Article

Two-Steps Identification of N-, S-, R- and T-cytoplasm Types in Onion Breeding Lines using High Resolution Melting (HRM)-Based Markers

Ludmila Khrustaleva [1,2,*], Mais Nzeha [3], Aleksey Ermolaev [3,4], Ekaterina Nikitina [4] and Valery Romanov [5]

[1] Center of Molecular Biotechnology, Department of Botany, Breeding and Seed Production of Garden Plants, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy (RGAU-MTAA), 49, Timiryazevskaya Str., 127550 Moscow, Russia
[2] Laboratory of Plant Cell Engineering, All-Russian Research Institute of Agricultural Biotechnology, Timiryazevskay 42 str., 127550 Moscow, Russia
[3] Center of Molecular Biotechnology, Russian State Agrarian University-Moscow Timiryazev Agricultural Academy (RGAU-MTAA), 49, Timiryazevskaya Str., 127550 Moscow, Russia; mais123nzeha@gmail.com
[4] Laboratory of Applied Genomics and Crop Breeding, All-Russia Research Institute of Agricultural Biotechnology, Timiryazevskaya str. 42, Moscow 127550, Russia, All-Russia Research Institute of Agricultural Biotechnology, Moscow, Russia ermol-2012@yandex.ru; Shhket@gmail.com
[5] Federal Scientific Vegetable Center, Selectionaya St. 14, VNIISSOK, Odintsovo region, Moscow oblast, 143072, Russia; romanov_valera@mail.ru
* Correspondence: ludmila.khrustaleva19@gmail.com or khrustaleva@rgau-msha.ru

Abstract: High resolution melting (HRM) analysis is a powerful detection method for fast, high-throughput post-PCR analysis. A two-step HRM marker system was developed for identification of the N-, S-, R- and T-cytoplasms of onion. In the first step for identification of N-, S-, and R-cytoplasms, one forward primer was designed to the identical sequences of both *cox1* and *orf725* genes and two reverse primers specific to the polymorphic sequences of *cox1* and *orf725* genes were used. For the second step breeding lines with N-cytoplasm were evaluated with primers developed from the *orfA501* sequence to distinguish between N- and T-cytoplasms. An amplicon with primers to the mitochondrial *atp9* gene was used as an internal control. The two-step HRM marker system was tested using 246 onion plants. HRM analysis showed that the most common source of CMS, often used by Russian breeders, is S-cytoplasm, the rarest type of CMS is R-cytoplasm, and the proportion of T-cytoplasm among the analyzed breeding lines was 20.5%.

Keywords: Cytoplasmic male-sterility; High resolution melting (HRM); molecular markers; mitochondrial genes; onion (*Allium cepa* L.)

1. Introduction

Onion (*Allium cepa* L.) is the second most valuable vegetable crop in the world following by tomato. A significant increase in yield through heterosis was achieved by the discovery of CMS (Cytoplasmic Male Sterility) in onion [1]. The seed propagation of male sterile lines has become an important tool for the creation of commercial F_1 hybrids. The discovery of CMS systems in onions allows breeders to avoid the laborious work of anther removal when creating new breeding lines and overcome the difficulties in producing hybrid onion seeds. Knowledge about cytoplasm types plays one of the important roles in onion breeding.

CMS is determined by aberrant mitochondrial genes and associated with the failure to produce functional pollen. Due to advances in plant cell molecular biology the knowledge gained over the past decade clarified the role of mitochondria in triggering death of the male reproductive organs. Mitochondria is the energy station of cells. Pollen development is an energy costly process, therefore disturbances in mitochondrial functions could have dramatic effects on male fertility [2–4]. CMS products may interrupt either the assembly

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or functions of complexes in the electron transport chain [5,6] and perhaps play roles for programmed cell death and an excessive accumulation of reactive oxygen species (ROS) that are byproducts during the operation of the electron transport chain in mitochondria [7–9]. It should be stressed that due to a unique natural phenomenon of CMS only male gametes are impaired while female gametes are functional. Also, CMS products do not show abnormal phenotypes in somatic cells, which may be due to modification of CMS products at different stages resulting in the neutralization of their effect on mitochondrial function [10].

In plants, each mitochondrion contains multiple DNA molecules of different sizes that are capable of recombination [11]. The plant mitochondrial genome (mtDNA) is much larger than that of other eukaryotes and evolves very rapidly in structure [12]. Moreover, the plant mtDNA is composed of a mixture of circular subgenomes and linear and branched molecules. Recent NGS sequencing and new technology of mitochondrial genome assembly revealed primarily non-circular forms, which could be intermediates in replication or recombination [13]. CMS-associated genes cloned so far are mostly created by repeat-mediated mtDNA rearrangements. The plant mtDNA is rich in repeated sequences which can be involved in homologous recombination events and consequently have a major impact in the structure of the mtDNA [12]. CMS-associated genes are often chimeric genes composed of partial sequences of known mitochondrial genes and unknown sequences [4,14,15]. In most cases, multiple recombination events involving known mitochondrial genes as well as sequences of unknown origin create new open reading frames (ORF) associated with cytoplasmic male sterility in higher plants [4,16]. These CMS associated ORFs have been used for producing molecular markers to identified type of CMS.

