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Phylogenetic Analysis of Strawberry, *Fragaria x ananassa* (Rosaceae: *Fragaria*) using Cytochrome C Heme Attachment Protein (ccsA) Gene

Kundansing Rajpalsing Jadhao[1*] and Prajjal Dey[2]

[1]Dept. of Agricultural Biotechnology, College of Agriculture, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha (751 003), India

[2]Dept. of Plant Physiology, College of Agriculture, Odisha University of Agriculture & Technology, Bhubaneswar, Odisha (751 003), India

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Corresponding Author

Kundansing Rajpalsing Jadhao

e-mail: kjadhao1987@gmail.com

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Abstract

Phylogenetic relationships and genetic diversity were inferred using nucleotide sequences of chloroplast ccsAgene (cytochrome c heme attachment protein, for c-type cytochrome synthesis) of strawberry, *F. x ananassa* and other *Fragaria* species. Genomic DNA was isolated from fresh and green leaves of strawberry cultivar 'Chandler' seedlings. The ccsA gene sequence was amplified and isolated by using gene specific primers designed from consensus sequences of *Fragaria* species. After sequencing, the isolated ccsA gene sequence was corrected using BioEdit programs. Then ccsA nucleotide sequence isolated from cultivated strawberry (*F. x ananassa*) cultivar 'Chandler' was deposited in GeneBank at NCBI with accession number MK872805.1. Based on the BLASTn hit results, two separate datasets were prepared, one for *Fragaria* genus and other for Rosaceae family species to study the diversity analyses. MEGA 7.0 program was then used for phylogenetic analyzes and the detection of evolutionary genetic divergence between the species. For *Fragaria* genus which also included cultivated strawberry (*F. x ananassa*) ccsA gene sequence, the mean nucleotide composition was estimated as 29.3% A, 37.8% T, 15.5% C and 17.4% G. The lowest divergence distance *i.e*., 0.00 was estimated for *Fragaris* species, while the highest distance 1.00 was observed only for *Fragaria nipponica* (KY769125.1). Overall, the molecular phylogenetic analysis revealed that no genetic variation was observed in *Fragaria* species that was due to the highly conserved chloroplast nucleotide sequences of ccsA gene during evolution. However, the ccsA nucleotide sequences can be useful to study intra genus evolutionary relationship between Rosaceae family members.

1. Introduction

The genus *Fragaria* is a flowering plants belongs to the economically important *Rosaceae* family, popularly known as strawberries for their edible fruits. The genus *Fragaria* comprises about 25 species as well as many hybrids and cultivars, which have been described on the basis of morphological features, geographic distribution and their ploidy level (Staudt, 1989; Folta and Davis, 2006). The modern cultivated strawberry is garden hybrid strawberry known as *Fragaria x ananassa* which is an economically important fruit crop grown in over 60 countries (DiMeglio *et al*., 2014). The cultivated strawberry (*F. x ananassa*) is octoploid originated in Europe in the 1700's from accidental hybridization between the ancestral octoploids *Fragaria chiloensis* and *Fragaria virginiana* (Hancock *et al*., 2008; Folta and Gardiner, 2009). It is one of the favourite berry fruits consumed worldwide for their pleasant aroma, flavour, taste, and texture. In addition, strawberry has rich source of vitamins, minerals as well as antioxidant compounds, which receives more and more attention from growers and consumers worldwide (Forbes-Hernandez *et al*., 2016; Patrick *et al*., 2019). Genetically, *Fragaria* species contains wide range of ploidy level like ranges from diploid (2n = 2x = 14), tetraploid (2n = 4x = 28), hexaploid (2n = 6x = 42), octoploid (2n = 8x = 56) to decaploid (2n = 10x = 70) with basic chromosome number is x = 7 (Ichijima, 1926). Currently there are very few studies has been done to establish the molecular evolutionary relationship between cultivated octoploid (2n = 8x = 56) strawberry and other *Fragaria* species distributed worldwide that can be

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use in further improvement of cultivated strawberry cultivars (Potter *et al*., 2000; Rousseau-Gueutin *et al*., 2008; Davis *et al*., 2010; Mahoney *et al*., 2010; DiMeglio *et al*., 2014).

