**, Stakhov Vl, Bittsánszky A, Yashina Sg, Heszky L (2007) Pleisztocén kori (Jégkorszak) Silene magvak morfológiai és molekuláris elemzése. VII. Magyar Genetikai Kongresszus-14dik Sejt- és Fejlődésbiológiai Napok, Balatonfüred, április 15-17. P064, 134.
33. Kis A, Gyulai G, Lágler R, Szabó Z, Gubin, **Tóth Z**, Stakhov Vl, Bittsánszky A, Yashina Sg, Heszky L (2007) Morfológiai és molekuláris elemzés jégkorszaki Silene magokban. XIII. Növénynemesítési Tudományos Napok, p.78.Budapest.
34. Lágler R, Gyulai G, Szabó Z, Bittsánszky A, **Tóth Z**, Kiss J, Gyulai F, Horváth L, Bock I, Holly L, Heszky L (2006) mtDNS RFLP-PCR, SSR és ISSR elemzés középkori köles (Panicum miliaceum L.) magvak DNS mintáiban, XII. Növénynemesítési Tudományos Napok, Budapest, p. 120.
35. Lágler R, Gyulai G, Szabó Z, **Tóth Z**, Lehoczky P, Bittsánszky A, Horváth L, Heszky L (2007) A köles (Panicum miliaceum) SSR- és ISSR szekvencia-stabilitás a kölesben (Panicum miliaceum) a középkortól napjainkig. XIII. Növénynemesítési Tudományos Napok, p.80. Budapest.
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6. ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor, Prof. Dr. G. Gyulai whose encouragement, approachability and willingness to help throughout the course of the PhD study has been very much appreciated.

I also would like to extend my thanks to Prof. Dr. F. Gyulai for the contribution in the analysis of archaeological *Citrullus* seed samples.

I am pleased to acknowledge the contribution of Prof. Dr. L Heszky head of the PhD School, and Prof. Dr. E. Kiss, Head of the Institute of Genetics and Biotechnology, and the staff especially Dr. Z. Szabó, Dr. A. Bittsánszky, and Predoc R. Lágler in the Szent István University, Gödöllő, Hungary.

My particular thanks are due to my family for making my study so enjoyable.