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Elucidating mitochondrial DNA-markers of *Ogura*-based CMS linesin Indian cauliflowers (*Brassica oleracea* var. *botrytis* L.) and their floral abnormalities due to diversity in cyto-nuclear interaction

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Abstract

Background: Mitochondrial markers can be used to differentiate diverse mitotypes as well as cytoplasms in angiosperms. In cauliflower, cultivation of hybrids is pivotal in remunerative agriculture and cytoplasmic male sterile lines constitute an important component of the hybrid breeding. Thus, the breeders look for utilizing diverse male sterile cytoplasms in hybrid progamme. In diversifying source of male sterility,it is essential to appropriately differentiate among the available male sterile cytoplasms in cauliflower.PCR polymorphism atthe key mitochondrial genes associated with male sterility will be useful in developing mitochondria specific markers for the different male sterile cytoplasms. Also, the auto and alloplasmic cytonuclear combinations result in complex floral abnormalities.Thus, the study aimed at developing mitotype specific markers of the sterile cytoplasms and to unravel thegenetic effects of the cytonuclear interactions on flower morphology in Indian cauliflowers.

Results: In PCR based analysis using a set of primers targeted to *orf-138*, 76 Indian cauliflower lines showed presence of *Ogura* cytoplasm though amplicons showed polymorphism within the *ofr-138* sequence. The polymorphic loci were found to be spanning over 200-280 bp and 410-470bp genomic regions of *BnTR4* and *orf125*, respectively. Sequence analysis revealed that such cytoplasmic genetic variations could be due to single nucleotide polymorphisms and insertion or deletions of31/51 nucleotides.The cytoplasmic effects on varying nuclear-genetic backgrounds led to varying degree of floral malformations ranging from reduction in flower size, stamens and style length, modification in position of styleand anthers, absence of non-functional stamens to other floral abnormalities. These floral malformations caused dysplasia of flower structure affecting female fertility and inefficient nectar production.

Conclusions: The mitochondria specific markers can distinguish *ogura*based male sterile cytoplasm. Large number of Indian cauliflower lines showed mitotype variations even within the *ogura* based cytoplasm. Variable nucleo-cytoplasmic interactions resulted into diverse type of floral malformationsin addition to pollen sterility even within the group of *ogura* based CMS lines. The finding provide important reference ameliorate understanding of mechanism of cytonuclear interactions in floral organ development in *Brassicas.* The study will help the breeders in selecting CMS lines without any floral abnormalities in *B. oleracea*.

Background

Mitochondria are primarily maternally inherited, semi-autonomous, endosymbiotic complex organelles in eukaryotic cells [1-2].Mitochondria play a central role in energy production by oxidative phosphorylation, apoptosis and other cellular processes [2, 3]. The migration of essential genes from mitochondrial genome to nuclear genome has been a consistent feature of genome evolution in eukaryotic organisms and thus mitochondrial genomes in many organisms comprise ofan incomplete set of essential genes required for their autonomous function [4-5]. Simultaneously, mitochondria participate in retrograde signaling and regulate the nuclear gene expression [5-6]. Hence, integrated action of mitochondrial and nuclear genomes is pivotal for many of the cellular functions. As compared to most of the metazoan mitochondrial genomes, the plant mitochondrial genomes are considerably larger with extensive variation in genomesize and are complex in structure. The substantial structural complexity and variation in genome size of plant mitochondrial genome is resulted from the accumulation of diverse repeat sequences, frequent homologous recombination of large repeat sequences and inclusion of foreign DNA fragments [2, 7-9]. The plant mitogenomes also have the remarkable features of presence of highly diverse inter-genetic regions, frequent mt-DNA rearrangements, slow evolution in mtDNA sequences and rapid structural evolution [2, 7-9]. On account of these characteristics, the extensive genome reorganization and shuffling in gene order may occur

in plant mitogenomes and unusual open reading frames (*ORFs*) are generated, some of which causes extreme phenotypes such as cytoplasmic male sterility (CMS) [2, 10-11].

The CMS is a maternally inherited trait which is characterized by inability to produce viable pollen or male reproductive organs for sexual fertilization [2, 5-6]. The mitochondrial genes determining CMS phenotype can be masked by fertility restorer genes (*Rf*) in nuclear genome. Presence of male sterile cytoplasm without the *Rf* gene in the nuclear genome results in CMS phenotype [2, 6, 11]. CMS is an important trait to provide new insights into plant nuclear-mitochondrial communication. Cytoplasm induced male sterility is a widespread phenomenon reported in over 150 plant species [2, 6, 12]. The CMS system is instrumental for utilization of heterosis in vegetables *Brassicas* for higher yield and quality traits [13-17]. Cauliflower (*Brassica oleracea* var. *botrytis* L.) is an important member of vegetable*Brassicas*grown worldwide. It has been an important contributor in human diet owing to high content of dietary minerals, vitamins, glucosinolates and other phytochemical compounds with antioxidant and anticancer properties [13-14, 18]. Availability of genetic mechanisms like sporophytic self-incompatibility (SSI) and CMS make the hybrid breeding in cauliflower remunerative [15-19]. However, non-availability of strong S alleles for reliable SSI system and instability of the S alleles under varied climatic conditions made this system less attractive. Therefore, presently cytoplasm induced male sterility are most widely used in hybrid breeding in cauliflower [13, 19]. The wild allies and other closely related *Brassica* coenospecies serve as the reservoir of different cytoplasms whichare being used to develop CMS throughintraspecific/interspecific/intergeneric hybridization mediated by sexual crossing or protoplast fusion [11, 20-24]. Among the different CMS types, *ogu*[25]and *pol*[26]CMS systems are widely used in hybrid breeding of vegetable*Brassicas*.Once developed the CMS phenotype and the associated mitochondrial genes exhibit a maternal inheritance pattern.Numerous CMS related genes (*orfs*) have been identified and well characterized in different plant species. The key mitochondrial genes (*orfs*) associated with different CMS typesin *Brassicaceae* are *orf138* correlated with *Ogura* CMS [21, 25], *orf125* associated with *kosena*CMS of Radish [27], *orf72* correlated with *mur*CMS of *Diplotaxismuralis*[28], *orf222* associated with'*nap CMS*' of *Brassica napus*[29], *orf224/atp6*correlated with *pol* CMS [26, 30], *orf288* linked with *hau*CMS of *B. juncea*[31], *orf263* correlated with '*tour CMS*' of *Brassica tournefortii*[32], and *orf220* associated with 'tuber mustard CMS' of *Brassica juncea*[33]. The gene specific primers for these mitochondrial genomic regions can beefficiently utilizedto differentiate different CMS types in *Brassica* crops.The complete mitochondrial genome sequence of many *Brassica species* and other important genera belonging to *Brassicaceae* (e.g. Arabidopsis, Radish) are available which can be mined for developing markers for differentiating differentcytoplasm responsible for male sterility [2, 10, 34-36]. Several mitochondrial DNA specific microsatellites(mt-SSR) markers have been developed to distinguish different CMS types and assessment of cytoplasmic diversity in different *Brassica* species like*B. oleracea* var. *italica*[23, 37], *B. oleracea* var. *capitata*[38], *B. rapa*[39-41], *B. napus*[39] and *B. juncea*[42].

The CMS cauliflowers and other *Brassica* crops with*Ogura* or other alien cytoplastsdisplay complex variations in reproductive features. These reproductive phenotypes are expressed as floral abnormalities such as homeotic- like transformation of stamens to pistil like structures (pistillody), homeotic modification of stamens to petal like structures (petaloidy), carpelloid stamens, adherence of functional stamens to style, partially opened flowers, splitted style with exposed ovule, splitted style, absence of nectaries, rudimentary nectaries and fused flowers [11, 21-22, 43-45].These floral malformations are result of incongruity between nuclear and mitochondrial genomes [11, 37, 43]. It is evident that loss of function, mutation or insertion/deletion in MADS-box genes *APETALA2, APETALA3* or *PISTILLATA*, which are class B genes of classic ABC model for flower development, renders homeotic transformation of stamens to carpels or petals to sepals [37, 46-47].The homeotic floral deformities have also been explained by the dosage imbalance of class B and class C genes of classic ABC model and aberrant mitochondrial gene expression [45, 48]. These floral malformations which are linked to genetic background and cytoplasm types,

results inefficient nectar production, impaired pollination, reduced female fertility, and consequently a drastic reduction in seed yield. The other alterations in flower phenotype and reproductive structures, such as aborted pollen, degenerative anthers, crooked or bent stigma, reduction in flower size, variation in filament size etc., are universal in autoplasmic and alloplasmic CMS systems of *Brassica* crops [6, 22-24, 49].In India, during the last three decades, a large number of CMS based breeding lines/material have been generated in Indian cauliflowers by repeated back crossing or somatic hybridization by exploiting various CMS sources. However, systematic characterization and identification of these floral malformations in CMS lines of the Indian cauliflowers have not been done so far.

Further, mitochondrial markers can be potentially be used to determine the CMS mitotypes and analyze genetic diversity in Indian cauliflower cytolines. Moreover, mitotype-specific SSRs can be reliably usedfordistinguishing normal cytolines from cytolines having varying degree of floral deformities in Indian cauliflowers.Identification of mt-SSRs and other mitochondria specific markersassociated with floral deformities will enable to screen out cauliflowers CMS lines with different types of floral abnormalities at an early stage. In the present investigation we have analysed a set of mitochondria specific SSRs across 76 cytoplasmic male sterile genotypes of Indian cauliflowers for distinguishing their cytotypes, assessing cytoplasmic genetic diversity and linking the polymorphism in these markers to CMS induced floral abnormalities.

Results

Identification of cauliflower CMS types and genetic divergence

The 76 CMS accessions (63 indigenously developed CMS lines and 13 CMS based hybrids) of cauliflower (Table 1) were screened using nineteen pairs of mitochondrial markers. These markers consist of 13 pairs of mitochondrial gene-specific primers: P1-P13 and six pairs ofmt-SSRs: P1-P13, designed based on the*B. napus*mitochondrial genome(Additional file 1: Table S1)[23, 38, 50]. Among the 13 pairs of mitochondrial gene specific primers, 7 pairs specific to *orf138* were amplified. Although, they did not show any amplification for the control non CMSline (Fig 1).However, none of the primers governing *pol, nap* and *tour* cytoplasm generate any amplification.The 6 pairs of mitochondrial SSRs were amplified across the studied Indian cauliflower CMS lines. The amplification pattern of the gene based primers indicated that the cytoplasmtype of all the cytolines in Indian cauliflower was derived from *Ogura* CMS system;however, there was variation at nucleotide level among the studied CMS accessions. Two pairs of mt-SSRs, P15and P16specific to*BnTR4* and *orf125* genomic regions respectively, exhibited striking polymorphism among the CMS accessions. The amplicon size for the primer P15 and P16 varied between 200-280 bp and 410-470bp, respectively.

The PCA (Principal component analysis) and Neighbour joining (NJ) cluster analysis based on sequence of the mitochondrial amplicons obtained from*Ogura*CMS and in case of *Brassica napus*mitochondrialgenome based mt-SSR markers was used to estimate the diversity in origin of male sterile cytoplasm in cauliflower accessions. In cluster analysis the CMS cauliflower lines could be categorized into three major distinct groups each with two sub-groups and further sub-clusters in each sub-group (Fig. 2a, 2b). Broadly, six distinct sub-groups were determined based on the origin of their cytoplasm source (Fig. 2b). The six CMS lines (A38 to A43) including four CMS based hybrids CFH1522, Kimaya, Pahuja and Snowpearl remained in one single group; the other eight CMS accessions (A44 to A51) belonged to different group. Ten CMS accessions (A52 to A61) belonged to third group and other fifteen CMS lines (A62 to A76) including six CMS based hybrids (SM, Indam, KTCF-10A, Casper, Ponder, Brahma) formed fourth group. The nine CMS accessions (A29 to A37) represented in fifth group and rest of the twenty-eight

CMS accessions (A1 to A28) including three CMS based hybrids of AcsenHyVeg private limited (HVCF-29, HVCF-18 and HVCF-16) formed the largest group (Fig. 2b).