The first source of onion CMS was discovered by Henry A. Jones, a geneticist who was known as the "father of the hybrid onion", in the cultivar 'Italian Red' in 1925 that later was determined as a S-cytoplasm CMS [1]. The second source of CMS (T-cytoplasm) was discovered by Berninger [17] in the cultivar 'Jaune paille des Vertus'. There are two hypotheses for the origin of S-cytoplasm. According to one hypothesis, mitochondria from another Allium species entered the cytoplasm of onion via the triploid top-setting onion 'Pran' [18]. Another hypothesis is based on the assumption of the existence of sympatric S- or N-cytoplasmic variants containing the corresponding mitochondrial DNA in the ancestral forms of the onion. S- and N-cytoplasm could be preserved during the domestication and dispersal of onion from its center of origin in Central Asia throughout the world [19]. S-cytoplasm is widely used by seed growers due to stable male sterility, no reduction in female fertility, and the relatively frequent occurrence of a recessive allele (*ms*) at the nuclear locus for restoring male fertility (*Ms*), allowing seed propagation of male-sterile lines [20]. T-cytoplasm is used less common than S-cytoplasm [21] and, presumably, it is a relatively recent cytoplasmic variant closely related to N-cytoplasm of onion [22].

Onion is a biennial crop and identification of cytoplasm types takes 4-8 years. However, the use of molecular markers capable to speed process and save time of breeders. Number of molecular markers to distinguish mitotypes in onion have been developed [23–27]. Most of the markers clearly distinguish normal N-cytoplasm and sterile S-cytoplasm. Sato [24] developed marker based on *cob* mitochondrial gene and chimeric gene that possessed an insertion of chloroplast DNA sequence into the upstream region of *cob* in the S-cytoplasm. The Sato [24] marker distinguishes between N- and S-cytoplasm. However, the 5' *cob*-marker did not distinguish T-cytoplasm from N-cytoplasm and in both cytoplasm types the 180-bp fragment is amplified [25]. For the first time, a markers capable of identifying N-, S- and T-cytoplasm was reported by [25]. The authors proposed the combination of the 5' *cob*-marker with the *orfA501* marker, which was developed from a CMS1-specific sequence in chives [28]. Later, Kim *et al.* [26] developed markers that also make it possible to identify three types of cytoplasm based on DNA sequences of other mitochondrial genes, *cox1* gene encoding cytochrome c oxidase subunit I and its chimeric *orf725* gene. Recently, Havey and Kim published, a comprehensive analysis of commercially used sources of CMS and nuclear male-fertility restoration *Ms* locus [19]. The authors made an important conclusion that there was a fourth type of cytoplasm, which they proposed to be labelled as 'R' cytoplasm, because it may have originated from the onion cultivar 'Rijnsburger' in the Netherlands. *Bona fide* T-cytoplasm was proposed to be labelled as 'T', which was genetically characterized by Berninger [17]. To add to this study and fill the gap covering the territory of the Russian Federation, we analyzed the types of CMS and genotyped for the nuclear *Ms* locus for fertility restoration across onion genetic collection kindly provided by Federal Scientific Vegetable Center and Timofeev breeding station of Russian State Agrarian University. In order to facilitate the monitoring of cytoplasmic type in large populations of breeding lines, HRM markers have been developed that clearly distinguish between N-, T-, R- and S-cytoplasm. Our study showed that S-cytoplasm is mainly used for onion breeding in Russian Federation, less often T-cytoplasm and rarely R-cytoplasm.

2. Results

2.1. Development of HRM markers for identification of N-, S- and R-cytoplasms (1st step)

Each plant cell contains many mitochondria [29] and each mitochondrion contains numerous DNA molecules [11]. Stoichiometries of *cox1* and *orf725* genes in plant cell define of N-, S- and R-cytoplasm types [19,26,30]. In N-cytoplasm, the copy number of normal *cox1* gene is significantly high and copy number of *orf725* is nearly undetectable. In contrast, S-cytoplasm is characterized by a higher number of *orf725* genes and significantly reduced numbers of the normal *cox1* genes. R-cytoplasm contains similar relative numbers of both *cox1* and *orf725* genes [19].

In order to distinguish between these three types of cytoplasm we developed markers on the *cox1* and *orf725* mitochondrial genes. To facilitate screening a large number of samples from onion breeding lines we applied high resolution melting (HRM) analysis, which is a powerful detection method for fast, high-throughput post-PCR analysis [31,32]. Previously, Kim and Kim [30] developed HRM markers based on *cox1* and *orf725* genes to identify the type of onion cytoplasm. However, the markers did not clearly distinguish between S- and T-type cytoplasm. According to later studies, the 'T-like' type of cytoplasm carrying the *orf725* gene was proposed to be the R-type and cytoplasm carrying *orfA501* as *bona fide* T-cytoplasm [19].

To develop HRM markers, we performed partial sequencing of regions with a length 594 bp based on known *cox1* gene sequences extracted from the complete mitochondrial genome of N-cytoplasm (GenBank: AP018390.1) and with a length 577 bp based on *orf725* gene sequences extracted from CMS-S complete mitochondrial genome (GenBank: NC_030100.1) in eleven breeding lines (Table 3). The *cox1* amplicons were obtained from eight breeding lines as expected (Table 2). The *orf725* amplicons were obtained for six breeding lines possessing CMS (Table 2). The amplicons were sequenced by Sanger using constructed primers (Table 5). Both *cox1* and *orf725* genes partial amplicons shows no difference from normal and CMS-S references sequences, respectively (Supplementary Figures 1 and 2).