In many areas of biology, phylogenetic analysis play an important role in studying species relatedness as well as it can also provide the initial starting information to study developmental genetics, genomics, taxonomy and biogeography (Wei *et al*., 2014). Currently, the most preferred markers used to study the plant phylogenetic relatedness is molecular markers in order to overcome the limitations of morphological and biochemical markers. It includes nuclear ribosomal DNA of the internal transcribed spacer (ITS), chloroplast DNA, inter-generic spacer (IGS), external transcribed spacer (ETS) and simple sequence repeat (SSR) technology which have been used in plant classification (Agarwal *et al*., 2008; Kim *et al*., 2015; Sun *et al*., 2015). However, compared to nuclear molecular markers used in phylogenetic study, the chloroplast genome based molecular markers are ideal system phylogeny studies as chloroplastis small genome with very low rate of nucleotide substitutions (Wei *et al*., 2005). Hence, chloroplast DNA (cpDNA) sequencing technology has been widely used to investigate phylogenetic relationships between various plant groups as it is an important research object in the field of molecular evolution, phylogeny, and molecular markers (Daniell *et al*., 2016; Sevindik and Okan, 2020). Particularly the non-coding regions of chloroplast DNA sequences such as cemA-petA, clpP-psbB, ndhF-rpl32, petA-psbJ, psbA-trnK, trnL-ccsA, rpl32-trnL, trnE-trnT, trnK-rps16, trnP-psaJ, trnT-trnL molecular markers are used in resolution of phylogenetic relationships of closely related species as it contained parsimony-informative (Liu *et al*., 2005). Among them, trnL intron and trnL-F spacer along with trnL-ccsA, has become one of the most widely used chloroplast genome based molecular marker to study the phylogenetic analyzes in plants (Pirie *et al*., 2007). In this context, the nucleotide sequence of isolated cytochrome c heme attachment protein (ccsA) gene of cultivated strawberry (*F. x ananassa*) cv. Chandler was sequenced from cpDNA and used to elucidate phylogenetic relationships and genetic diversity among the investigated taxa. Our Study provides the comprehensive understanding of the molecular evolutionary patterns in *Fragaria* and other related species at the cpDNA level.

2. Materials and Methods

2.1 Plant Samples and DNA Extractions

The plant sample of strawberry (*Fragaria x ananassa*.) cv. Chandler was collected from Department of Fruit science and Horticultural technology, College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha. Total cellular DNA of strawberry was extracted from young leaf tissues of seedlings using modified CTAB method (Doyle and Doyle, 1987). Later the DNA sample was stored at -20 °C for further use.

2.2 PCR Amplification and Sequencing

The purified DNA of strawberry (*Fragaria x ananassa*) cv. Chandler was subjected for PCR amplification using designed set of gene specific oligos developed from consensus ccsA nucleotide sequences of *Fragaria* species. The forward and reverse primers designed from consensus sequences was ccsAFW: 5'GATATTTTCAACTTTAGAGC3' and ccsARW: 5'AACCAATGCGATTGGAGAGC3' (Merck Bioscience, India) respectively. PCR amplification was carried out in 25 µl PCR reaction with the template DNA (20~50 ng), 2.5 µl 10X PCR assay buffer (Hi-media, India), 1.5 µl each of 10 mMdNTPs (M/S Merck Bioscience, India), 1 µl (5µM) of both forward and reverse primers and 0.5 µl of 3U Taq DNA polymerase (Hi-media, India). Bio-Radthermal cycler was used for the PCR amplification of ccsA gene, consisted of a total of 35 cycles of initial denaturation (95 °C for 5 min) followed by denaturation (95 °C for 1 min), annealing (57 °C for 1 min), elongation (72 °C for 2 min) and final elongation (72 °C for 10 min). The PCR product was visualized on in 2.0% (w/v) agarose gel (Merck Bioscience, India) by using the 1X TAE buffer. Then the single bright band of 400 bp was eluted using Gel Extraction Kit (Genei™). The purified PCR product then subjected for two way sequencing to minimize the error in sequencing and was sequenced by using both 96 capillary high throughput sequencer; ABI 3730 XL system to generate sequences with accurate base calling which is an extension and refinement of Sanger's dideoxy method (Sanger *et al*., 1977) at SciGenom, Cochin, Kerala, India. Further, DNA sequence was edited both manually and using BioEdit (Hall, 1999) program which was then checked through BLASTn search tool in GeneBank (NCBI) for correctness of nucleotide sequence.