Sequence features of polymorphic amplicons

The fragments amplified by primer P15 were of 251 bp for 46 CMS accessions under study and 220 bp for the remaining cytolines.The polymorphic amplicons of both these groups depicting 251 and 220 bp size were purified, cloned in pGEM-T vector and sequenced. The obtained sequences were subjected to multiple sequence alignment with each other and reference mitochondrial genome sequences of *Brassicaceae* crops exhibiting high degree of sequence similarity (Table 2). The accession numbers for the sequenced fragments were obtained from GenBank NCBI as described in the method section. The sequence analysis revealed that MN549523shared highly conserved region with MN549524 and high degree of similarity with other reference genomic regions of mitochondrial genome sequences of KU831325.1, KJ820683.1, AB627043.1, Sequence 3 P15, AP012988.1 and JF920286.1(Fig.3; Table 2).While, the MN549525 exhibited high degree of similarity with MN549526 and reference mitogenome sequences used in sequence alignment analysis such as Sequence 4 P15, AP018472.1 and AP012989.1 (Fig.3). The sequence alignment revealed single nucleotide polymorphism (SNP) at position 78 (C/T) and in addition, the deletion of 31 nucleotide between positions 78 and 110 for MN549525 and MN549526 (Fig. 3).

The amplicons generated by primer P16 were of size 420 bp in 42 CMS accessions under study and 471 bp in remaining. The obtained sequences were subjected to multiple sequence alignment with other mitogenome sequences of *Brassicaceae* crops available in NCBI database (Table 3). The sequence similarity analysis revealed that amplicons of MN549527 to MN549530shares a high degree of sequence similarity with each other and CMS related proteins in other *Brassicaceae* accessions AP018472.1, MG872827.1, JF920287.1, Seq6 P16, AP012990.1, AP012989.1 and AB694744.1 (Fig.4). Likewise, MN549531 exhibited highlyconserved regionwith Seq5 P16, KU831325.1 and KJ820683.1 (Fig. 4). The sequence alignment analysis revealed a deletion of size 51 bp between positions 371 to 423 in the exonic regions of *ORFs* for MN549527 to MN549530. While normal sequence without deletion was observed for MN549531.

Cytoplasmic genetic variations and floral malformations

We sought to investigate whether cytoplasmic genetic diversity associated with SNPs or InDels at loci *BnTR4* and *orf125*(Fig. 3; Fig .4) has specifically impacted floral phenotypes of CMS lines. The polymorphism at these loci categorized the cytolines into two distinct groups (Fig. 2). It was evident that the cytolines exhibiting SNPs and deletions of 31 and 51 nucleotidesin the *ORFs* exonic region was associated with varying degree of floral abnormalities (Fig. 5; Table 4). The CMS lines with the absence of these InDels and SNPs identified by polymorphic markers had normal flower structure with no deformities which indicated the role ofcytoplasmic genetic variations in determining floral phenotypes.The major deformities recorded in the CMS lines were (i) adherence of functional stamens with style (ii) homeotic-like floral transformationpetaloidy condition of stamens (iii) partial petaloidy of functional stamens (iv) splitted style along with adherence of stamens (v) stigma hidden inside the petals (vi) splitted style along with exposed ovules (vii) unopened flower (viii) stamens adherence with style and crooked stigma (ix) partially opened flowers (x) absence of non-functional stamens (xi) fused flower (xii) curved functional stamens with crooked stigma and (xiii) absence of nectaries. Most of the CMS lines with none of the above floral deformities had normal female fertility and seed set(Fig. 5; Table 4).

Analysis of *ORFs* and similarity of genes, phylogenetic relationships

The open reading frame (*ORF*) analysis revealed nucleotide deletion in the *orf125* coding region ofthe male sterile cytoplasm. The protein sequence analysis of polymorphic amplicons and reference mitotypes showed deleted nucleotide encoded 17 amino acids (Fig. 6).Therefore, polymorphism of P15 (*BnTR4*) and P16 (*orf125*) could be ascribed to SNPs or fragment insertion/deletion near the mt-SSR loci. The amino acid sequence analysis in the region of *ORF* depicted the polymorphic region amplifiedby P16 mt-SSR is located in the coding region of *orf125* protein of *B. oleracea* (wild cabbage), *B. juncea, B. rapa*ssp. *oleifera, Erucavesicaria*subsp. s*ativa*, *B. oleracea* var. *capitata*and *B. oleracea* var. *botrytis* (Additional file 1: Table S2)besides its location in *B. carinata*exonic region of *orf108c.* The protein sequence analysis revealed deletion of amino acids related to array of floral deformities withhigh degree of similarity with *orf108c* in *B. carinata*(Additional file 1: Table S2).The protein sequences devoid of deletion exhibited high similarity with *orf125* in *B. oleracea.*

Polymorphism exhibited with with P15mt-SSR and mt-DNA sequences of other 9 *Brassicaceae* mitotypes were broadly grouped into two major groups (Fig.7a). The phylogenetic analysis revealed close affinity of cauliflower CMS lines,Ogu16A with Ogu12A and Ogu307-33A with Ogu33-1A. These cytoplasmic sources were clustered in one major group with one diploid accession of *B. oleracea.* This clustering in one group concurs with*rapa/ oleracea* lineage propounded by Warwick and Black [51] based on chloroplast genome. Another group represented diploid *B. oleracea* var. *botrytis, B. oleracea* var. *capitata, B. oleracea* var. *italica, Raphanus sativus* var. *kosena*and *B. nigra.* This grouping also corresponded to *rapa/ oleracea* lineage as proposed by Warwick and Black [51]except one species of *nigra*lineage.Another group also confined to *rapa/ oleracea* lineage with the exception of one species of *nigra*lineage [51]. Similarly, the polymorphic amplicons based on P16 mitochondrial marker and corresponding mt-DNA sequences of ten *Brassicaceae* mitotypes were clustered two major groups (Fig.7b). The selected CMS sources, Ogu50A and Ogu17A were distantly placed from other CMS sources. OguHL-3A, Ogu12A and Ogu121-2A exhibited close affinity with each other and *B. oleracea* var. *capitata*based on phylogenetic analysis. The CMS line Ogu50A depicted high affinity with KJ820683.1 (*B.oleracea*var. *botrytis*L.)and Ogu17A showed high affinity with mitogenome of *B. nigra*and *R. sativus*var. Kosena,and clusteredin one major group along with *Raphanus sativus* cv. Ms-gensuke, Ogu50A and *B. oleracea* var. *botrytis* (KJ820683.1). The black radish distantly placed itself from other members of its group.

Effects of mito-nuclear genomic interactions on floral-nectar phenotypes in different nuclear backgrounds

Effect of cytonuclear interactions on floral qualitative traits

In the present investigation we sought to analyze whether cytonuclear interactions specifically affected floral qualitative traits in the cytolines of Indian cauliflowers. Cauliflower cytolines along with their respective maintainers were characterized for five traitsnamelystyle shape, petal color, presence of floral nectaries, presence of viable pollen and type of ovary (Additional file 1: Table S3,Additional file 2: Fig.S1-S2).All the CMS lines had normal ovary and varied nectaries. None of the CMS line showed the presence of viable pollen grains and few lines were devoid of anther and pollen grains. The CMS accessions had straight to slightly curved or fully curved stigma. Yellow colored petals were predominant however white petals were recorded in Ogu33A, Ogu134-8A, Ogu13A, Ogu118-6A, OguKt-2-6A and Ogu118-2A.

Cytonuclear interactions influencing floral reproductive whorls

Comparative analysis for floral reproductive whorls and floral phenotypes of cauliflower cytolines and their respective male fertile counterparts was conducted to determine the effects of mito-nuclear co-adaptaion and disruption. The *per se* performance of several cytolines lines and their respective maintainers recorded significant variation for different floral phenotype (Additional file 1: Table S4, Additional file 2: Fig. S2).Petal length varied from 12.91 mm (Ogu76-4A) to 17.92 mm (Ogu2-6A). The longest petal was recorded in the CMS line Ogu2-6A followed by Ogu126-1A and Ogu310-8A. The petal width ranged from 4.18 mm (Ogu13-85-2A) to 7.83 mm (Ogu2-6A). The CMS line, Ogu2-6A followed by Ogu178-8A and Ogu33A-1301 had the widest petals. The ratio of petal length to petal width varied from 1.88 (Ogu33A-1301) to 3.82 (Ogu13-85-2A) and ratio was>2 except Ogu-13-01-5A, Ogu307-1A and Ogu-13-01-33A.The considerable differences were also observed for sepal size,and the sepal length and width varied from 6.23 mm (Ogu15A) to 10.28 mm (Ogu2-6A) and 2.21 mm (Ogu13-85-2A) to 3.53 mm (Ogu115-8A), respectively. The ratio of sepal length to sepal width ranged from 2.19 (Ogu15A) to 3.88 (OguKt-2-1A). Like petal size, sepal size too was significantly reduced in the male sterile lines. The length of short (non-functional) and long (functional) filament ranged from 2.30 mm (Ogu16A) to 6.38 mm (Ogu310-8A) and 3.40 mm (33A-1301) to 7.52 mm (Ogu310-8A), respectively. The introgression of sterile cytoplasm resulted in elimination of non-functional filaments in 9CMS lines. Marked differences were also observed for stamen length, as the short stamen (non-functional) length varied from 3.41 mm (Ogu16A) to 8.13 mm (Ogu310-8A) and long stamen (functional) length from 4.79 mm (33A-1301) to 9.48 mm (Ogu2-6A). The ratio of functional (long stamen) to non-functional anthers (short stamen) was determined to detect any changes in CMS types and it varied from 1.03 (OguKt-2-1A) to 2.07 (Ogu77-4A) and it varied from 1.03 to 1.26 in maintainer lines. The 9 CMS lines *viz*. Ogu121-1A, Ogu122-1A, Ogu126-1A, OguKt-9-2A, OguKt-8-2A, Ogu118-3A, Ogu2A, Ogu3A, and Ogu118-2A were completely devoid of nonfunctional stamens. The significant differences were also observed in all the CMS lines for style length and it varied from 5.32 mm (Ogu14A) to 9.44 mm (Ogu16A). However, in the male fertile counterparts the style length varied from 6.33 mm to 10.86 mm. These results clearly indicated the effect of cytonuclear genomic incompatibilities on reproductive whorls. Reduction in style length, stamen length and even absence of short stamens was also recorded in the cytolinesin different nuclear backgrounds. Generally, the position of style was higher than the stamens in majority of the cytolines except Ogu-Kt-2-1A, Ogu122-5A, Ogu118-4A, Ogu33-1A, Ogu33A, Ogu77-4A, OguKt-9-2A, Ogu3A and Ogu14A (Additional file 1: Table S4). Introgression of *ogura* cytoplasm invariably reduced the stamen length in call Indian cauliflower lines. The relative position of stamens and stigma was determined by estimating the ratio between functional stamens and style length. The ratio varied from 0.62 (33A-1301) to 1.30 (Ogu14A) in CMS lines and 0.98 to 1.46 in male fertile counterparts.

