ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization 7(2); 127–134
doi:10.1017/S1479262108098481

Molecular diversity in the Ukrainian melon collection as revealed by AFLPs and microsatellites

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Received 1 July 2008; Accepted 21 September 2008 – First published online 10 October 2008

Abstract

Thirty-eight melon accessions, which are of primary breeding importance in the Ukraine, were analysed for diversity. These collections represent a major non-US and non-western Europe source of melon germplasm that have not yet been subjected to molecular characterization. Molecular diversity was estimated based on a robust set of 465 polymorphisms gathered by amplified fragment length polymorphisms and simple sequence repeats (SSR). In this paper, we report 12 newly developed polymorphic SSR primer pairs, and their use for molecular characterization in the Ukrainian melon collections. Based on these polymorphisms, we estimated similarity indices that ranged from 0.70 to 1.00 among various accessions. The phylogenetic tree based on the similarity indices and a three-dimensional plot of the first three vectors of the principal component analysis corresponded fairly well with the existing three classical morphotypes namely *aestivalis*, *europeus* and *hiemalis*, under the *convar Europeus*, which is also known as *adana*. The polymorphisms generated in the current study, which are specific to the grouping of fruit types and days to maturity will be very useful for further genetic studies and marker-assisted selections.

Keywords: AFLP; *Cucumis melo*; diversity; melon; SSR

Introduction

Melon (*Cucumis melo* L.) is an economically important horticultural crop of the *Cucurbitaceae* family. It is diploid ($n = 12$) with genome size of 450 Mb (Arumuganathan and Earle, 1991) and with wide diversity in fruit characteristics, plant type, floral structure and sex expression (Robinson and Decker-Walters, 1997).

Fossilized melon seeds from excavations near the ancient Greek colony Chersonese, located at the outskirts of the Sevastopol, Crimean peninsula of Ukraine (founded approximately 2500 years ago on the shore of the Black Sea), were dated back to the 2nd century BC (Pangalo, 1958). Melons were known to be cultivated in the territory of Ukraine and Russia for about 15 centuries, and the genealogy records of these melon types have been traced to eastern and central Asian lineages (Pyzhenkov and Malinina, 1994). Environmental differences across a wide spread agroecological zone

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diverged this group from the other melon morphotypes grown in western and southern Europe (Pyzhenkov and Malinina, 1994; Urina *et al.*, 1998). Melon production is predominantly spread throughout the large river basins: Don, Volga, and Dnepr and the coast of Azov and Black seas. The diverse melon germplasm that exists in Ukraine and the neighbouring countries Russia, Uzbekistan, Tajikistan and Kazakhstan evolved as one of the secondary centres of melon diversity (Fig. 1; Pangalo, 1958; Robinson and Decker-Walters, 1997). Currently, this particular market group of melons occupies about 50,000 ha throughout eastern Europe.

A number of studies have used molecular markers to examine genetic diversity of melons from the USA, western Europe, Africa, India and Japan (Silberstein *et al.*, 1999; Stepansky *et al.*, 1999; Oliver *et al.*, 2000; Mliki *et al.*, 2001; Decker-Walters *et al.*, 2002; López-Sesé *et al.*, 2002, 2003; Monforte *et al.*, 2003; Ritschel *et al.*, 2004; Staub *et al.*, 2000, 2004; Nakata *et al.*, 2005; Szabó *et al.*, 2005; Yashiro *et al.*, 2005; Dhillon *et al.*, 2007; Sensoy *et al.*, 2007; Tanaka *et al.*, 2007). On the other hand, there is little information about melon germplasm from east Europe, particularly from Ukraine.