2.3 Multiple Sequence Alignment and Phylogenetic Analyzes

The isolated strawberry (*Fragaria x ananassa*) nucleotide sequence was queried in BLASTn search tool of GeneBank database at NCBI for the preparation of datasets for MSA and phylogenetic analyses. The BLASTn hit result sequences obtained against nucleotide sequence of cytochrome c heme attachment protein (ccsA) as query were retrieved for preparation of *Fragaria* genus containing species dataset and sorted according to ploidy level (Table 1). The second dataset of closely related species was prepared by downloading all the possible sequences from the same BLASTn hit result, particularly for the species of Rosaceae family. For the first dataset of *Fragaria* genus, an alignment of 16 *Fragaria* species ccsA nucleotide sequences along with cultivated strawberry (*F. x ananassa*) cv. Chandler was toggled using multiple sequence alignment (MSA) in muscle program (www.ebi.ac.uk/Tools/MSA/muscle/). Whereas, for second dataset, all the possible related species of *Fragaria* genus and Rosaceae family members were also aligned (MSA) by muscle program in MEGA7.0 (Molecular Evolutionary Genetic Analysis) tool. Further, both the MSA data sets were used for phylogenetic analysis and calculations of pair wise distances for diversity

Table 1: Details of *Frgaria* species used in present study

Sl. No.	Accession Number	Species Name	Ploidy level	Chromosome Number	Habitat
1	MK872805.1	*F. x ananassa*	Octoploid	56	Cultivated
2	KY358226.1	*F. x ananassa* cv. *Benihoppe*	Octoploid	56	Cultivated
3	NC048474.1	*Fragaria viridis*	Diploid	14	Central Asia, Europe
4	KY769126.1	*Fragaria orientalis*	Tetraploid	28	China, Mongolia, Russia
5	KY085911.1	*Fragaria virginiana*	Octoploid	56	North America
6	KY434061.1	*Fragaria pentaphylla*	Tetraploid	28	China
7	KC507760.1	*Fragaria mandshurica*	Diploid	14	NA
8	KC507759.1	*Fragaria iinumae*	Diploid	14	Japan, Eastern Russia
9	KC507757.1	*Fragaria vesca*	Diploid	14	Northern Hemisphere
10	JN884817.1	*Fragaria virginiana*	Octoploid	56	North America
11	JN884816.1	*Fragaria chiloensis*	Octoploid	56	Pacific Ocean coasts of North and South America and Hawaii
12	JQ396172.1	*Fragaria mandshurica*	Diploid	14	NA
13	JQ396171.1	*Fragaria vesca* subsp. *bracteata*	Diploid	14	Northern Hemisphere
14	JF345175.1	*Fragaria vesca* subsp. *vesca*	Diploid	14	Northern Hemisphere
15	GU363535.1	*Fragariavesca* subsp. *americana*	Diploid	14	Northern Hemisphere
16	KY769125.1	*Fragaria nipponica*	Diploid	14	Western side of the Japanese island of Honshū, Japanese island of Yakushima

analysis (http://www.megasoftware.net) (Tamura *et al*., 2013). The Maximum Likelihood (ML) phylogenetic tree for *Fragaria* genus was inferred with 1000 replications of bootstrap test of Tamura-Nei model to evaluate the evolutionary history among *Fragaria* species (Tamura and Nei, 1993). *Arabidopsis thaliana* (MK380723.1) was used as out group in ML phylogenetic tree construction for stabilization of tree nodes. For second dataset, Neighbour joining (NJ) method with Kimura two-parameter model was used to draw phylogenetic tree based on MSA of all closely related species ccsA nucleotide sequences of Rosaceae family (Kimura, 1980). The stability of internal nodes of NJ tree was assessed by bootstrap test with 1000 replicates. In NJ tree of Rosaceae family members, *Arabidopsis thaliana* (MK380723.1) and *Solanum lycopersicum* (MK380723.1) ccsA gene sequences was included as out group in phylogenetic tree construction retrieved from NCBI. Both the phylogenetic trees were constructed in MEGA7.0 tool (Kumar *et al*., 2016).