Impact of cytonuclear interactions on nectar production

Comparative nectar quantity in the cytolines and their respective male fertile maintainers was conducted to determine nuclear-cytoplasmic interaction for this trait. Significant variation was observed in the cytolines lines for nectar quantity (Table 5). The nectar quantity varied from 0.28 µl (Ogu15A) to 5.69 µl (Ogu1-8A) in the CMS lines, while in the male fertile counterpart the nectar quantity varied from 0.60 µl to 13.94 µl.The highest quantity of nectar was found in the CMS line, Ogu1-8A followed by Ogu308-6A and Ogu2-6A. The results (Table 5) revealed significant reduction in the nectar quantity ofcytolinesas compared to their respective male fertile counterparts.

Cluster analysis

The CMS lines were grouped into different clusters based on floral reproductive whorls and phenotypic traits and 2 major clusters (CI and CII) formed with two sub-clusters in cluster II (Fig.8). The sub-clusters (C-IIA and C-IIB) in the cluster-II were further grouped into sub-clusters C-IIA-1, C-IIA-2 and C-IIB-1, C-IIB-2. Majority of CMS lines were

grouped into cluster II and cluster I had only 9 CMS lines. All the CMS accessions with the absence of non functional stamens remained in cluster-I. The 6 CMS lines with the functional stamens of length 8.5 mm to 9.48 mm remained in the sub-cluster C-IIA-1. The nine CMS accessions grouped into sub-cluster C-IIA-2 with nonfunctional filament length 3.1-3.8 mm and majority of the CMS lines in this cluster had sepal width < 2.45 mm. The sub-cluster C-IIB-1a contained five CMS lines with petal length < 14.5 mm, style length 6.28-6.6 mm and functional stamen length of 6.49-6.61 mm .The sub-cluster C-IIB-1b had ten CMS lines and majority of them (60%) have petal length 15-17 mm and sepal width 2.9-3.4 mm. The sub-cluster C-IIB-2a comprised of eight CMS lines and majority of them had sepal length > 8.7 mm (i.e. 8.7-9.8 mm). The remaining thirteen CMS lines were clustered into sub-cluster C-IIB-2b with petal length of >13 mm to < 15 mm in majority of the lines.

Discussion

Mitochondrial markers in identifying *B. oleracea* CMS lines with floral deformities

The CMS phenotype is a common feature in higher plants controlled by mt-DNA and widely used to facilitate hybrid development in *Brassica* vegetables [11, 13, and 23]. The propensity to frequent recombination, mt-DNA rearrangements and encoding of numerous genes makes plant mitogenome more complex relative to their metazoan counterpart.In this context, the role of mitochondrial markers in quick identification and differentiation of different CMS types has been reported earlier in other members of *Brassicaceae* [23, 37-40, 52]. We are reporting the usefulness of mitochondria specific markers in selecting CMS lines with normal flower structure and extent of mitogenome diversity for the first time in Indian cauliflower. In this study mt-DNA specific primers associated with different CMS genes,*orf138, orf222, orf224, orf222-224, orf263, atp6-orf224* and mt-SSRs were utilized. The results revealed that all the cytolines of Indian cauliflowerpossessed *orf138* related fragment specific to*Ogura* CMS eatablishing wide use of *Ogura* cytoplasm in the development of CMS [10-11, 23, 37, 53-55]. Therefore, it is urgently needed to diversify the source of the CMS in Indian cauliflowers to avoid any imminent threat of epidemicwhich may collapse the entire hybrid seed industry of *Brassica oleracea* vegetables. The male sterile cytoplasm available in other Brassicas like *Trachystomaballii*, *Diplotaxiscatholica*, *D. berthaultii*, *B. tournefortii*, *Moricandia arvensis*, *Erucasativa*couldbe utilized to diversify CMS system in Indian cauliflowers. However, the polymorphic mt-SSRs loci indicated the presence of cytoplasmic genetic variation among *Ogura*CMS based male sterile genetic stock of Indian cauliflowers.The PCA and NJ cluster analysis based on combined analysis of mt-DNA specific and mt-SSR markers classified the 76 cytolines into six different groups (Fig.2b).

The polymorphic amplicons generated with primer P15 were of size between 220 to 280 bp, while for P16 of 410-470 bp size. The length of polymorphic products of A13 to A17, A24 to A27, A29 to A37, A69, A73, A2, A4-A5, and A52-A58 were identical to as reported in *B.oleracea*var. *capitata*[38], which can differentiate *OguCMSHY*, $OguCMSR_{1-2}$ (420bp) from *pol*CMS and $OguCMSR_3$ (471bp). Similar findings were reported for 5 CMS lines of broccoli [23]. Therefore, the original source of *Ogu*CMS in the studied cauliflower cytolines could have come from $OguCMSR_{1-2}$ and *OguCMSHY*. The sequence analysis revealed that SNPs and insertion/deletions could explain the cytoplasmic genetic variations among cytolines of Indian cauliflowers. Polymorphic amplicons targeting *orf125* coding regions of mitochondrial genome demonstrated a deletion of 51 nucleotides in the CMS lines with varied floral deformities. Similarly, the deformed CMS lines had a deletion of 31 nucleotides in the coding region of *BnTR4.* Wang et al. [38] successfully reported the use of chloroplast (cpSSRs) and mitochondrial (mt-SSRs) primers in demonstrating the polymorphism in alloplasmic CMS lines of cabbage. Shu et al [23, 37] demonstrated the role of organelle SSRs, mt-DNA specific and mt-SSR markers in distinguishing different CMS types, differentiating CMS lines with carpelloid stamens in broccoli. Cytoplasmic genetic variations associated with deletions of 31/51 nucleotides in the *ORFs*

exonic region demonstrated that insertions/deletions of nucleotides can explain the wide range of floral abnormalities in the CMS lines of Indian cauliflowers.

Mito-nuclear genomic incompatibilities and introgression of alien cytoplasm have been reported to display complex floral structure variations in auto-and alloplasmic CMS lines of *Brasssica*crops. The flowering regulation in higher plants is highly complex process controlled by interactions of genes or genes × environment. MADS-box genes are crucial player in plants for vegetative and reproductive growth development [44-48, 56]. The identity and development of each floral reproductive whorl is determined by varying combinations of genes of classical flowering ABCDE model [56]. It is well documented that loss of function, mutations or insertions/deletions in MADS-box transcription factors such as *APETALA2, APETALA3* or *PISTILLATA, AGAMOUS, AGL11, SEP1, SEP2, SEP3, SEP4, SHP2, SHP2* etc causes varying degree of floral malformations [44-48, 56].Different types of floral abnormalities observed in the cauliflower cytolines (Fig. 5) such as homeotic- like modification of stamens to petal like structures (petaloidy), adherence of functional stamens to style, partially opened flowers, splitted style with exposed ovule, splitted style, absence of nectaries, rudimentary nectaries and fused flowers could be explained by absence of amino acids causing dysfunction of one of MADS-box genes. Further, the dosage imbalance of class B and class C genes of flowering model, aberrant mitochondrial gene expression results homeotic-like floral deformitiesobserved in the present investigation [45, 48]. Previously, the role of deletions of nucleotide in the *ORFs* coding region of broccoli cytolines was suggested to be associated withcarpelloid stamen phenotype [23, 37], however, their role in cause an array of floral deformities is not reported. Identification of these specific deletion associated with several floral deformities would be instrumental in selection of desirable CMS types for hybrid breeding and will enhance the understanding about evolutionary relationships in *Brassicas.* Analysis of *ORFs,* similarity of genes and *protein* sequence revealed that polymorphic loci were located at exonic regions of *orf125* and *orf108c* of mitochondrial genomes of *Brassicaceae* species. It is quite likely that *orf125* and *orf108c* mitochondrial genes are playing pivotal role in development of flower organs. The floral abnormalities which are associated with aberrant mitochondrial gene expression, mutation in mitochondrial genomes, cytoplasmic-nuclear conflict, cytoplasm types and genetic backgrounds, renders scanty nectar production, impaired pollination and affects female fertility [22, 37, 43, 57]. These characteristics consequently results poor seed yield. Identification and roughing out undesirable cytolines in the initial stage of breeding programme will save huge time efforts in developing CMS lines for their successful use in hybrid development.

In the present investigation and our earlier reports [43, 58] we observed floral abnormalities in CMS lines in successive nuclear backgrounds. Initially the expression of floral deformities may be weak or incomplete because of partial substitution of nuclear genome. The exact expressions were observed only after complete nuclear substitution. The identification of specific insertion/deletion and SNPs associated with floral abnormalities in the initial stage of back-cross introgression will help the breeders to use only those CMS types which will later results in normal flower structures. The polymorphism in the cytolines of varying nuclear backgrounds carrying identical cytoplasmsuggests possiblepaternal leakage in certain cases. However, further investigation is needed to establish the role of the paternal genome in inheritance of the mitochondrial genome controlled male sterility in *B. oleracea* lines.The proclivity of plant mitochondrial genome to frequent recombination, genomic rearrangements and rapid structural evolution leading to heteroplasmy results in polymorphism in cauliflower cytoplasmic sources [59-60]. Predominantly, the uni-parental, which is maternal inheritance of chloroplast and mitochondrial genome is reported in most of the angiosperms [2, 61] including *Brassica* crops such as broccoli, cabbage and cauliflower [37, 62-63]. Recently, the paternal [60, 64-66] and biparental [67-68] inheritance of mitogenome is also reported. Therefore, the findings of the present study need to be analyzed further to detect any possible paternal inheritance of mitochondrial genome.

Nucleo-mitochondrial interactions influencing floral-nectar phenotypes

Pollinators play an important role in hybrid breeding of *Brassicas* and pollinator visit is determined by flower phenotype like size, color and form [57].Usually, the large flower size is positively linked with more numbers of pollinator visits, as large floral organ size is generally assumed to contain more nectar [57, 69-71]. Floral nectar is the primary reward besides pollen, floral oils, resins and scents, offered to pollinators and their proportionate ratio is vital in determining plant-pollinator interactions [71]. Floral organ size, morphology, proper development of nectaries, efficient nectar production, fruiting featuresand pollinator attracting ability are considered as potent indexes to evaluate cytolines [57]. The cytoplasmic effects on floral phenotypes are well documented in *Brassicas* which are manifested through cyto-nuclear allelic interactions.The role of additive mito-nuclear genetic effects on altering floral phenotypes is rare, while epistatic cytonuclear genetic effects are larger in magnitude [72-73]. In the present investigation, the effects of cytonuclear interactions on floral qualitative traits were insignificant in different nuclear backgrounds (Additional file 1: Table S3; Fig. S1-S2). However, varying degree of cytoplasmic effects on floral reproductive whorls, floral size, structure and other floral-nectar phenotypes were observed over the successive backcross generations in the development of cauliflower cytolines (Additional file 1: Table S4).The introgression of sterile cytoplasm significantly reduced the flower size in the form of reduction in petal length, petal width, and sepal length across the nuclear background series. The other predominant type of structural changes in reproductive whorlswere flowers devoid of non-functional stamens, alteration in style length, reduction in functional stamens length, modification in position of style and anthers (style position was higher than anthers across the nuclear background series with a few exceptions) and inefficient nectar production. The genetic conflict of sterile cytoplasm- nuclear genome of *B.oleracea*var. *botrytis* could explain the floral deformities [2, 6, 11]. In *Arabidopsis*, the role of cytonuclear epistasis on various adaptive traits is suggested [73].Singh and Srivastava [74] observed the narrowing of petals, reduction of filament length, increase in style length in the *B. juncea*male sterile lines with the introgression of *ogura, tournefortii, trachystoma*and *siifolia*wild cytoplasm. They also obtained the erratic results with the *ogura*cytoplasm in the different nuclear backgrounds because ofmito-nuclear epistasis. Although, it is also suggested that rigorous recurrent manual or honey bee- mediated selection during backcross breeding cycles could reduce floral deformities to a limited extent [43, 75]. However, it is not possible to ameliorate these deformities through conventional back-crossing and different selection strategies.