The eastern European melon varieties were classified under the *convar Europeus*, which is also known as *adana* (Pangalo, 1958; Pyzhenkov and Malinina, 1994). The *convar Europeus*, is further divided into three different morphotypes: var. *europeus* – Skorospelka (early melon); var. *aestivalis* – Letniai (summer melon); and var. *hiemalis* – Zimniai (winter melon). Our objective of this study was to estimate genetic diversity in the Ukrainian melon collection using the polymorphisms generated by the amplified fragment length polymorphisms (AFLP) and simple sequence repeats (SSRs) and to further determine whether this molecular diversity corroborates the existing classification.

Materials and methods

The source of seed includes various accessions collected from the melon breeding program at Dnepropetrovsk State Agrarian University (DSAU) and from the Institute of Vegetable and Melon Crops at Ukrainian Academy of Agrarian Science. The list of pertinent accessions is presented in Table 1.

DNA was extracted from frozen leaf tissues using the method described in the DNeasy plant mini kit (Qiagen, Hilden, Germany). AFLP analysis was carried out using the protocols and kits developed by LI-COR Biosciences, Lincoln, NE, USA (www.licor.com). The *Eco*RI and *Mse*I enzyme-digested products were ligated to respective restriction-site-specific adapters and diluted 10-fold. Diluted adapter ligated templates were preamplified using adapter-specific primers with overhangs of A and C for *Eco*RI and *Mse*I, respectively. Preamplified products were further diluted 20-fold and subjected to selective amplification using IR-700 or IR-800 labelled *Eco*RI-AXX primers and unlabelled *Mse*I-CXX primers using standard touchdown polymerase chain reaction (PCR) conditions (Vos *et al.*, 1995). Amplified products were denatured and resolved on a LICOR-4500 genotyper. New SSRs were developed

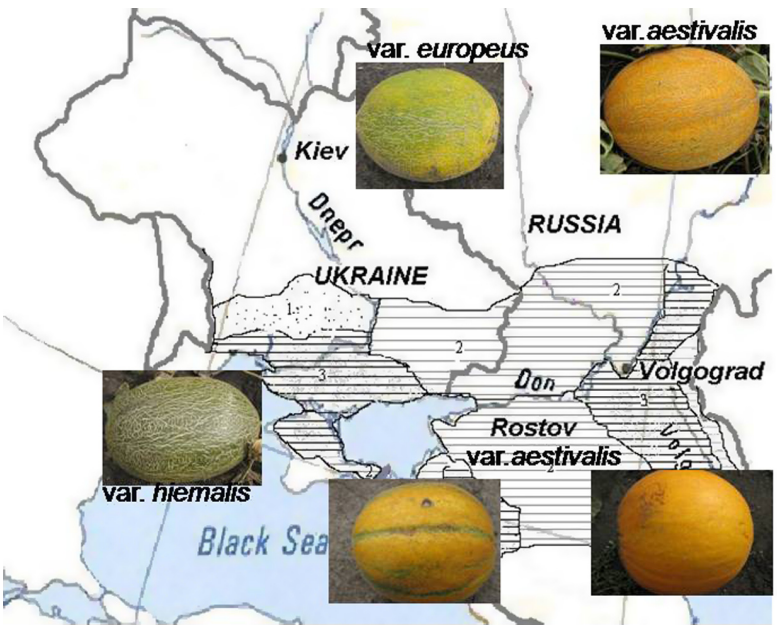

Fig. 1. Distribution of secondary centre of melon diversity across the agroecological zones of Ukraine and neighbouring countries (a colour version of this figure can be found at journals.cambridge.org/pgr).

https://doi.org/10.1017/S1479262108098481 Published online by Cambridge University Press