3. Results and Discussion

3.1 Sequence Characteristics

Among the various approaches used in molecular systematic and phylogenetic analyses, DNA sequences has become one of the most widely used starting point of most of the experiments, particularly for identification of genetic variations at genus level (Patil *et al*., 2015). Variation in nucleotide sequences of the coding as well as spacer noncoding regions of chloroplast, mitochondrial and nuclear genes has been utilized in molecular systematic. These variations in coding and non-coding region of DNA associated with any gene region or gene region in the genome have significant advances in plant biotechnology as molecular markers (Yeşiltaş and Kolören, 2019). Particularly in plant systems, the chloroplasts are inherited from maternal cytoplasm, that comprise genes encodes for many chloroplast specific components that are highly conserved for more variable regions and informative for evolutionary history of plants (Filiz *et al*., 2018).

In the present investigation, sharp and bright band of 400 bp of plastid ccsA gene fragment from template DNA of strawberry (*Fragaria x ananassa*) cv. Chandler variety was amplified by using gene specific primers. After sequencing, the strawberry sequence was used for MSA along with other *Fragaria* species to prepare first dataset for its downstream analysis. The isolated chloroplast gene sequence of ccsA from strawberry (*Fragaria x ananassa*) was submitted to Gene bank at NCBI database with accession number MK872805.1. An average nucleotide composition of ccsA gene sequences was 29.3% A, 37.8% T, 15.5% C and 17.4% G. The average AT content (67.07%) and GC content (32.93%) was observed for ccsA gene sequences of genus *Fragaria* was constant for all the species except for an out group *Arabidopsis thaliana* (Table 2). The genetic pair wise distances of *Fragaria* genus containing species was calculated in MEGA 7.0 software

Table 2: Gene Length and nucleotide composition of *Fragaria* species ccsA gene sequences including out group *A. thaliana* used in the present study

Sl. No.	Species Name	Length (bp)	A (%)	T (%)	G (%)	C (%)	A+T (%)	G+C (%)
1	*Fragaria x ananassa*	398.0	29.1	37.7	15.6	17.6	66.83	33.17
2	*Fragaria viridis*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
3	*Fragaria x ananassa* cv. *Benihoppe*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
4	*Fragaria orientalis*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
5	*Fragaria virginiana*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
6	*Fragaria pentaphylla*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
7	*Fragaria mandshurica*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
8	*Fragaria iinumae*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
9	*Fragaria vesca*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
10	*Fragaria virginiana*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
11	*Fragaria chiloensis*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
12	*Fragaria mandshurica*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
13	*Fragaria vesca* subsp. *bracteata*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
14	*Fragaria vesca* subsp. *vesca*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
15	*Fragaria vesca* subsp. *americana*	397.0	29.2	37.5	15.6	17.6	66.75	33.25
16	*Fragaria nipponica*	397.0	29.2	37.5	15.4	17.9	66.75	33.25
17	*Arabidopsis thaliana*	404.0	30.7	41.3	13.6	14.4	72.03	27.97
	Avg.	397.5	29.3	37.8	15.5	17.4	67.07	32.93

based on their nucleotide sequences and it was observed that divergence values were varied from 0.00 to 1.00 (Table 3). The highest divergence value *i.e.*, 1.00 was observed for *Fragaria nipponica* (KY769125.1) against the all the studied *Fragaria* species. Whereas, the lowest *i.e.*, 0.00 divergence value was observed between other *Fragaria* species. These results showed that there was no nucleotide variations in *Fragaria* species at ccsA gene level, indicates that the coding

Table 3: Pairwise sequence distance matrix among *Fragaria* species based on ccsAcp DNA data calculated by MEGA 7.0 software