Understanding the molecular mechanisms and pathways of cytoplasmic male sterility in cauliflower associated with floral abnormalities along with determining the paternal leakage (if any) need further in depth investigation. Identified molecular markers associated with different kind of floral deformities will facilitate the selection of CMS lines with normal flower structure at the initial stage of breeding.

Methods

Plant materials and nucleic acid isolation

The basic plant material consisted of 76 CMS accessions of cauliflower with different nuclear backgrounds, developed after more than nine generations of backcrossing (Table 1). Among the 76 CMS lines, 71 lines were developed at ICAR-Indian Agricultural Research Institute, Regional Station, Katrain, Kullu, HP-175129, India through back-crosss introgression. Four CMS based F_1 hybrids, Casper, KTCF-10A, Ponder and SM were obtained from by RijKZwaan private limited and one CMS based F_1 hybrid, Indam was obtained from Indo Americal Hybrid Seeds company. (Table 1). In addition, one non CMS inbred line of cauliflower ‘Sel-27’ was used as a control the CMS accessions were grown in pro-trays under glasshouse conditions of Baragram Experimental Farm of ICAR-Indian

Agricultural Research Institute (IARI), Regional Station, Katrain, Kullu Valley, Himachal Pradesh, India for extraction of DNA. DNA was isolated from fully expanding leaves (100 mg) from 25-30 days old seedlings using cetyltrimethyl ammonium bromide (CTAB) method with minor modifications [76].The genomic DNA samples were adjusted to 25-50 ng DNA/µl and were stored at -80 ºC till use.

PCR amplification

Nineteen pairs of mitochondrial markers were used to detectnucleotide diversity among male sterile mitotypes of Indian cauliflowers(Additional file 1: Table S1) [23]. One pair of primers (P1) was specific to *orf138* determining *Ogura* CMS, two pairs of primers specific to *orf222* (P2 and P3), one pair specific to *orf222-orf224* (P4), then *orf224* (P5), *atp6-orf224* (P6) and *orf263* (P7). The six pairs of primers (P8-P13) were based on *orf138* related genomic sequences and other six pairs (P14-P19) were mitochondrial simple sequence repeat markers (mt-SSRs) [23].The PCR amplifications were carried out in a reaction mixture consisted of 2 µl of genomic DNA template (50 ng), 1 µl of each forward and reverse primers, 12.50µl of 2×PCR Green master mix (GoTaq DNA polymerase; Promega, USA) and 8.50µl nuclease free water. The Eppendorf Mastercycler Nexus GSX1 was used for PCR amplification. Amplification was done following PCR cycling programme : an initial denaturation of 95 ºC for 5 min, then35 cycles of denaturation at 95 ºC for 30s, annealing of primers at suitable temperature for 30s and extension at 72 ºC for 1min, then a final extension of 72ºC for 7min. The amplification by each polymorphic locus was repeated three times for the confirmation and the PCR products were separated on 3% agarose gel electrophoresis in 1X TBE buffer (pH 8.0) at 100 mA voltage for 120 min. Ethidium bromide (EtBr) of 0.5 mg/ml was used for gel staining and gel pictures were captured using digital gel documentation unit (BioSpectrum® Imaging System™, UK). The determination of fragment sizes was done using PromegaTM 100 bp DNA step ladder.

Cluster analysis based on mitochondrial markers

To categorize the CMS accessions into distinct groups, the molecular data generated by combining the two types of analytical methods (mitochondrial DNA-specific markers and *B. napus*based mt-SSRs) was subjected to cluster analysis *via* neighbor-joining (NJ) unweighted pair group method with arithmetic mean (UPGMA) using DARwin v6.0.017 [77]. The simple matching (SM) coefficient was computed to calculated genetic distance.

Sequence analysis and genome annotation

The PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). For each of the polymorphic loci the representative purified PCR products were cloned into *pGEM®-T Easy* vector system (Promega Corporation, USA) following the supplier's guidelines. The products were transformed into *E. coli* (*DH5α*) competent cells. The positive clones were confirmed by colony PCR and restriction digestion. The five positive clones for each of the selected polymorphic fragments weresubjected to Sanger Dideoxy sequencing (Eurofins Genomics India Pvt., Ltd.). The per cent sequence identity and similarity analysis was carried out with NCBI (National Center for Biotechnology Information) web based BLAST service (https://blast.ncbi.nlm.nih.gov/) [78] for the comparative analysis of obtained sequences with available relevant mitochondrial genome sequences in *Brassicaceae.* The Sanger sequences obtained were assembled and subjected to multiple sequence alignment with reference mitochondrial genomes to determine any variation at nucleotide level using SeqMan Pro tool of DNASTAR version 15.3 (Lasergene Inc. Madison, WI, USA, https://www.dnastar.com/).The phylogenetic tree was constructed with MEGA X software [79] based on different datasets of polymorphic primers. NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/)was used to determine *ORF* numbers in the representative DNA sequence of polymorphic loci and to screen hypothetical protein translations. To verify predicted proteins, NCBI BLASTP or

SMART BLAST [78] was used. The amino acid multiple sequence alignment was performed using CLUSTAL Omega programme [80].

Accession numbers of selected polymorphic amplicons

The sequences of selected representative polymorphic amplicons of Ogu307-33A, Ogu33-1A, Ogu16A, Ogu12A, Ogu12A-orf125, Ogu121-2A, Ogu-HL-3A, Ogu17A and Ogu50A have been submitted to the GenBank nucleotide sequence database of NCBI under the accession numbers of MN549523, MN549524, MN549525, MN549526, MN549527, MN549528, MN549529, MN549530 and MN549531, respectively.

Impact of mito-nuclear interactions on floral-nectar phenotype

The field study was carried out at Baragram Experimental Farm of ICAR-IARI, Regional Station, Katrain, Himachal Pradesh, India, situated along the river Beas. The recommended package of practices, suggested for growing a healthy crop at the Baragram Experimental Farm, were followed for better agronomic and phenotypic expression of crop [15]. To determine the cytoplasmic effect inducing alteration in floral reproductive traits, a comparative phenotypic analysis of a set of 60 CMS lines (A line) of snowball group of Indian cauliflower along with their respective male fertile counterparts (B line)was done based on important floral morpho-physiological traits viz. (i) petal color (ii) shape of style (iii) presence of floral nectaries (iv) presence of viable pollen (v) type of ovary (vi) petal size : petal length (mm) and petal width (mm) (vii) ratio of petal length to petal width (viii) sepal size : sepal length (mm) and sepal width (mm) (ix) ratio of sepal length to sepal width (x) short filament length (mm) (xi) long filament length (mm) (xii) short stamen length (mm) (xiii) long stamen length (mm) (xiv) style length (mm) (xv) ratio of stamen to style (xvi) ratio of long stamen to short stamen and (xvii) nectar quantity(µl) [22, 43, 74, 81-82]. The petal length to petal width ratio was estimated to determine changes in petal size. The long stamen to style ratio was determined to estimate relative position of stigma to stamen. The long to short stamen ratio was estimated to determine the effect of cytoplasm on stamen length [74]. The presence or absence of pollen grains was determined on the basis of visual observation and pollen viability was tested by staining with 2% acetocarmine [74]. All the CMS lines and their respective maintainers were evaluated for different floral traits in Randomized Block Design (RBD) design with three replications to quantify the modifications in flower reproductive features because of sterile cytoplasm introgression. Observations were recorded from 12 flowers per genotype (4 flowers from 3 plants) in each replication. The CMS accessions (A lines) were compared with their respective B lines for detection of floral abnormalities (e.g. pistillody, carpelloid stamen, petaloid stames, splitted style, partially opened flowers, fused flowers, absence of nectaries etc.). Floral nectar is an important trait for comprehending the plant-pollinator mutualisms. For the analysis of effect of nuclear-genomic conflict on nectar production, the quantification of nectar production was done from CMS accessions and their respective maintainers. The data was taken from 10 flowers of each genotype during early morning hours with graduated capillary of 10 µl. The average data was used for further statistical analysis.

Statistical analysis for floral-nectar traits

All the CMS lines were clustered into different groups based on cluster analysis and dendrogram construction based on Euclidean distance was done with the PCA and neighbor joining (NJ) UPGMA method using DARwin software version 6.0.017 [77]. For testing the reliability of NJ dendrogram, a bootstrap value of 1000 replicates was used. To compare the A and B lines, average of CMS lines and their male fertile counterparts (maintainers) for different floral traits was compared using paired t-test by subjecting the data to ‘R’ statistical software.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All the data sets generated and analysed during this investigation are presented in this published research article and its supplementary additional information files.

Competing interests

All the authors declare that they have no competing interests with respect to any aspect of this research.

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Authors' Contribution

SSD and RB conceived and designed the study. SS and SSD performed the experiment. SS did the data recording and lab experiments with the input from KS, HG, and KK. SS and SSD analyzed the data. AP helped in recording of data related to the reproductive structure of the CMS lines. RK, SSD, RB monitored and contributed the materials. SS performed the sequencing and other molecular analysis. SS and SSD wrote the original draft manuscript. SSD, TKB, RK and RCB edited thefinal manuscript. All the authors read and approved the final manuscript.

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Abbreviations

PCR: Polymerase chain reaction

CMS: Cytoplasm male sterile

DNA: deoxyribonucleic acid

Rf: Restorer of fertility

SSI: Sporophytic self-incompatibility

ORF: Open reading frame

SSR: Simple sequence repeats

NCBI: National centre for biotechnology information

Mt-DMA: Mitochondrial DNA

PCA: Principal component analysis

NJ: Neighbour joining

SNP: Single nucleotide polymorphism

Tables

Table 1 Cauliflower cytoplasmic male sterile lines used in the present investigation

Sr. No	CMS accessions	Status	Developmental Stage	Source	Leafiness/Riceyness
A1	Ogu122-5A	CMS line	BC_{12}	IARI Katrain	Absent
A2	Ogu115-33A	CMS line	BC_{9}	IARI Katrain	Absent
A3	Ogu118-6A	CMS line	BC_{12}	IARI Katrain	Absent
A4	Ogu307-33A	CMS line	BC_{9}	IARI Katrain	Absent
A5	Ogu33-1A	CMS line	BC_{9}	IARI Katrain	Absent
A6	Ogu1A	CMS line	BC_{9}	IARI Katrain	Absent
A7	Ogu309-2A	CMS line	BC_{13}	IARI Katrain	Absent
A8	OguKt-2-6A	CMS line	BC_{12}	IARI Katrain	Absent
A9	Ogu13-85-6A	CMS line	BC_{12}	IARI Katrain	Absent
A10	Ogu16A	CMS line	BC_{9}	IARI Katrain	Absent
A11	Ogu3A	CMS line	BC_{9}	IARI Katrain	Absent
A12	Ogu2-6A	CMS line	BC_{11}	IARI Katrain	Absent
A13	Ogu2A	CMS line	BC_{9}	IARI Katrain	Absent
A14	Ogu14A	CMS line	BC_{9}	IARI Katrain	Absent
A15	Ogu122-1A	CMS line	BC_{13}	IARI Katrain	Absent
A16	OguKt-9-2A	CMS line	BC_{13}	IARI Katrain	Absent
A17	Ogu121-1A	CMS line	BC_{12}	IARI Katrain	Absent
A18	Ogu126-1A	CMS line	BC_{13}	IARI Katrain	Absent
A19	Ogu134-8A	CMS line	BC_{12}	IARI Katrain	Absent
A20	Ogu12A	CMS line	BC_{9}	IARI Katrain	Absent
A21	Ogu119-1A	CMS line	BC_{13}	IARI Katrain	Absent
A22	Ogu34A	CMS line	BC_{9}	IARI Katrain	Absent
A23	Ogu178-8A	CMS line	BC_{12}	IARI Katrain	Absent
A24	Ogu118-2A	CMS line	BC_{11}	IARI Katrain	Absent
A25	Ogu33A	CMS line	BC_{9}	IARI Katrain	Absent
A26	HVCF-29	Hybrid	-	AcsenHyVeg	Absent
A27	HVCF-18	Hybrid	-	AcsenHyVeg	Absent