Table 1. List of the Ukrainian melon collections

S. No.	Samples	Origin	Convar[a]	Var.[a]
1	Gorkovska 310	Russia	*Europeus*	*aestivalis*
2	Lipneva	Ukraine	*Europeus*	*aestivalis*
3	Piepsha	Russia	*Europeus*	*aestivalis*
4	Samarskaia	Ukraine	*Europeus*	*aestivalis*
5	Titovka 1r. ex.station	Ukraine	*Europeus*	*aestivalis*
6	Titovka ex.station	Ukraine	*Europeus*	*europeus*
7	Ananas	Russia	*Europeus*	*aestivalis*
8	Zlata	Ukraine	*Europeus*	*aestivalis*
9	Kubanka 93	Russia	*Europeus*	*aestivalis*
10	Titovka original seed	Ukraine	*Europeus*	*europeus*
11	Titovka 1r. DSAU	Ukraine	*Europeus*	*europeus*
12	Titovka from Zaporogie	Ukraine	*Europeus*	*europeus*
13	Ingulka	Ukraine	*Europeus*	*europeus*
14	Gruntovaia gribovskaia	Russia	*Europeus*	*europeus*
15	Selena	Ukraine	*Europeus*	*aestivalis*
16	Lada	Ukraine	*Europeus*	*aestivalis*
17	Tavrichanka	Ukraine	*Europeus*	*aestivalis*
18	G-14	Ukraine	*Europeus*	*aestivalis*
19	Dneprianka 163	Ukraine	*Europeus*	*hiemalis*
20	Bronzovka	Russia	*Europeus*	*aestivalis*
21	Desertnaia 5	Russia	*Europeus*	*aestivalis*
22	Kynpou	Japan	*Chinensis*	*makuwa*
23	N38	Ukraine	*Europeus*	*europeus*
24	Koy Bash	Uzbekistan	*Rigidus*	*zard*
25	Promitey	Ukraine	*Europeus*	*hiemalis*
26	Dachnitza	Ukraine	*Europeus*	*europeus*
27	ZhZL	Ukraine	*Europeus*	*hiemalis*
28	KZhT	Ukraine	*Europeus*	*hiemalis*
29	KRL	Ukraine	*Europeus*	*europeus*
30	Beregina	Ukraine	*Europeus*	*aestivalis*
31	Ineia	Ukraine	*Europeus*	*aestivalis*
32	Diana	Ukraine	*Europeus*	*hiemalis*
33	Musa	Ukraine	*Europeus*	*aestivalis*
34	Blue sweet	Taiwan	*Orientalis*	*zhukowskii*
35	L.20/1	Ukraine	*Europeus*	*aestivalis*
36	L.22/1	Ukraine	*Europeus*	*aestivalis*
37	Rannia 133	Russia	*Europeus*	*europeus*
38	Krinichanka	Ukraine	*Europeus*	*europeus*

[a] According to Pyzhenkov and Malinina (1994).

using the enrichment procedure developed by Connell *et al.* (1998). Genomic DNA was digested with a set of restriction enzymes. Purified digested DNA was ligated to AP-11 and AP-12 adapters and hybridized with biotin labelled oligos, which contain the repeat motifs. DNA fragments that are hybridized with the repeat oligos were separated using streptavidin beads. These repeat-motif-enriched fragments were separated from the beads in an alkaline buffer for purification using a QIAGEN PCR purification kit. These enriched fragments were cloned (TOPO cloning kit; Invitrogen, Carlsbad, CA, USA) and 96 randomly picked clones were sequenced. The sequences with repeat motifs were identified and used for designing SSR primer pairs. PCR conditions for SSRs were used as per Reddy *et al.* (2001) and gel electrophoresis was carried out using SFR high-resolution agarose (www.amresco-inc.com).

Minor AFLP polymorphisms that were not uniformly amplified, such as being faint or not distinct in some genotypes, were eliminated from the analysis. Similarly, the stutter and background bands were not considered, while scoring SSR markers. The presence or absence of each fragment was scored as a binary unit character (1 = present and 0 = absent). Genetic similarities based on Jaccard's coefficients (Jaccard, 1908) were calculated using the SIMQUAL program of the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) Version 2.0 software package (Rohlf, 1998). The resulting genetic similarity indices were used to generate a tree using the neighbour joining method (Saitou and Nei, 1987). The robustness of the clustering was verified by bootstrapping (Felsenstein, 1985) using PAUP*4.0. Principal component analysis (PCA) based on the genetic

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similarity matrices were performed using DCENTER and EIGEN algorithms of the NTSYS-pc software package.