HRM marker was developed using a combination of one common primer (AcM-HRM-F1) anchored to the identical sequence of both *cox1* and *orf725* genes and two specific primers (AcM-HRM-R1 and AcM-HRM-R2) anchored to the polymorphic sequences of *cox1* and *orf725* genes, respectively (Figure 1). The melting temperature (T_m) and size of the expected amplicon from *cox1* gene were 80 °C and 128 bp, respectively. T_m and size of the expected amplicons from *orf725* gene were 89 °C and 132 bp, respectively. Normalized melting curve of amplicons belonging to normal cytoplasm were clearly separated from that of CMS cytoplasm (Figure 2). The curve with one peak at T_m = 80 °C corresponds to N-cytoplasm, the curve with one peak at T_m = 89 °C corresponds to S-cytoplasm and the curve with two peaks corresponds to R-cytoplasm. The sequenced breeding lines were evaluated using the developed HRM marker system. The results of the HRM analysis of the cytoplasm types completely coincided with the results of morphological and cytological analysis (Table 3).

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Table 1. Conventional PCR marker system in the mitochondrial DNA distinguishing of onion cytoplasm according to the classification proposed by Heavy and Kim [19].

Reference	Marker on the gene	Cytoplasm[1]/Amplicon size, bp			
		N	S	R	T
[24]	*cob*	180	414	180	180
[25]	*orfA501*	absent	473	473	473
[26]	*cox1*	833	absent	833	833
	orf725	absent	628	628	absent

1 - Normal (N) male-fertility cytoplasm and male-sterile cytoplasm (S), (T) and (R).

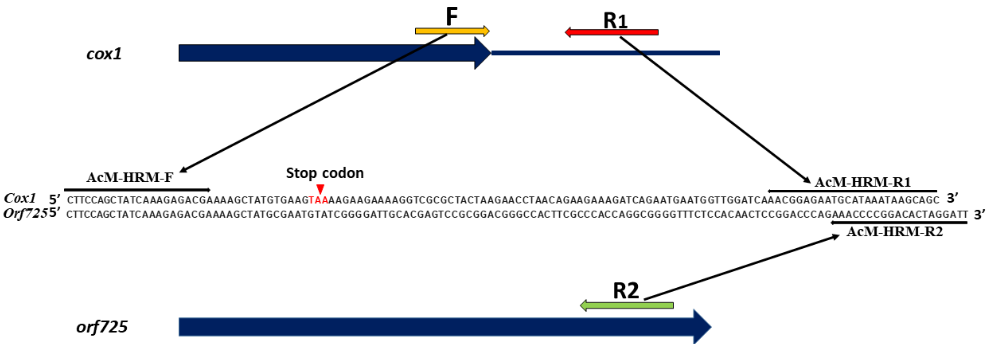

Figure 1. HRM primer annealing sites on *cox1* and *orf725* gene sequences. Primer binding sites are indicated by horizontal arrows. The red triangle indicates the stop codon of the *cox1* gene.

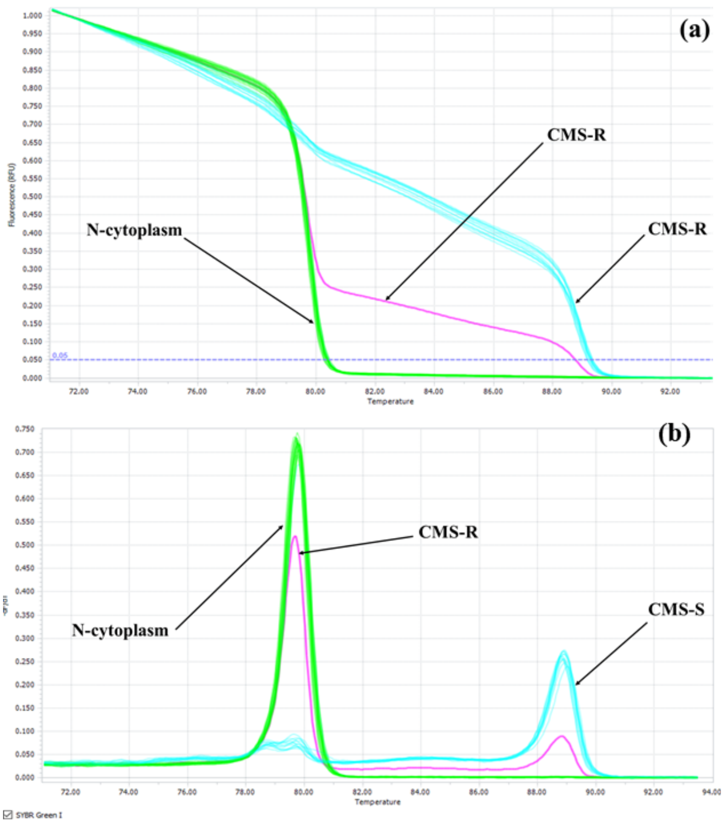

Figure 2. HRM analysis of cytoplasm type in onion using three AcM-HRM primers based on *cox1* and *orf725* mitochondrial genes: A – normalized melting curve of amplicons; B – normalized melting peaks of amplicons.