A28	HVCF-16	Hybrid	-	AcsenHyVeg	Absent
A29	OguKt-2-1A	CMS line	BC_{12}	IARI Katrain	Absent
A30	Ogu309-1A	CMS line	BC_{11}	IARI Katrain	Absent
A31	Ogu-HL-1A	CMS line	BC_{11}	IARI Katrain	Absent
A32	Ogu307-1A	CMS line	BC_{12}	IARI Katrain	Absent
A33	Ogu Kt-8-2A	CMS line	BC_{12}	IARI Katrain	Absent
A34	Ogu119-2A	CMS line	BC_{11}	IARI Katrain	Absent
A35	Ogu121-2A	CMS line	BC_{11}	IARI Katrain	Absent
A36	Ogu-HL-3A	CMS line	BC_{11}	IARI Katrain	Absent

Table 1 Continue Cauliflower cytoplasmic male sterile lines used in the present investigation

Sr. No	CMS line	Status	Developmental Stage	Origin of CMS sources	Leafiness/Riceyness
A37	Ogu13-85-3A	CMS line	BC_{11}	IARI Katrain	Absent
A38	Ogu119-6A	CMS line	BC_{12}	IARI Katrain	Absent
A39	Snowpearl	Hybrid	-	Syngenta	Absent
A40	CFH-1522	Hybrid	-	Syngenta	Absent
A41	Kimaya	Hybrid	-	Syngenta	Absent
A42	Pahuja	Hybrid	-	Pahuja seeds	Absent
A43	Ogu13A	CMS line	BC_9	IARI Katrain	Absent
A44	Ogu34-1A	CMS line	BC_9	IARI Katrain	Absent
A45	Ogu1-2A	CMS line	BC_{12}	IARI Katrain	Absent
A46	Ogu13-85-2A	CMS line	BC_{11}	IARI Katrain	Absent
A47	Ogu118-3A	CMS line	BC_{12}	IARI Katrain	Absent
A48	Ogu307-6A	CMS line	BC_{12}	IARI Katrain	Absent
A49	Ogu-HL-6A	CMS line	BC_{11}	IARI Katrain	Absent
A50	Ogu308-6A	CMS line	BC_{12}	IARI Katrain	Absent
A51	Ogu13-01-5A	CMS line	BC_9	IARI Katrain	Absent
A52	Ogu13-85-4A	CMS line	BC_{11}	IARI Katrain	Absent
A53	Ogu118-4A	CMS line	BC_{12}	IARI Katrain	Absent
A54	Ogu76-4A	CMS line	BC_9	IARI Katrain	Absent
A55	Ogu33-4A	CMS line	BC_9	IARI Katrain	Absent
A56	Ogu77-4A	CMS line	BC_{12}	IARI Katrain	Absent
A57	Ogu13-85-33A	CMS line	BC_{11}	IARI Katrain	Absent
A58	Ogu76-33A	CMS line	BC_{11}	IARI Katrain	Absent
A59	Ogu122-8A	CMS line	BC_{12}	IARI Katrain	Absent
A60	Ogu1-8A	CMS line	BC_{12}	IARI Katrain	Absent
A61	Ogu309-8A	CMS line	BC_{13}	IARI Katrain	Absent
A62	Ogu115-8A	CMS line	BC_{13}	IARI Katrain	Absent
A63	Ogu310-8A	CMS line	BC_{11}	IARI Katrain	Absent
A64	Ogu34-1-8A	CMS line		IARI Katrain	Absent

			BC_{12}		
A65	Ogu34-8A	CMS line	BC_{12}	IARI Katrain	Absent
A66	Ogu-HL-50A	CMS line	BC_{11}	IARI Katrain	Absent
A67	Brahma	Hybrid	-	Sakata	Absent
A68	Ogu15A	CMS line	BC_9	IARI Katrain	Absent
A69	Ogu17A	CMS line	BC_9	IARI Katrain	Absent
A70	Ogu50A	CMS line	BC_9	IARI Katrain	Absent
A71	Ogu60A	CMS line	BC_{10}	IARI Katrain	Absent
A72	Casper	Hybrid	-	RijkZwaan	Absent
A73	KTCF-10A	Hybrid	-	Private seed	Absent
A74	Ponder	Hybrid	-	RijkZwaan	Absent
A75	SM	Hybrid	-	RijkZwaan	Absent
A76	Indam	Hybrid	-	IAHS	Absent

Table 2 Sequence similarity percentage of the selected polymorphic amplicons of cauliflower CMS accessions generated by mt-SSR (*BnTR4*) with corresponding mitogenome sequences

Accession no	Species	Description	Sequence identity (Ogu307-33A)	Sequence identity (Ogu33-1A)	Sequence identity (Ogu16A)	Sequence identity (Ogu12A)
KU831325.1	*Brassica oleracea* var. *capitata*	mitochondrion, complete genome	97.12%	97.12%	96.60%	95.24%
KJ820683.1	*Brassica oleracea* var. *botrytis*	mitochondrion, complete genome	97.12%	97.12%	96.60%	95.24%
AB627043.1	*Brassica oleracea*	mitochondrial DNA, minisatellite: BnTR4	99.47%	98.95%	99.12%	99.10%
AP012988.1	*Brassica oleracea*	mitochondrial DNA, complete sequence, cultivar: Fujiwase	97.12%	97.12%	96.60%	95.24%
JF920286.1	*Brassica oleracea*	mitochondrial DNA, complete genome	97.12%	97.12%	96.60%	95.24%
AP018472.1	*Raphanus sativus*	mitochondrial DNA, complete genome, cultivar: Kosena	95.86%	95.77%	96.79%	95.87%
AP012989.1	*Brassica nigra*	mitochondrial DNA, complete sequence	96.55%	95.86%	96.33%	95.41%

Table 3 Sequence similarity percentage of the selected polymorphic amplicons of cauliflower CMS accessions generated by mt-SSR (*orf125*) with corresponding mitogenome sequences

Accession no	Species	Description	Sequence identity (Ogu50A)	Sequence identity (Ogu12A)	Sequence identity (Ogu121-2A)	Sequence identity (Ogu17A)
KU831325.1	*Brassica oleracea*var. *capitata*	mitochondrion, complete genome	99.34%	99.06%	99.06%	99.36%
KJ820683.1	*Brassica oleracea* var. *botrytis*	mitochondrion, complete genome	99.34%	99.06%	99.06%	99.36%
AB694744.1	*Raphanus sativus*	mitochondrial DNA, complete genome, cultivar: MS-gensuke	99.08%	98.82%	98.58%	96.88%
AP012990.1	*Raphanus sativus*	mitochondrial DNA, complete sequence, cultivar: Black radish	99.39%	98.58%	98.35%	96.63%
JF920287.1	*Brassica carinata*	mitochondrial DNA, complete genome	99.39%	98.58%	98.35%	96.63%
AP018472.1	*Raphanus sativus*	mitochondrial DNA, complete genome, cultivar: Kosena	99.08%	98.82%	98.58%	96.88%
AP012989.1	*Brassica nigra*	mitochondrial DNA, complete sequence	99.39%	98.58%	98.35%	96.63%
MG872827.1	*Brassica juncea*	isolate 93 mitochondrion, complete genome	99.39%	98.58%	98.35%	96.63%

Table 4 Impact of cytoplasmic genetic variations based on InDels on floral phenotypesin varying nuclear backgrounds

Code	CMS accessions	Type of nucleotide variation (SNPs/Deletions)		Flower Phenotype of cytolines (CMS)	
		(*BnTR4*)	(*Orf125*)	Normal	Varying Abnormalities
A1	Ogu122-5A	-	-	NCMS	
A2	Ogu115-33A	-	1 (51)		A
A3	Ogu118-6A	-	-	NCMS	
A4	Ogu307-33A	1 (T/C)	1 (51)		A, B F, G
A5	Ogu33-1A	1 (T/C)	1 (51)		A, B, G
A7	Ogu309-2A	1 (31)	1 (51)		A, F, G
A8	OguKt-2-6A	-	-	NCMS	
A9	Ogu13-85-6A	-	-	NCMS	
A10	Ogu16A	1 (C/T), 1 (31)	1 (51)		B, F, J
A11	Ogu3A	1 (31)	1 (51)		B, J
A12	Ogu2-6A	-	-	NCMS	
A13	Ogu2A	1 (31)	1 (51)		F, I, J
A14	Ogu14A	1 (31)	1 (51)		J
A15	Ogu122-1A	1 (31)	1 (51)		J
A16	OguKt-9-2A	1 (31)	1 (51)		J, K
A17	Ogu121-1A	1 (31)	1 (51)		J
A18	Ogu126-1A	1 (31)	1 (51)		J
A19	Ogu134-8A	-	-	NCMS	
A20	Ogu12A	1 (C/T), 1 (31)	1 (51)		F, I, J
A21	Ogu119-1A	-	1 (51)		A
A22	Ogu34A	-	1 (51)		A
A23	Ogu178-8A	-	-	NCMS	
A24	Ogu118-2A	1 (31)	1 (51)		G, J
A25	Ogu33A	-	1 (51)		A
A29	OguKt-2-1A	1 (31)	1 (51)		J, K
A30	Ogu309-1A	1 (31)	1 (51)		I, J
A31	Ogu-HL-1A	1 (31)	1 (51)		L
A32	Ogu307-1A	1 (31)	1 (51)		A
A33	Ogu Kt-8-2A	1 (31)	1 (51)		J, K
A34	Ogu119-2A	1 (31)	1 (51)		A

A35	Ogu121-2A	1 (31)	1 (51)	I, J, K
A36	Ogu-HL-3A	1 (31)	1 (51)	A, C

(A) Adherence of functional stamens with style; (B) Homeotic-like floral transformation: petaloidy condition of stamens; (C) partial petaloidy of functional stamens; (D) splitted style along with adherence of stamens; (E) stigma hidden inside the petals; (F) splitted style along with exposed ovules; (G) unopened flower; (H) stamens adherence with style and crooked stigma; (I) partially opened flowers; (J) Absence of non-functional stamens; (K) Fused flower; (L) Very curved functional stamens with crooked stigma; (M) absence of nectaries; (NCMS) Cytoplasmic male sterile sources with normal flower phenotype and devoid of other abnormalities as mentioned in A-M. Although, the conventional changes in flower morphology owing to cyto-nuclear interaction are evident; (+) sign denotes presence; (51)The number in parenthesis under the column deletions denote the number of base pair deletions

Table 4 Impact of cytoplasmic genetic variations based on InDels on floral phenotypes in varying nuclear backgrounds