Results

Twenty AFLP primer combinations collectively amplified 2773 bands, out of which 423 bands were polymorphic among the Ukrainian melon collections. The polymorphism level of AFLPs among the collections in the current study was 15.8%. On average, 138 bands were amplified per primer combination. The range of polymorphisms for various primer combinations was from 7.5 to 30.3%. Polymorphic bands ranged from 5 to 52 for various primer combinations. Information pertaining to primer-pairwise amplification pattern and the respective number of polymorphic bands is presented in Table 2.

We made an SSR-enriched library and sequenced 96 clones and have obtained 40 SSR containing sequences. When we amplified, 12 of these were found to be polymorphic among the Ukrainian collections. Twelve polymorphic SSRs amplified a total of 42 alleles (Table 3). A diversity analysis using these 42 alleles produced a dendogram that did not resolve varietal relationships (tree not shown). Therefore, these 42 alleles were added to 423 AFLPs and a combined diversity analysis was carried out (Fig. 2).

Genetic diversity among the Ukrainian cultivars ranged from 0 to 38%. Similarity indices within the groups of *europeus*, *aestivalis* and *hiemalis* were 88, 74 and 85%, respectively. The phylogenetic tree contained three distinct clusters (Fig. 2). We estimated the bootstrap values (BV), and the clusters that were supported with 50 or above BV were considered as well supported. The first two clusters were on the top of the phenogram contained the *aestivalis* and *europeus* groups, respectively, and these clusters were supported by BV of 74 and 65, respectively, indicate a robust clustering pattern. A basal mixed group mainly consisted of the *hiemalis* along with the other exotic collections and was not supported by BV. The *aestivalis* cluster further resolved into six sub-clusters. Some of these sub-clusters were highly supported with BVs ranging from 51 to 98. A sub-cluster containing of Desertnaia 5, Lipneva, Samarskaia, Zlata Kubanka 93 and Pepsha were *aestivalis* types with furrowed fruit types. This was supported with the highest BV of 98. Two other sub-clusters were supported with BVs of 81 and 60 and contained *aestivalis* types with netted fruits. The fourth sub-cluster of *aestivalis* with the varieties of half-sibs Lada and Tavrichanka was supported with the BV of 52. A fifth sub-cluster of *aestivalis* varieties were L22/1–25, Musa, G-14 and Bronzovka, which have high sucrose content. The sixth subcluster of *aestivalis* types L20/1 and Bereginia was rooted from the bottom.

The second major clade containing *europeus* types resolved into sub-clusters based mainly on their maturity or fruit type. For example, the heirlooms KRL, Krinichanka and Rannia 133 were in one cluster and had similar fruit types, which were oval shape, white flesh colour, yellow exocarp and netted exocarp surface.

Table 2. Total number of amplified and polymorphic fragments generated using 20 AFLP primer combinations

S. No.	Primer combination	Amplified fragments	Polymorphic fragments	Polymorphism (%)
1	M-CAC/E-ACA 700	99	30	30.3
2	M-CAC/E-ACC 800	162	13	8.0
3	M-CAC/E-ACG 700	99	13	13.1
4	M-CAC/E-AGG800	120	9	7.5
5	M-CAG/E-ACG700	232	39	16.8
6	M-CAG/E-ACA700	427	52	12.2
7	M-CAG/E-AGG800	75	7	9.3
8	M-CAG/E-ACC800	176	24	13.6
9	M-CTC/E-ACG700	52	5	9.6
10	M-CTC/E-AAC700	149	21	14.1
11	M-CTC/E-ACC800	162	20	12.3
12	M-CTC/E-AGG800	87	15	17.2
13	M-CTG/E-AAC700	106	19	17.9
14	M-CTG/E-ACG700	54	13	24.1
15	M-CTG/E-AGG800	83	18	21.7
16	M-CTG/E-ACC800	176	31	17.6
17	M-CTT/E-ACA700	151	30	19.9
18	M-CTT/E-ACG700	149	33	22.1
19	M-CTT/E-ACC800	162	23	14.2
20	M-CTT/E-AGG800	52	8	15.4
	Total (mean)	2773 (138.7)	423 (21.19)	316.9 (15.8)