2.2. Developing of HRM markers for distinguishing between N- and T- cytoplasm types (2nd steps)

The third type of CMS is T-cytoplasm, which is closely related to N-cytoplasm of onion [19]. The first PCR markers based on mitochondrial DNA sequences [24] did not distinguish between N- and T-cytoplasms, while T-cytoplasm showed the same PCR product as N-cytoplasm. A PCR marker was developed to distinguish between N- and T-types of the cytoplasm by Engelke *et al*. [25]. This marker was developed from a CMS_1-specific sequence in chives (*Allium schoenoprasum* L.), a closely related species of onion [28]. In chives the sequence is of chimerical nature consisting the *atp9* homologous sequence interrupted at position 147 bp a 623 bp insertion of unknown origin at its 5′-end. Assuming that *A. cepa* CMS is of alloplasmic origin [18], the authors designed primers based on the chives sequence, which span nearly the complete *orfA501*. With this *orfA501* marker, in combination with markers developed by Sato [24], it became possible to distinguish between N-, T- and S- cytoplasm types. Later, a new open reading frame mitochondrial chimeric gene (*orf725*) was isolated containing almost the entire *cox1* gene sequence at the 5′ end, a 473 bp sequence homologous to the chives *orfA501* gene, and a unique sequence at the 3′ end [26]. Although, the authors reported that it was impossible to distinguish between N- and T-cytoplasm with *orf725* marker. Therefore considering all the knowledge described above, we may conclude that the *orf725* chimeric gene is the result of an insertion into *cox1* gene and this chimeric gene present in S- and R-cytoplasm. However, it is still unknown in which gene the *orfA501* was inserted in the onion mitochondrial genome.

In order to test the hypothesis that the *orfA501* inserted into the *atp9* gene in *A. cepa*, as was found in *A. schoenoprasum* [28], we constructed a putative chimeric gene by inserting a 473 bp *orfA501* sequence of the *A. cepa* from CMS-S complete mitochondrial genome (GenBank: NC_030100.1) into *atp9* gene of the *A. cepa* N-cytoplasm from complete mitochondrial genome (GenBank: AP018390.1) after the position 147 at 5′-end. HRM marker was developed using a combination of one common primer atp9-HRM anchored to the identical sequence of both *atp9* and a putative chimeric *orfA501* genes and two specific primers atp9-R1 and orfA501-R2 anchored to the polymorphic sequences. A PCR product with expected size and T_m was obtained only for *atp9* gene suggesting its intact nature at least in amplified region. So, HRM-markers were developed separately using two T-HRM primers designed on *atp9* gene as an internal control and two atp9-HRM primers designed on the *orfA501* sequence (Table 5; Figure 3).

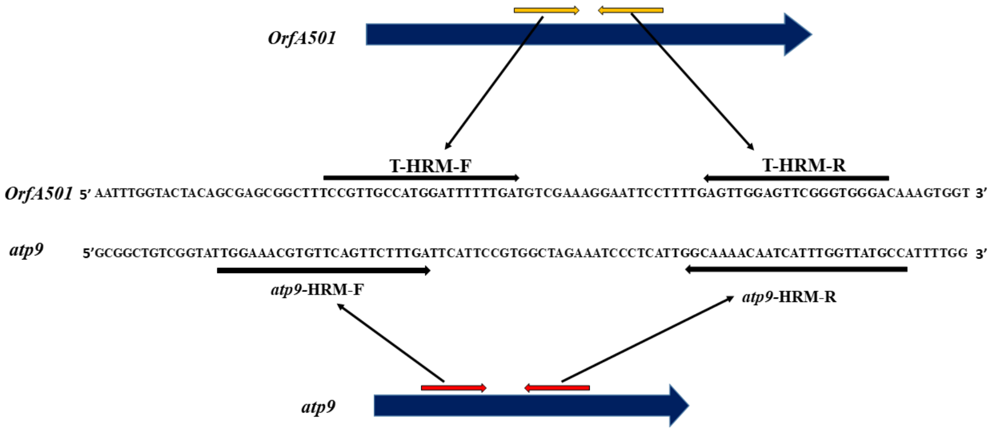

Figure 3. HRM primer annealing sites on *orfA501* and *atp9* gene sequences. Primer binding sites are indicated by horizontal arrows.

A total of 130 individual plant DNA samples with N-cytoplasm as determined by the first step were evaluated simultaneously with *orfA501* and *atp9* primers using the HRM assay. Thirty DNA samples among the 130 analyzed detected T-cytoplasm. The normalized melting curve of amplicons belonging to the N-cytoplasm was clearly separated from that of the CMS-T cytoplasm (Figure 4a). The N-cytoplasm is characterized by only one

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normalized melting peak that is obtained on *atp9* gene, and T-cytoplasm is characterized by two normalized melting peaks that is obtained on *orfA501* and the intact *atp9* (Figure 4b).