Sr. No	CMS line	Type of nucleotide variation (SNPs/Deletions)		Flower Phenotype of cytolines (CMS)	
		(*BnTR4*)	(*Orf125*)	Normal	Varying Abnormalities
A37	Ogu13-85-3A	1 (31)	1 (51)		B, I
A38	Ogu119-6A	-	-	NCMS	
A43	Ogu13A	-	-	NCMS	
A44	Ogu34-1A	-	1 (51)		A
A45	Ogu1-2A	1 (31)	1 (51)		I
A46	Ogu13-85-2A	1 (31)	1 (51)		B, J
A47	Ogu118-3A	1 (31)	1 (51)		B, J
A48	Ogu307-6A	-	-	NCMS	
A49	Ogu-HL-6A	-	-	NCMS	
A50	Ogu308-6A	-	-	NCMS	
A51	Ogu13-01-5A	-	-	NCMS	L
A52	Ogu13-85-4A	1 (31)	1 (51)		M
A53	Ogu118-4A	1 (31)	1 (51)		B, K
A54	Ogu76-4A	1 (31)	1 (51)		A, K
A55	Ogu33-4A	1 (31)	1 (51)		A, K
A56	Ogu77-4A	1 (31)	1 (51)		B, C, E
A57	Ogu13-85-33A	-	1 (51)		A
A58	Ogu76-33A	-	1 (51)		A
A59	Ogu122-8A	-	-	NCMS	
A60	Ogu1-8A	-	-	NCMS	
A61	Ogu309-8A	-	-	NCMS	
A62	Ogu115-8A	-	-	NCMS	
A63	Ogu310-8A	-	-	NCMS	
A64	Ogu34-1-8A	-	-	NCMS	
A65	Ogu34-8A	-	-	NCMS	
A66	Ogu-HL-50A	-	-	NCMS	
A68	Ogu15A	-	-	NCMS	
A69	Ogu17A	1 (31)	1 (51)		L
A70	Ogu50A	-	-	NCMS	

A71	Ogu60A	-	-	NCMS

- Adherence of functional stamens with style; (B) Homeotic-like floral transformation: petaloidy condition of stamens; (C) partial petaloidy of functional stamens; (D) splitted style along with adherence of stamens; (E) stigma hidden inside the petals; (F) splitted style along with exposed ovules; (G) unopened flower; (H) stamens adherence with style and crooked stigma; (I) partially opened flowers; (J) Absence of non-functional stamens; (K) Fused flower; (L) Very curved functional stamens with crooked stigma; (M) absence of nectaries; (NCMS) Cytoplasmic male sterile sources with normal flower phenotype and devoid of other abnormalities as mentioned in A-M. Although, the conventional changes in flower morphology owing to cyto-nuclear interaction are evident; (+) sign denotes presence; (51)The number in parenthesis under the column deletions denote the number o
- f base pair deletions

Table 5 Impact of cytonuclear interactions on nectar production of cytolines in varying nuclear backgrounds

A and B lines	Nectar Quantity (µl)	% Nectar Reduction in cytolines	A and B lines	Nectar Quantity (µl)	% Nectar Reduction in cytolines	A and B lines	Nectar Quantity (µl)	% Nectar Reduction in cytolines
Ogu33-1A	0.44**	73.17	Ogu1A	1.53**	85.57	Ogu2-6A	3.66**	65.50
Kt-33B1	1.64		Kt-1B	10.61		Kt-1B	10.61	
Ogu33A	0.38**	78.28	Ogu12A	2.24**	83.93	Ogu115-33A	2.02*	30.34
Kt-33B	1.75		Kt-12B	13.94		Kt-15B	2.90	
Ogu34-1A	0.60**	75.6	Ogu16A	0.44*	84.82	Ogu307-33A	0.87*	73.47
Kt-34B (WF)	2.46		Kt-16B	2.90		Kt-307-33B	3.28	
Ogu34A	0.65*	67.82	OguKt-2-1A	0.49*	84.82	Ogu1-8A	5.69**	46.37
Kt-34B (YF)	2.02		Kt-2B	3.23		Kt-1B	10.61	
Ogu15A	0.28*	86.13	Ogu121-1A	1.42	15.97	Ogu13-85-3A	0.55*	49.54
Kt-15B	2.02		Kt-121B	1.69		Kt-1385B	1.09	
Ogu17A	0.66**	76.76	Ogu121-2A	0.33*	76.76	Ogu2A	2.24*	30.65
Kt-17B	2.84		Kt-121B	1.42		Kt-2B	3.23	
Ogu119-1A	1.37*	32.17	Ogu1-2A	0.38*	88.23	Ogu3A	0.55*	81.35
RSK-119B	2.02		Kt-2B	3.23		Kt-3B	2.95	
Ogu307-1A	1.48**	54.87	Ogu-77-6A	0.55*	56.34	Ogu13A	1.59**	53.09
Kt-307B	3.28		Kt-77	1.26		Kt-13B	3.39	
Ogu309-1A	0.60**	64.49	Ogu-HL-6A	0.40**	90.74	Ogu14A	0.55**	85.21
Kt-309B	1.69		HL	4.32		Kt-14B	3.72	
Ogu-HL-1A	0.49**	88.65	Ogu122-5A	1.91**	74.66	Ogu119-2A	0.55*	72.77
HL	4.32		Kt-22B	7.54		RSK-119B	2.02	
Ogu309-2A	0.93*	44.97	Ogu122-8A	0.98**	87.00	Ogu13-85-2A	0.55	49.54
Kt-309B	1.69		Kt-22B	7.54		Kt-	1.09	

						1385B		
Ogu1301-5A	3.61*	24.15	Ogu309-8A	2.35	10.30	Ogu118-2A	0.71*	73.99
Kt-1301B	4.76		Kt-309-8B	2.62		Kt-18B	2.73	
Ogu-HL-3A	0.55**	87.26	Ogu118-4A	0.44*	83.88	Ogu119-6A	0.38*	81.18
HL	4.32		Kt-18B	2.73		RSK-119B	2.02	
Ogu76-4A	1.09*	70.21	Kn81.1301	0.54**	88.65	Ogu13-85-6A	0.98	10.09
DC-76	3.66		Kt-1301B	4.76		Kt-1385B	1.09	
Ogu76-33A	1.04*	71.58	Ogu13-85-33A	0.44*	59.63	Ogu118-6A	0.87*	68.13
DC-76	3.66		Kt-1385B	1.09		Kt-18B	2.73	
Ogu77-4A	0.66*	47.61	Ogu122-1A	0.49**	93.50	OguKt-2-6A	0.86*	68.13
Kt-77B	1.26		Kt-22B	7.54		Kt-2B	2.73	
Ogu310-8A	0.60	26.82	Ogu126-1A	2.95**	78.76	Ogu-13-01-33A	1.37*	71.21
Kt-310B	0.82		Kt-126B	13.89		Kt-1301B	4.76	
Ogu178-8A	1.86*	35.86	OguKt-9-2A	1.04**	83.75	Ogu115-8A	1.05*	48.01
Kt-178B	2.90		Kt-9B	6.40		Kt-15B	2.02	
Ogu-HL-50A	0.66**	84.72	OguKt-8-2A	1.04**	91.61			
HL	4.32		Kt-8B	12.41				
Ogu13-85-4A	0.66*	39.44	Ogu118-3A	0.87*	68.13			
Kt-1385B	1.09		Kt-18B	2.73				
Ogu34-1-8A	0.60*	75.60	Ogu308-6A	5.30*	17.18			
Kt-34B (WF)	2.46		Kt-308B	6.40				

Figures

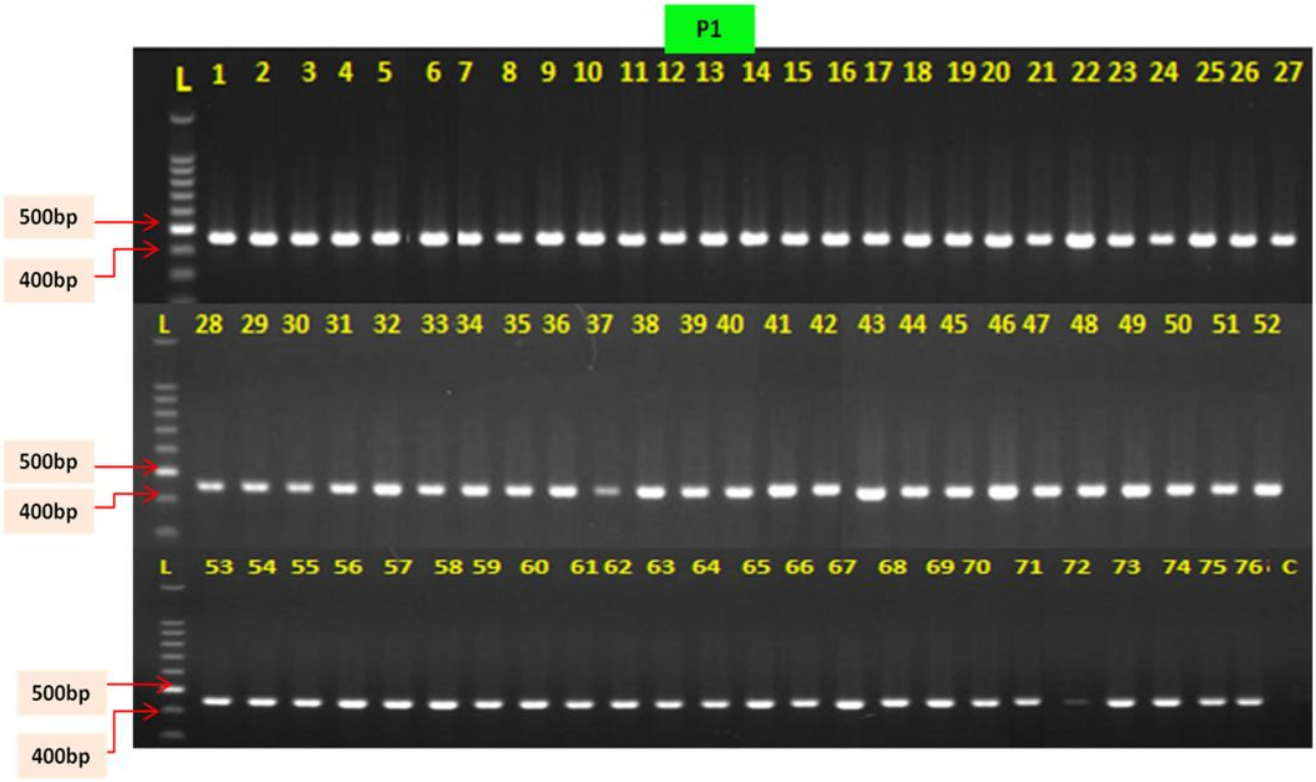

Figure 1

PCR amplification profile of 76 cytolines of cauliflower. The P1 depicts the PCR amplification with primer P1 specific to orf-138, L: ladder, C: control (Sel-27)