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Table 3. Details of microsatellites generated and used to amplify melon collections in the current study

S.No.	Forward primer	Reverse primer	Basic allele size	No. of alleles
1	ACGTACTTGCCGCAGACCAT	CCATTACCGCCATGCTCCAC	156	3
2	GCAGTTGCAGTTCGTGGAGAAG	ATGGAGCGCTCCCACAGAAC	129	2
3	ACCTGGGAAAAAGGCGATGC	CGGACGTTTTTCCTGCGTAAC	140	2
4	CTGCTAAGGGAGTACACCTCA	GAGTCCGTTGGTGTTTTTATACC	134	2
5	AATCTCCAACATCCGCGAAC	TGTAGACCTCAGCTTATCGGCTT	149	2
6	CCTGGAGTGCGGCAACATCA	ATCCCTCTGCTGGAGCGCA	114	3
7	CCATTACGTTACCCACGCCT	ATTCTCAACGACCTCTCCGGT	94	3
8	CCCTACATCCATATCCTTCATGT	TGGTCTGATCATCTCAACATGT	118	2
9	GAGCTGCATGTTCTTCGAGGTT	CATCGCGTCGAGACATCCTG	198	5
10	GACGGACGGCTCTATCCCA	GTCTTCATCAAATCATCGCCC	196	4
11	GCTTTTCTGCTTCCCGCAGA	GGTTCGCCAGAGTGGATAAGAGA	167	2
12	CTATTTGCAGCGAGAGATCTCC	GATAAGCGAGTCTGGTTTCGG	211	1
13	TCCAGGTTTGCCCTTCCATC	GCGCACATGGAAGTTGAAGC	267	4
14	AGAAGCTGCATCGCATTTAG	CTCAAGTTCCTGGACTTTTAAGT	177	2
15	GATTCCCAATTCACTCATGA	GTGGTAGCTGGACCCAGTC	240	3
16	CGTGTCGTGAGGCTAAGGC	GTATCGGGGAGCTGGCAAC	212	2

Second sub-cluster had many early maturing landraces of Titovka along with Ingulka, a core collection of the early type from the melon breeding station Khersone, Ukraine.

Third major clade of *hiemalis* along with some exotic collections resolved into two sub-clusters of several diverse melon types (Blue sweet from Taiwan, Kynpou from Japan and Koy Bash from Uzbekistan) and *hiemalis* (Dianna, Promitei, Dneprianka 163). Morphological marker accessions for Bush type KZhT and virescent marker ZhZl were also clustered with the mixed group