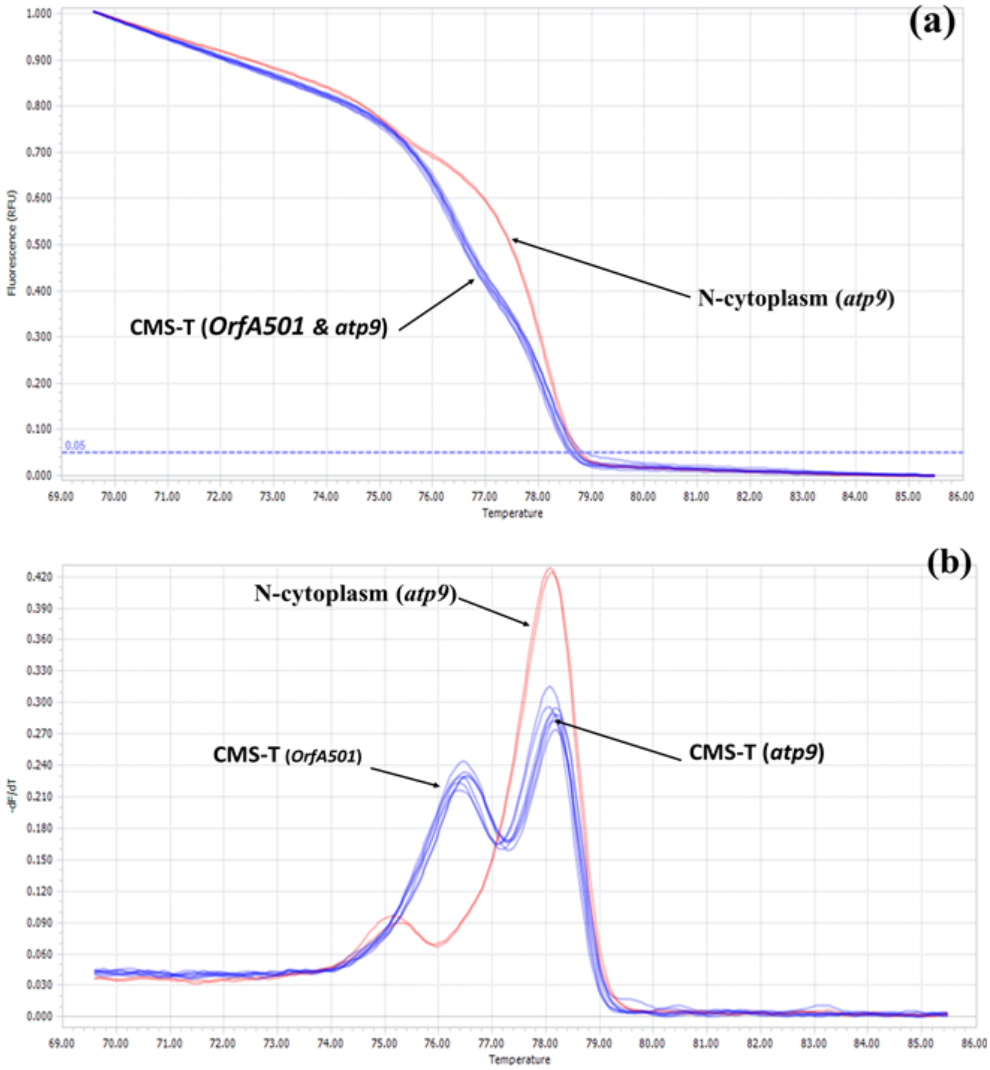

Figure 4. Difference between N and T-cytoplasms detected using T-HRM primers based on orfA501 and atp9-HRM primers based on atp9 gene (internal control): (a) normalized melting curve of amplicons; (b) normalized melting peaks of amplicons.

2.3. Validation of the reliability of two-steps HRM marker system by comparison with existing PCR marker systems

In order to evaluate the HRM markers developed in this study, a conventional PCR was carried out with markers reported earlier by Sato [24], Engelke *et al.* [25] and Kim *et al.* [26]. The PCR-markers used for determination of cytoplasm types are presented in Table 5. The Sato [24] marker distinguished N- and S-cytoplasm. Combination of the Sato [24] marker with the *orfA501* marker proposed by Engelke *et al.* [25] distinguished three types of cytoplasm: N-, S-, and *bona fide* T-cytoplasms. Primers developed by Kim *et al.* [26] identified N-, S-, and R-cytoplasms [19]. Using the combination of three marker systems is possible to distinguish four cytoplasm types of onion (Table 1). Analysis of eleven breeding lines with conventional PCR showed that the results (Table 2; Figure 5) completely agreed with the HRM results.

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Table 2. Comparative analysis of eleven breeding lines using conventional PCR and HRM-analysis.

Breeding line	Markers				Cytoplasm type	
	cob	*orfA501*	*cox1*	*orf725*	PCR	AcM-HRM
Banko	180	absent	833	absent	N	N
Sibirskiy	180	absent	833	absent	N	N
Derek-8	180	absent	833	absent	N	N
Odintsovets-28	180	absent	833	absent	N	N
LFK	180	473	833	628	R	R
CM Banko	180	473	833	628	R	R
Ivashka	180	473	833	628	R	R
Rawhide-17	414	473	absent	628	S	S
Sandra-276	414	473	absent	628	S	S
Derek-3	414	473	absent	628	S	S
Odintsovets-37	180	473	833	absent	T	T