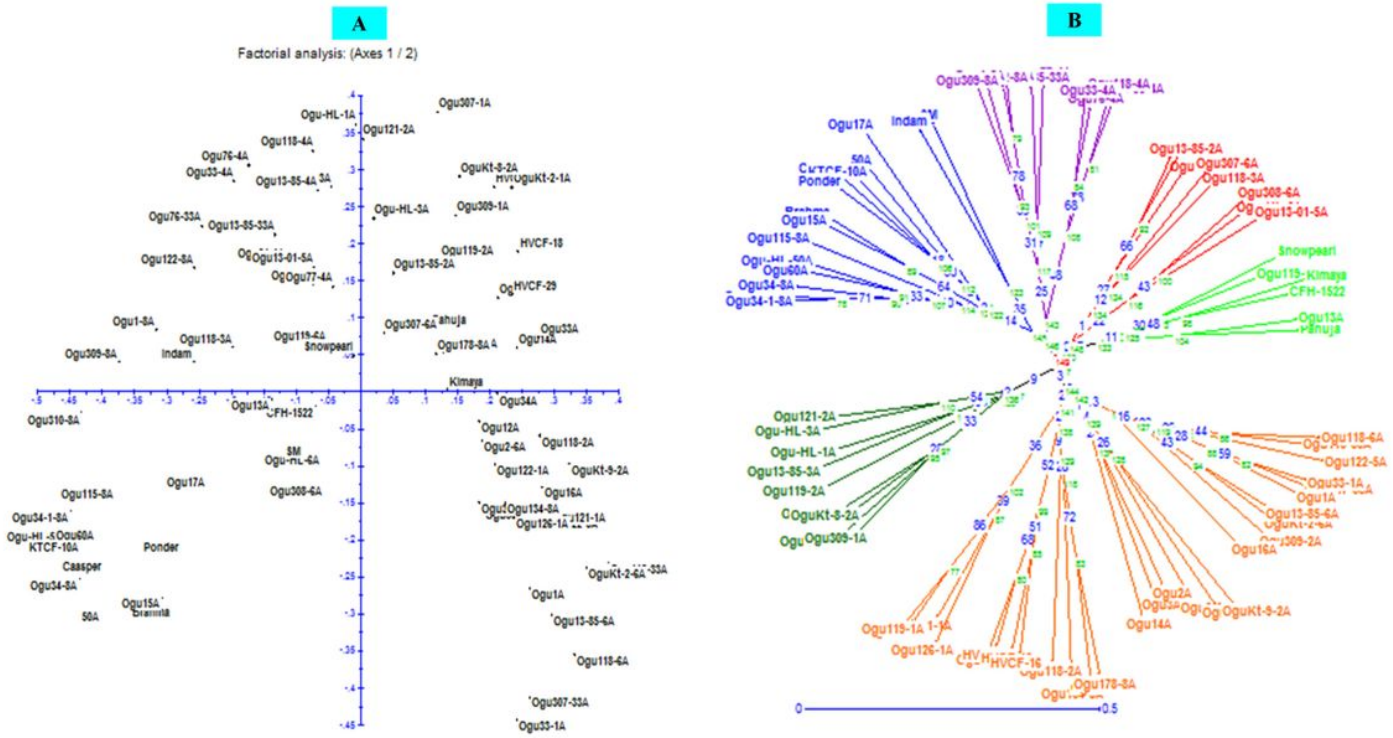

Figure 2

Principal component analysis and Neighbour joining cluster analysis. The cluster analysisof cauliflower cytolines of varying nuclear genome background based on combined analysis of mt-DNA specific and mt-SSR primers is presented here.(a) Principal component analysis of 76 cytolines based on molecular data (b) NJ dendrogram of cytolines depicting 6 distinct groups in different colors

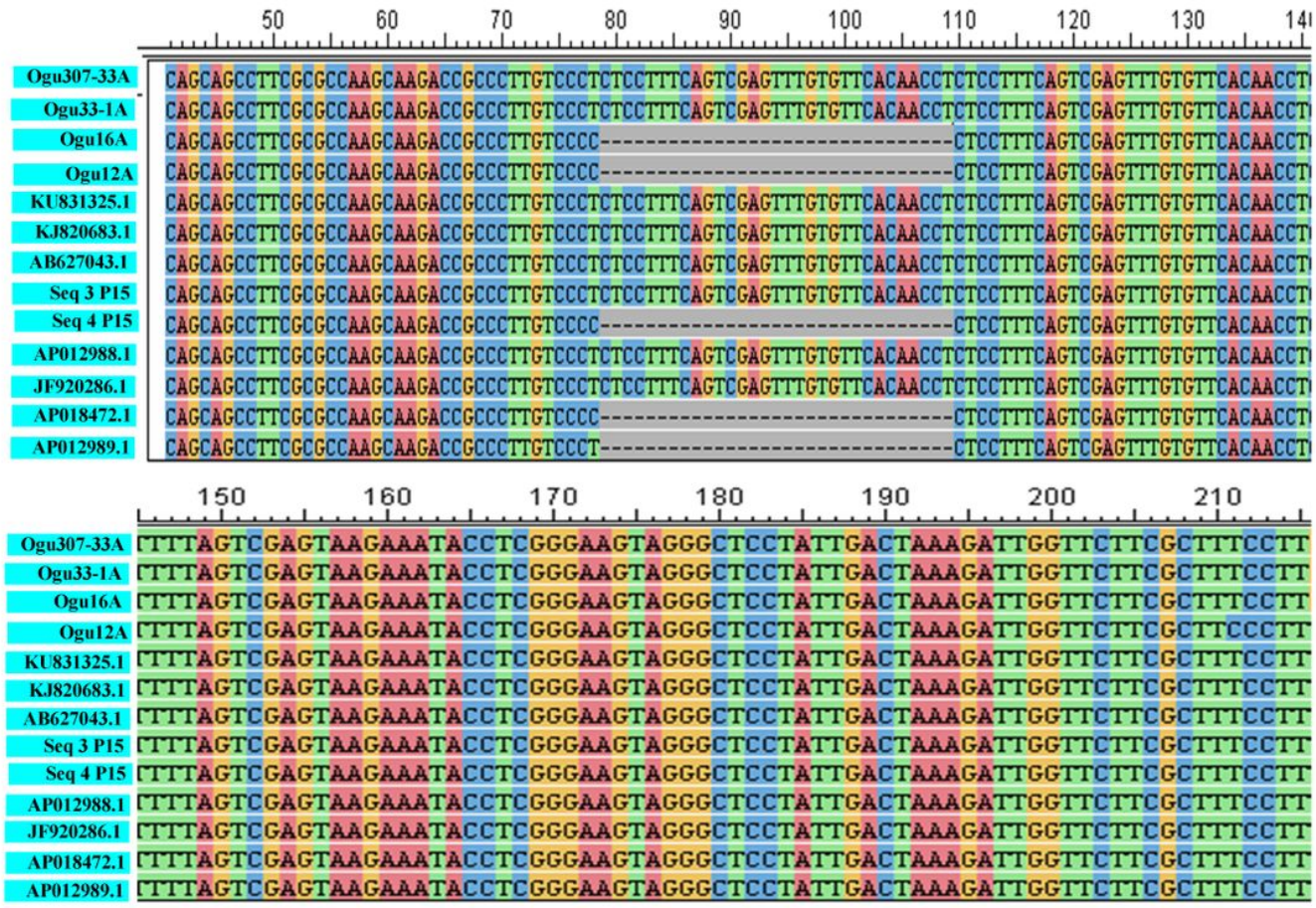

Figure 3

Sequence analysis and alignment of polymorphic amplicons generated by primer P15.Ogu307-33A (MN549523), Ogu33-1A (MN549524), Ogu16A (MN549525) and Ogu12A (MN549526) are selected polymorphic amplicons of CMS mitotypes. Numbers in parenthesis are accession numbers obtained for the respective fragment sequence submitted to GenBank. KU831325.1 (Brassica oleracea var. capitata), KJ820683.1 (Brassica oleracea var. botrytis), AB627043.1(Brassica oleracea), AP012988.1 (Brassica oleracea), JF920286.1 (Brassica oleracea), AP018472.1 (Raphanus sativus) and AP012989.1 (Brassica nigra) are related reference mitochondrial genome sequences of Brassicaceae crops available in NCBI gene bank database.

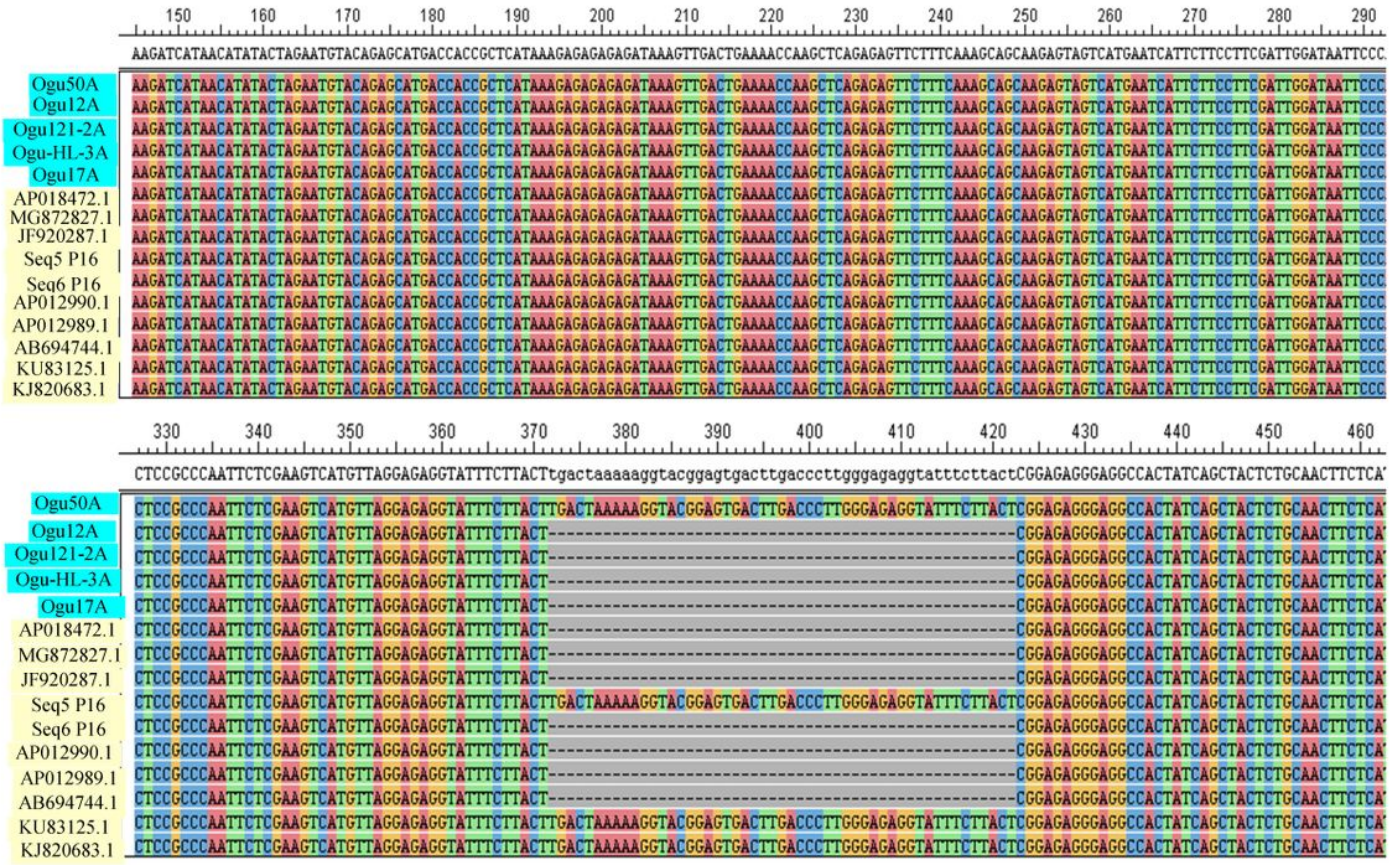

Figure 4

Sequence analysis and alignment of polymorphic amplicons generated by primer P16. Ogu50A (MN549531), Ogu12A-orf125 (MN549527), Ogu17A (MN549530), OguHL-3A (MN549529) and Ogu121-2A (MN549528) are selected polymorphic amplicons of cauliflower CMS mitotypes. Numbers in parenthesis are accession numbers obtained for the respective fragment sequence submitted to GenBank.KU831325.1 (Brassica oleracea var. capitata), KJ820683.1 (Brassica oleracea var. botrytis), AB694744.1 (Raphanus sativus), AP012990.1 (Raphanus sativus), JF920287.1 (Brassica carinata), AP018472.1 (Raphanus sativus), AP012989.1 (Brassica nigra), and MG872827.1 (Brassica juncea) are related reference mitochondrial genome sequences of Brassicaceae crops available in NCBI gene bank database.