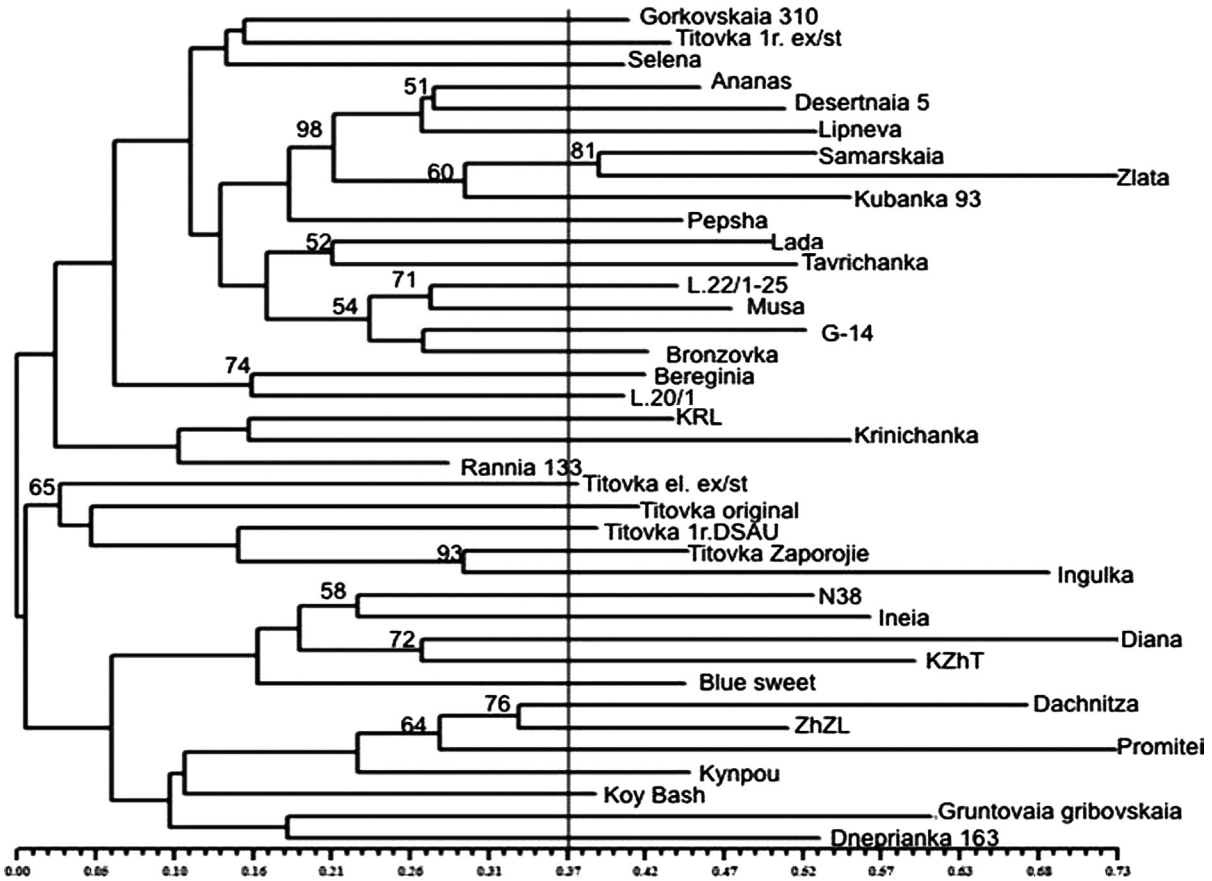

Fig. 2. Phenogram obtained with the joint analysis of amplified fragment length polymorphisms and simple sequence repeats. The numbers adjacent to some nodes indicate bootstrap confidence values (1000 bootstrap replicates).

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of melons. The collection Ineia, which is a typical *aestivalis* group, clustered with the mixed group of melons as an exception from the other *aestivalis* collections. The collections N38 and Gruntovaia gribovskaia are mixed-melon types, which appear to have undergone extensive introgression with the exotic melon types.

To corroborate the results of diversity analysis, we also carried out a PCA using the first three eigen vectors that cumulatively absorbed 66.04% of the variation (vector I = 32.15, vector II = 21.63 and vector III = 12.39). The extent of the cumulative variation absorbed by the first three vectors indicates robustness of the analysis. A three-dimensional graph of PCA was made using these three vectors (Fig. 3). Interestingly, this analysis also resolved three groups with the boundaries defining the classical morphotypes with few exceptions and more or less in agreement with the neighbor joining (NJ) analysis. A group of *aestivalis* types were clustered with an exception of Rannia and KRL, which are of typical *europeus* types. Another exception being two of the *aestivalis* types Kubanka 93 and Zlata, which were clustered with the mixed-melon group at the bottom of the PCA graph. All the *europeus* types were grouped together on top of the PCA graph. A cluster of mixed melons were at the bottom of the groups as topologies resolved in the NJ tree.