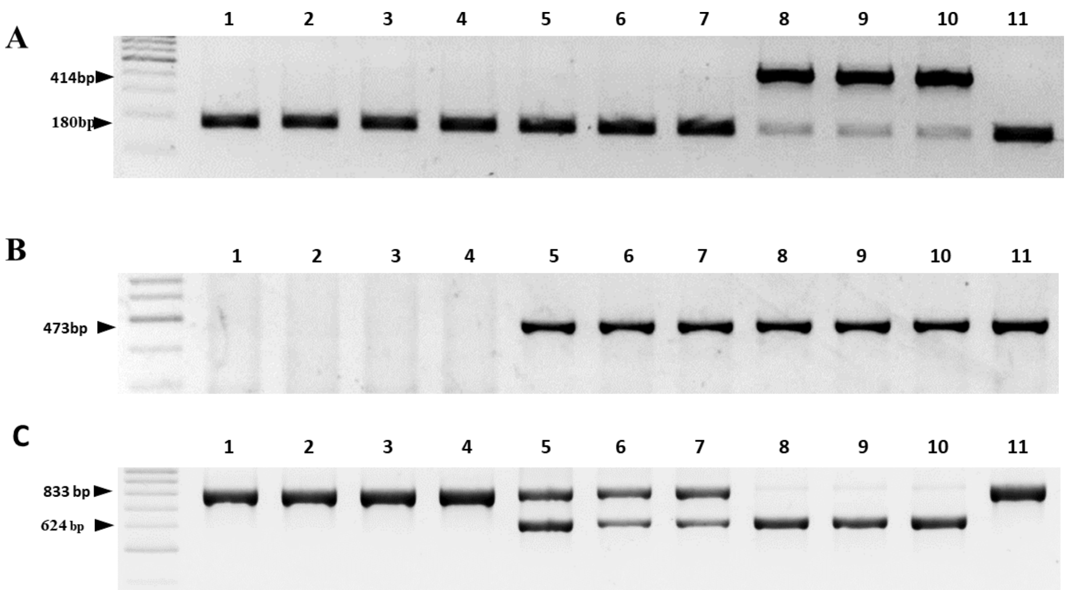

Figure 5. Identification of cytoplasm types in onion breeding lines using conventional PCR marker system developed by Sato [24] — A, Engelke *et al.* [25] — B and Kim *et al.* [26] — C. Lines: 1 — Banko; 2 — Sibirskiy; 3 — Derek-8; 4 — Odintsovets-28; 5 — LFK; 6 — CM Banko; 7 — Ivashka; 8 — Rawhide-17; 9 — Sandra-276; 10 — Derek-3; 11 — Odintsovets-37.

2.4. Analysis of the cytoplasm types and Ms *locus in breeding lines used by Russian breeders*

A total of 246 individual plant DNA samples from 77 breeding lines were analyzed using the two-step HRM marker system developed in this study. For genotyping of *Ms* locus we used Rf-HRM7 markers developed by Kim and Kim [30]. The authors constructed Rf-HRM7 markers based on InDel in partial sequences of *AcPMS1* alleles. *AcPMS1* gene encoding the PMS1 protein involved in DNA mismatch repair in other plant species was suggested to be a causal gene for restoration of male-fertility [33].

Identification of cytoplasm type and genotyping at *Ms* locus showed that 73 plants possessed N-cytoplasm and were homozygous recessive at the *Ms* locus (*msms*), and therefore are maintainer lines (Table 4). Twelve plants among 100 analyzed plants with N-cytoplasm were scored as homozygous dominant (*MsMs*) and 15 plants as heterozygous (*Msms*) at *Ms* locus. A total of 72 individual plans possessed S-cytoplasm and homozygous recessive at *Ms* locus (*msms*), and therefore could be used female parents for production of hybrid seed. Only 5 plants with S-cytoplasm were scored as homozygous dominant at *Ms* (*MsMs*) and 32 plants as heterozygous (*Msms*). R-cytoplasm was identified in seven plants, of which three were homozygous recessive at *Ms* locus (*msms*), two were homozygous

Table 3. Breeding lines used for partial sequencing of *cox1* and *orf725* genes and HRM analysis of cytoplasm types.

Breeding line	Fertility / sterility[1]	Cytoplasm Type[2]	*Ms* locus *AcPMS1*
Banko	fertile	N	*msms*
Sibirskiy	fertile	N	*Msms*
Derek-8	fertile	N	*msms*
Odintsovets-28	fertile	N	*msms*
LFK	sterile	R	*msms*
CM Banko	sterile	R	*msms*
Ivashka	fertile	R	*MsMs*
Rawhide-17	sterile	S	*msms*
Sandra-276	sterile	S	*msms*
Derek-3	sterile	S	*msms*
Odintsovets-37	fertile	T	*msms*

1 - Pollen fertility was established by morphological analysis of anther development and microscopy of pollen grains using acetocarmine staining.
2 - Normal (N) male-fertility cytoplasm and male-sterile cytoplasm (S), (T) and (R).

Table 4. Comparative analysis of eleven breeding lines using conventional PCR and HRM-analysis.

	Genotype of *Ms* locus			
Cytoplasm type	***MsMs***	***Msms***	***msms***	**Total**
N	12	15	73	100
S	5	32	72	109
R	2	2	3	7
T	0	1	29	30
Total	19	50	177	**246**
Percentage	**7.7%**	**20.3%**	**72.0%**	

dominant (*MsMs*) and two heterozygous (*Msms*). T-cytoplasm was found in 30 plants of which 29 of them were scored as homozygous recessive at *Ms* locus (*msms*), and only one plant was heterozygous (*Msms*).

3. Discussion

To provide information on distribution of CMS-systems and to facilitate the monitoring of cytoplasmic type of large breeding populations, we developed a two-step HRM marker system for identification of N-, S-, R- and T-cytoplasms. The identification of the cytoplasm of a single plant takes from 4 to 8 years and is complicated by the segregation of the nuclear gene that restores fertility. The HRM marker system clearly distinguished the four types of cytoplasm. The availability of reliable markers for the selection of CMS and maintainer lines will accelerate the development of locally adapted hybrids [23]. The use of the HRM closed tube marker system with high sensitivity and high throughput greatly facilitates the work of breeders.