Figure 5

Floral deformities associated with cyto-nuclear conflict. (a) Adherence of functional stamens with style (b) petaloidy condition of stamens (c) partial petaloidy of functional stamens (d) splitted style along with adherence of stamens (e) stigma hidden inside the petals (f) splitted style (g) unopened flower (h-i) stamens adherence with style and bent stigma

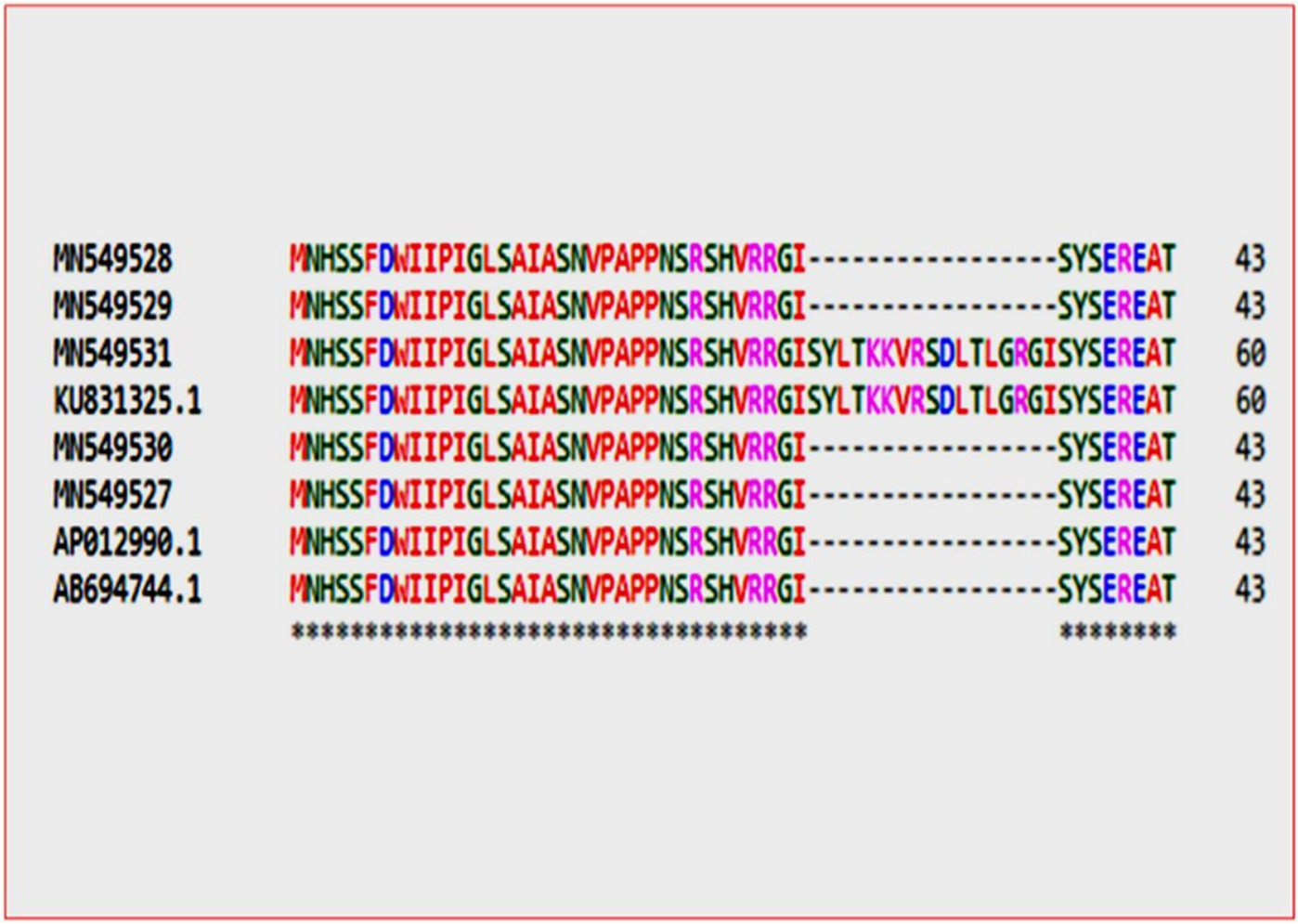

Figure 6

Amino acid sequence analysis of ORFs of P16 polymorphic amplicons. MN549531, MN549527, MN549530, MN549529 and MN549528 are selected protein sequences of ORFs of cauliflower CMS mitotypes.KU831325.1 (Brassica oleracea var. capitata), AB694744.1 (Raphanus sativus), AP012990.1 (Raphanus sativus) are reference amino acid sequences of Brassicaceaeobtained by NCBI ORF finder.

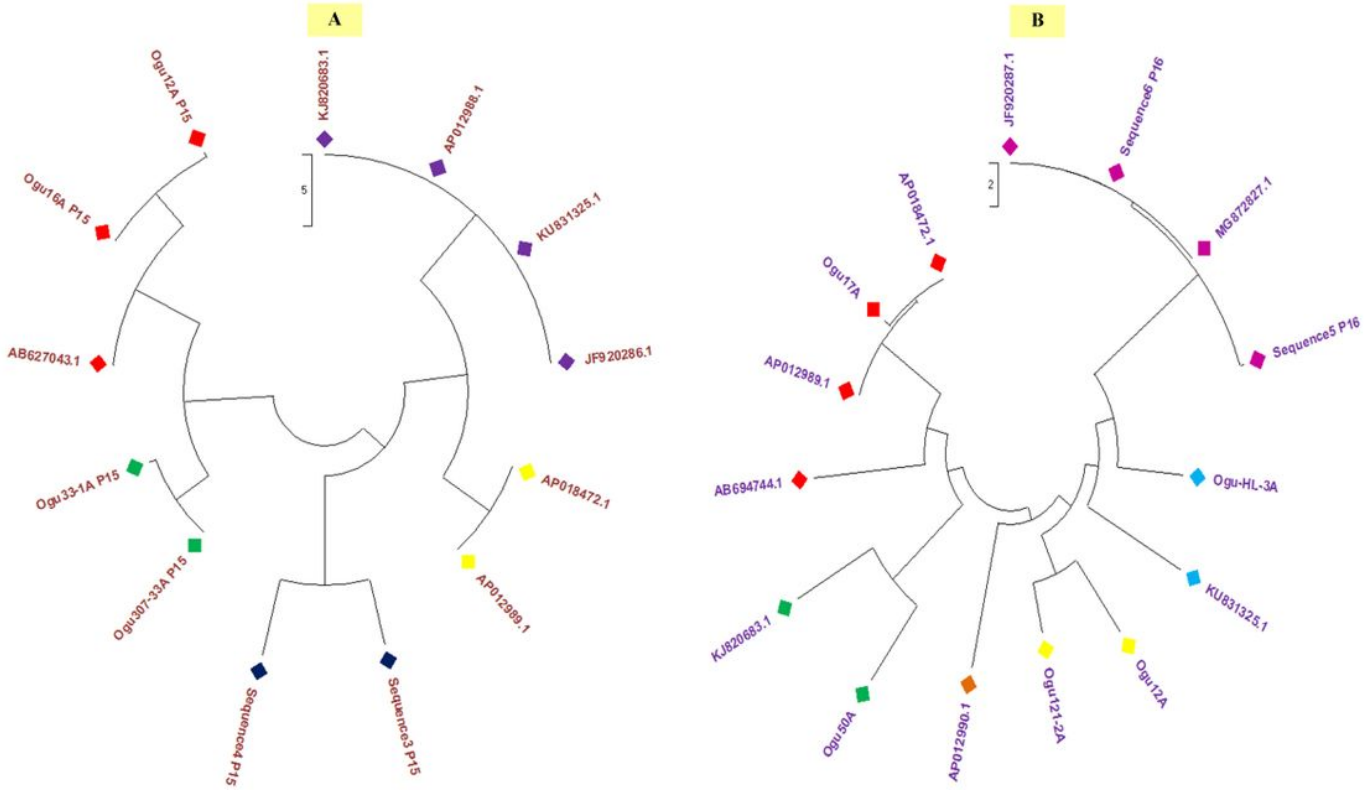

Figure 7

Phylogenetic analysis(a) Phylogenetic tree based on sequence analysis of selected polymorphic fragments generated with primer P15 and related reference mitogenome sequences from NCBI gene bank database. (b) Phylogenetic tree based on sequence analysis of selected polymorphic amplicons generated with primer P16 and related reference mitogenome sequences from NCBI gene bank database.

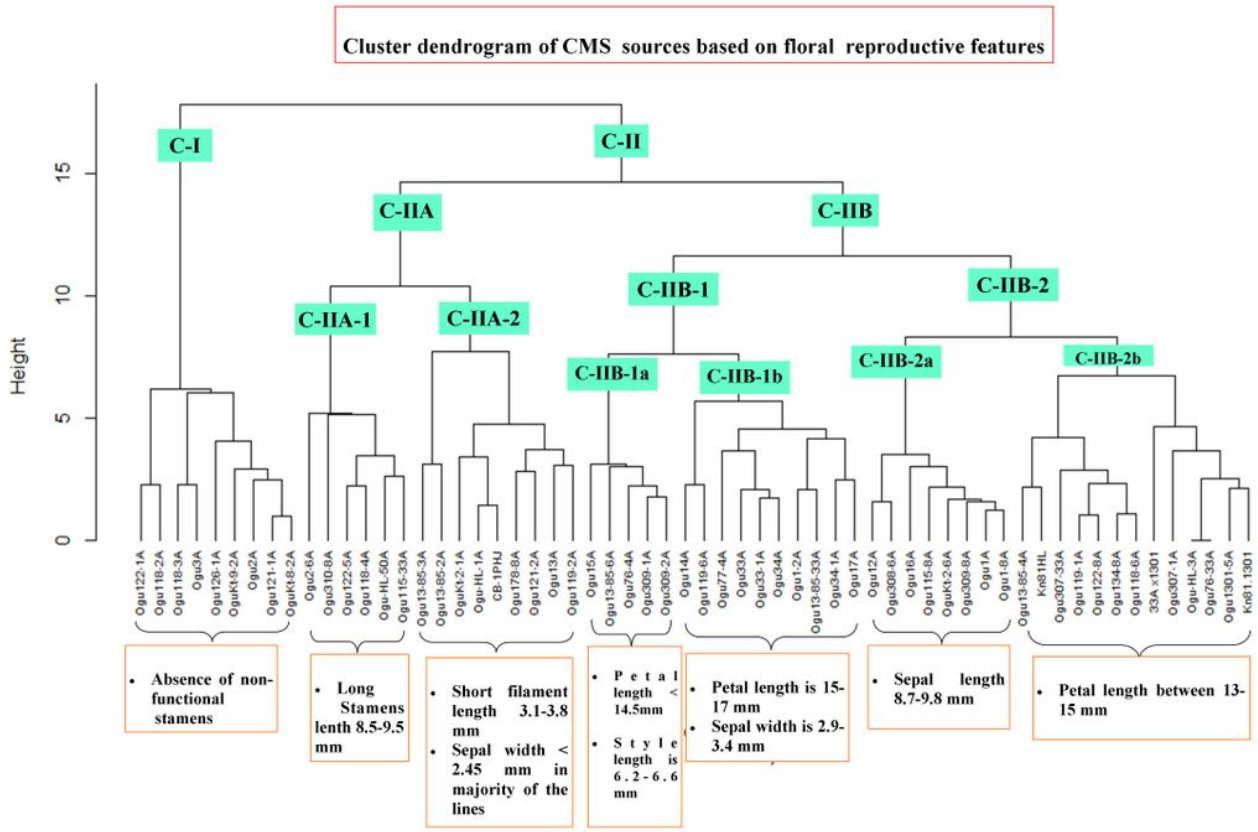

Figure 8

Cluster dendrogram of cauliflower cytoplasmic sources based on floral traits. Cluster C-I harbor all the CMS mitotypes without non functional stamens. Cluster C-II comprises different sub-clusters with varying degree of floral phenotypic variability and abnormalities.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS2.JPEG
- FigureS1.TIF
- Additionalfile1.docx