Discussion

Our study identified a robust set of AFLP polymorphisms that defined morphotype boundaries within the Ukrainian collections and was in agreement with previous studies. Therefore, the AFLPs are very informative in melon genome analysis. In melons, Garcia-Mas *et al.* (2000), after using random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and AFLPs, indicated that the AFLP markers are highly polymorphic and more informative than the other marker systems. Périn *et al.* (2002) used 346 AFLPs to generate a genetic map using melon RIL populations, indicating that the AFLPs are definitely useful for understanding cultivar relationships and mapping endeavours in melons.

The SSRs reported in the present study should prove to be very useful for the Ukrainian melon breeding program. They are simple to use in laboratories, which are equipped with horizontal gel electrophoresis but where no high-throughput facilities available. Several melon geneticists have used SSRs for understanding genetic diversity, species relationships, synteny with the other cucurbit species, quantitative trait loci (QTL) identification and genetic mapping (Staub *et al.*, 2000; Danin-Poleg *et al.*, 2001; Decker-Walters *et al.*, 2002; López-Sesé *et al.*, 2002; Monforte *et al.*, 2003; Garcia-Mas *et al.*, 2004; Ritschel *et al.*, 2004; Gonzalo *et al.*,

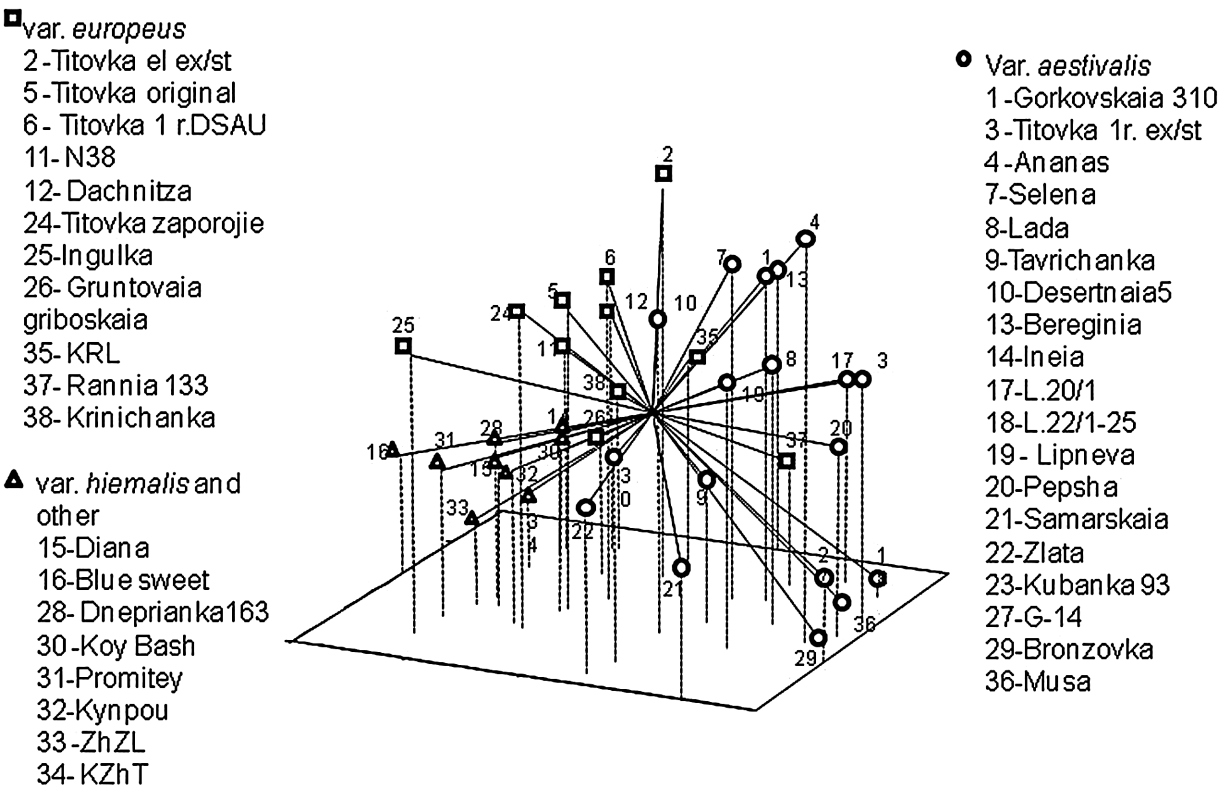

Fig. 3. Principal component analysis depicting relationships of the Ukrainian melon collections.

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2005). Three-hundred and twenty-four SSRs are currently available from various studies for public use to date. It is important to develop more SSR markers as these are technically facile and highly informative markers for various genetic studies. The small number of clones ($n = 96$) sequenced in this study yielded a high percentage of SSRs ($n = 40$), suggesting that significantly more SSRs might be obtained by sequencing a larger portion of the library.

In the current study, the phenogram resolved *aestivalis* and *europeus* groups with the strong support of significant BV. All the collections in the *aestivalis* group (cluster I) had smooth fruit surfaces except for five varieties (Desetnaia 5, Dachnitza, Lipneva, Pepsha and Samarskaia) which had fruits with a furrowed surface. The *europeus* group (cluster II) had accessions with predominantly early types. The fruit colour, while still immature varied from the green to dark green for the collections in cluster II. Further, when these fruits attained maturity, the