Analysis of 77 breeding lines (2-5 plants per breeding line) showed the presence of individual plants that were heterozygous at *Ms* locus within breeding lines. In total 20.3% of individual plants were heterozygous at *Ms* (Table 4). One explanation could be that the dominant allele at *Ms* can show reduced penetrance, complicating efforts to purge dominant alleles from an inbred line or population [34]. Another reason for the high percentage of heterozygosity may be the non-use of molecular markers by breeders and selection by the classical crossing and scoring segregation.

Eleven breeding lines were used for development of the two-step HRM marker system. The fertility/sterility of the breeding lines were established by analysis of anther's morphology and microscopy of acetocarmine stained pollen grains (Table 3).

An interesting observation was that the breeding line 'Ivashka' with R-cytoplasm was scored as homozogous dominant at *Ms* and is male fertile. This agrees with previous

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observation that the R-CMS is restored by the *Ms* locus, and this is consistent with the conclusion made by Havey and Kim [19]. We observed another scenario in the breeding line 'Odintsovets-37' with T-cytoplasm, which was scored as homozogous recessive at *Ms* and fertile. However, if the gene/genes restoring the fertility of the T-cytoplasm were in the *Ms* locus, then the plant should be sterile with *msms* nuclear genotype. [35] reported that male sterility of T–cytoplasm is conditioned by the interaction of T-cytoplasm with three male fertility restoration loci. Havey [36] showed by crossing the dominant restoration allele at *Ms* onto T-cytoplasmic male-sterile plants that T-cytoplasm is not restored by *Ms* locus. Apparently, the three restoration loci which restore the fertility of the T-cytoplasm have yet to be discovered and markers developed.

Analysis of breeding lines from two germplasm collections showed that S-cytoplasm is the most common source of CMS used by Russian breeders. R-cytoplasm, a CMS developed from 'Rijnsburger' onion and discovered by De Vries and Wietsma [37], appeared to be the rarest type of CMS among the Russian breeding lines. While R-cytoplasm is widely used to produce commercial hybrid seeds [19]. Of the 146 samples with sterile cytoplasm, 30 were with T-cytoplasm, which amounted to 20.5%. Most probably T-cytoplasm in analyzed breeding lines was originated from Chalcedon variety created by breeders from the Pridnestrovian Agricultural Research Institute in the late 80s and then spread through the territory of the former Soviet Union. Most likely, the Chalcedon variety was created using the T-cytoplasm described by Berninger [17].

4. Materials and Methods

4.1. Plant materials

Sixty-eight breeding lines (237 individual plants) from the genetic collection of the Federal Scientific Vegetable Center and 9 breeding lines from the genetic collection of the Timofeev Breeding Station were analyzed. In the third week of April, under favorable weather conditions and an air temperature of 14-16 °C, selected bulbs 5-6 cm in diameter were planted in soil on ridges 140 cm wide in 4 rows (25 cm between rows and 15-20 cm between bulbs in a row). In the second week of May, when growth of the leaves from the bulbs began, samples were taken from young leaves. The leaves were stored in a freezer at -80 °C until DNA isolation.

4.2. Total DNA extraction

Frozen samples were dried in Labconco Drying Chamber and then pulverized in TissueLyser II, Qiagene with stainless steel beads. Total genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) method according to the protocol of [38].

4.3. Partial sequencing of cox1 and orf725

Primers (EX-cox1-F, EX-cox1-R, EX-orf725-F and EX-orf725-R) were designed using Primer3Plus (https://www.primer3plus.com/) to amplify gene fragments with a length 594 bp based on known *cox1* gene sequences extracted from N-cytoplasm complete mitochondrial genome (GenBank: AP018390.1) and with a length 577 bp based on *orf725* gene sequences extracted from CMS-S complete mitochondrial genome (GenBank: NC_030100.1). Since both genes are present in stoichiometric copies in the CMS mitochondrial genome, amplicons for each gene were obtained in separate PCR. The amplicons were Sanger sequenced (Evrogen, Moscow) using designed primers. Poorly sequenced regions on the ends of amplicons were trimmed based on peaks in chromatograms. Only high quality regions were used in following analysis. Multiple alignments were created using Clustal Omega v1.2.4 [39] and visualized using Jalview v2.11.2.4 [40]. *cox1* and *orf725* gene sequences of *A. cepa* were extracted from normal (GenBank: AP018390.1) and CMS-S (GenBank: NC_030100.1) reference mtDNA sequences of *A. cepa*, respectively, based on annotation in GenBank database.

4.4. AcM-HRM analysis of cytoplasm type

High Resolution Melting (HRM) PCR was performed using the primer combination of one common forward primer (AcM-HRM-F) and two specific reverse primers (AcM-HRM-R1, AcM-HRM-R2). AcM-HRM-F primer hybridizes in a conserved upstream region of both the *cox1* gene and the chimeric *orf725* gene derived from the *cox1* gene. The AcM-HRM-R1 primer anchors in the downstream region in the *cox1* gene and the AcM-HRM-R2 anchors in the downstream region of the *orf725* gene (Table 5).