colour changed to yellowish brown in all the accessions. The *europeus* varieties Ananas, Titovka original, Diana, Samarskaia, Gruntovaia gribovskaia, Koy Bash and Titovka Zaporojie had netted fruit surfaces with white coloured flesh. Ukrainian consumers prefer melons with white coloured flesh (Tomason, 2002). The polymorphisms generated in the current study, which are specific to the grouping of fruit types and days to maturity would be very useful to pursue further genetic studies and marker-assisted selections.

The third major clade of *hiemalis*, along with some exotic collections resolved into two sub-clusters of several diverse melon types (Blue sweet from Taiwan, Kynpou from Japan and Koy Bash from Uzbaekistan) and *hiemalis* (Dianna, Promitei and Dneprianka 163). The morphotype *hiemalis* has non-climacteric fruits and hence long shelf life. Another distinguishing feature among these groups is the differences in the sex expression. The sex expression in *europeus* and *hiemalis* groups is andromonoecious or monoecious, whereas in *aestivalis*, a majority collections are andromonoecious except in two collections. The two collections – L20/1 and L22/1–25 that are exceptions in sex expression from the rest of the *aestivalis* group are interestingly gynomonoecious (Tomason, 2002).

Our study provides useful information pertaining to morphological and classical morphotype characterizations of melons specific to Ukrainian collections. However, the current analysis would not shed any light on the cultivar relationships in the broader perspective of collections from other regions of the world. It would be very interesting to extend this study along with the existing international reference collections to draw relationships with the other melon morphotypes such as *agrestis, flexuosus, conomon, cantalupensis, inodorus, chito, dudaim and momordica* (Munger and Robinson, 1991; Robinson and Decker-Walters, 1997, Greuter *et al.*, 2000, Staub *et al.*, 2000, 2004). The relationships of various Ukrainian collections and knowledge about the extent of genetic diversity among them would aid planning the melon improvement programs in the Ukraine.

Acknowledgements

Authors would like to profusely thank NATO Science Foundation (Expert Visit: Ref. 982309) for funding the visit of Dr Yan Tomason and USDA-CSREES Research Grant (2007-03 466) Agreement Number (2007-38 814-18 472) for the funding support. We acknowledge Drs Mark Chatfield, Robert Harris and Gerald Hankins for their critical comments.

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