A total volume of 20 μL of PCR mixture contained the following components: 2.5× RT-PCR reaction mix containing 2.5× PCR buffer B (KCl, TrisHCL, pH 8.8), 6.25 mM $MgCl_2$, Syn Taq polymerase, dNTPs, Glycerol, Tween 20 and EVA Green (Syntol, Moscow, Russia), 0.5 μM of AcM-HRM F, 0.25 μM AcM-HRM R1 primer, 0.25 μM AcM-HRM R2 primer, 5 μl of DNA (0.05 μg) and sterile distilled water.

PCR conditions were as follow: an initial denaturation step at 95 °C for 10 min and 45 cycles at 95 °C for 10 s, 60 °C for 5 s, and 72 °C for 5 s. The PCR products were then heated to 95 °C with a ramp rate of 4.4 °C/s, cooled to 40 °C with a ramp rate of 2.2 °C/s, and heated again to 65 °C with a ramp rate of 2.2 °C/s. By melting from 65 to 97 °C at a ramp rate of 0.07 °C/s melting curves were obtained.

Table 5. PCR markers used for determination of cytoplasm types and *Ms* locus in onion.

Primer names	Primer sequence (5'-3')	The marker based on genes	Reference
ACM-HRM-F ACM-HRM-R1 ACM-HRM-R2	GCTATCAAAGAGACGAAAAGCT GCTGCTTATTTATGCATTCTCCGT AATCCTAGTGTCCGGGGTTT	*cox1* & *orf725*	This study
MK-F MK-R1 MK-R2	CATAGGCGGGCTCACAGGAATA AATCCTAGTGTCCGGGGTTTCT CAGCGAACTTTCATTCTTTCGC	*cox1* & *orf725*	[26]
	ATGGCTCGCCTTGAAAGAGAGC CCAAGCATTTGGCGCTGAC	*orfA501*	[25]
Forward primer (S)-specific (N)-specific	CTTTTCTATGGTGACAACTCCTCTT GTCCAGTTCCTATAGAACCTATCACT TCTAGATGTCGCATCAGTGGAATCC	*cob*	[24]
EX-cox1-F EX-cox1-R EX-orf725-F EX-orf725-R	TATCCAGATGCTTACGCCGG ACTCGAACCTGCACTTCTGG TTACGCCGGATGGAATGCTC ACTGGGCGAATCACCACTTT	*cox1* & *orf725*	This study
T-HRM-F T-HRM-R atp9-F atp9-R	TTTCCGTTGCCATGGATTTT CCGAACTCCAACTCAAAAGG TGGAAACGTGTTCAGTTCTTTGA GCATAACCAAATGATTGTTTTGCCA	*orfA501* *atp9*	This study
Rf-HRM7-F Rf-HRM7-R	CCTATTCAATCCCTGGACATTT GAGTTTGAAGGGCTATCTTTACTTG	*AcPMS1*	[30]

4.5. T-HRM and atp9-HRM

A total volume of 20 μL contained the following components: 2.5× RT-PCR reaction mix containing 2.5× PCR buffer B (KCl, TrisHCl, pH 8.8), 6.25 mM $MgCl_2$, Syn Taq polymerase, dNTPs, Glycerol, Tween 20 and EVA Green (Syntol, Moscow, Russia), 0.2 μM of dNTPs, 0.25 μM of T-HRM forward and reverse primers and 0.125 μM forward and reverse atp9-HRM primers, 5 μL of DNA (0.05 μg) and sterile distilled water.

PCR conditions were as follow, an initial denaturation step at 95 °C for 10 min and 45 cycles at 95 °C for 10 s, 61 °C for 10 s, and 72 °C for 10 s. The PCR products were then heated to 95 °C with a ramp rate of 4.4 °C/s, cooled to 40 °C with a ramp rate of 2.2 °C/s, and heated again to 65 °C with a ramp rate of 2.2 °C/s. By melting from 65 ° to 97 ° C at a ramp rate of 0.07 C/s melting curves were obtained.

High-resolution analysis of DNA melting curves was performed using a real-time PCR system on a Roche LightCycler 96 amplifier. Measurement of the kinetics of the dissociation of DNA molecules was performed using the EvaGreen intercalating dye (Moscow, Russia, https://ru.lumiprobe.com/).

4.6. Conventional PCR

To check the HRM markers developed in this study PCR was carried out with markers reported earlier by Engelke [25], Sato [24] and Kim *et al*. [26]. Primers which anchor in the upstream region to the *cob* gene were used [24] to distinguish between N-cytoplasm and CMS-S. Primers that span nearly the complete *orfA501* were used in combination with primers to the *cob* gene [24] as suggested from Engelke *et al*. [25] for the differentiation of all three types of cytoplasm. All primers are listed in Table 5.

PCR amplification was performed in a 25 μL reaction mixture containing 50 ng template, 2.5 μL 10× PCR buffer, 0.2 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL dNTPs (10 mM each), and 0.5 U of Taq polymerase. PCR products were visualized on 2% agarose gel after ethidium bromide staining.

4.7. HRM analysis of Ms locus

Genotyping of *Ms* locus was carried out with HRM markers developed on full-length genomic DNA of *AcPMS1* sequences obtained from both homozygous dominant and recessive alleles reported by Kim and Kim [30](Table 5). PCR mix and condition were the same as described above for AcM-HRM analysis of cytoplasm type.

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Abbreviations

The following abbreviations are used in this manuscript:

HRM	High Resolution Melting
CMS	Cytoplasmic Male Sterility

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