Sl. No.	Species Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Fragaria x ananassa*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	*Fragaria viridis*	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	*Fragaria x ananassa* cv. *Benihoppe*	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	*Fragaria orientalis*	0.00	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-
5	*Fragaria virginiana*	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-	-
6	*Fragaria pentaphylla*	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-
7	*Fragaria mandshurica*	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-	-	-
8	*Fragaria iinumae*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-	-

Sl. No.	Species Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
9	*Fragaria vesca*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-	-
10	*Fragaria virginiana*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-	-
11	*Fragaria chiloensis*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-	-
12	*Fragaria mandshurica*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-	-
13	*Fragaria vesca* subsp. *bracteata*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-	-
14	*Fragaria vesca* subsp. *vesca*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
15	*Fragaria vesca* subsp. *americana*	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-
16	*Fragaria nipponica*	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-

region in ccsA gene was highly conserved in *Fragaria* genus during the evolution. Further, Tajma's Neutrality Test of 16 *Fragaria* species including strawberry (*F. x ananassa*) cv. Chandler was calculated on the basis of ccsA gene sequences using MEGA 7.0. The result also revealed that the sixteen numbers of sequences (m) (16 *Fragaria* species) gave one segregation site (S) only which indicates very low nucleotide diversity (π) of 0.000315 (Table 4). Moreover, Tamura and Nei (1993) model was used to estimate substitution pattern and rates based on relative values of instantaneous r that should

Table 4: Tajima's Neutrality Test results based on ccsA gene of *Fragaria* species

No. of sequences "m	No. of segregating sites "S"	P_s = S/n	$\Theta = p_s/a_1$	Nucleotide diversity ''π''	Tajima test statistic ''D"
16	1	0.002519	0.000759	0.000315	-1.162213

be considered when evaluating them. For simplicity sum of r values is made equal to 100. The nucleotide frequencies were A = 29.22%, T/U = 37.53%, C = 17.65%, and G = 15.60%. The result showed that there was no transitional substitution in sixteen *Fragaria* species studied. While, higher rate of transversionsal substitutions was observed in *Fragaria* species sequences (Table 5). For estimating ML values, a tree topology was automatically computed and the maximum Log likelihood for this computation was -533.355. Similarly, Jadhao and Patra (2019) also showed that the variation in transitional and transversionsal nucleotide substitutions in MatK gene (chloroplast gene) of *Oryza* genus were useful to study the nucleotide diversity and evolutionary history.

Table 5: Maximum likelihood estimate of substitution matrix

	A	T/U	C	G
A	-	*18.77*	*8.82*	0.00
T/U	*14.61*	-	0.00	*7.80*
C	*14.61*	0.00	-	*7.80*
G	0.00	*18.77*	*8.82*	-

*Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics.

3.2 Phylogenetic Analysis

The last two decades, the plant molecular systematics has been rapidly advancing by analyzing the sequences and using that sequences in discovery of new phylogenetic analyzes methods (Sevindik and Okan, 2020). This advancement in sequence analysis methods is proving very useful in plant systematics studies and phylogenetic analyzes when there are insufficient morphological traits/ parameters in terms of phylogenetic knowledge (İnal *et al*., 2017). In the present study, maximum likelihood method was used to draw the molecular phylogeny to study the genetic diversity between 16 *Fragaria* species with different ploidy level based on the ccsA gene of chloroplast (Table 1). Interestingly, the phylogenetic

tree of *Fragaria* species revealed that there was no significant diversity between *Fragaria* species despite of their diversity in ploidy level (Figure 1). It indicates that ccsA gene was highly conserved during evolution in all *Fragaria* species. Similarly, Potter *et al*., (2000) also observed low variability in both ITS (nuclear) and trnL-trnF spacer (chloroplast) regions, but comparatively much lower in the chloroplast sequences than in the nuclear sequences while studying the phylogenetic relationships among 14 species of *Fragaria* genus (43 accessions).

Furthermore, the evolutionary history of closely related species of *Fragaria* genus particularly species belongs to the Rosaceae family was inferred using the Neighbor-Joining method with 1000 replications (Figure 2). The model plant such as *Arabidopsis thaliana* and *Solanum lycopersicum* were also included in phylogenetic analysis as out group for stabilization

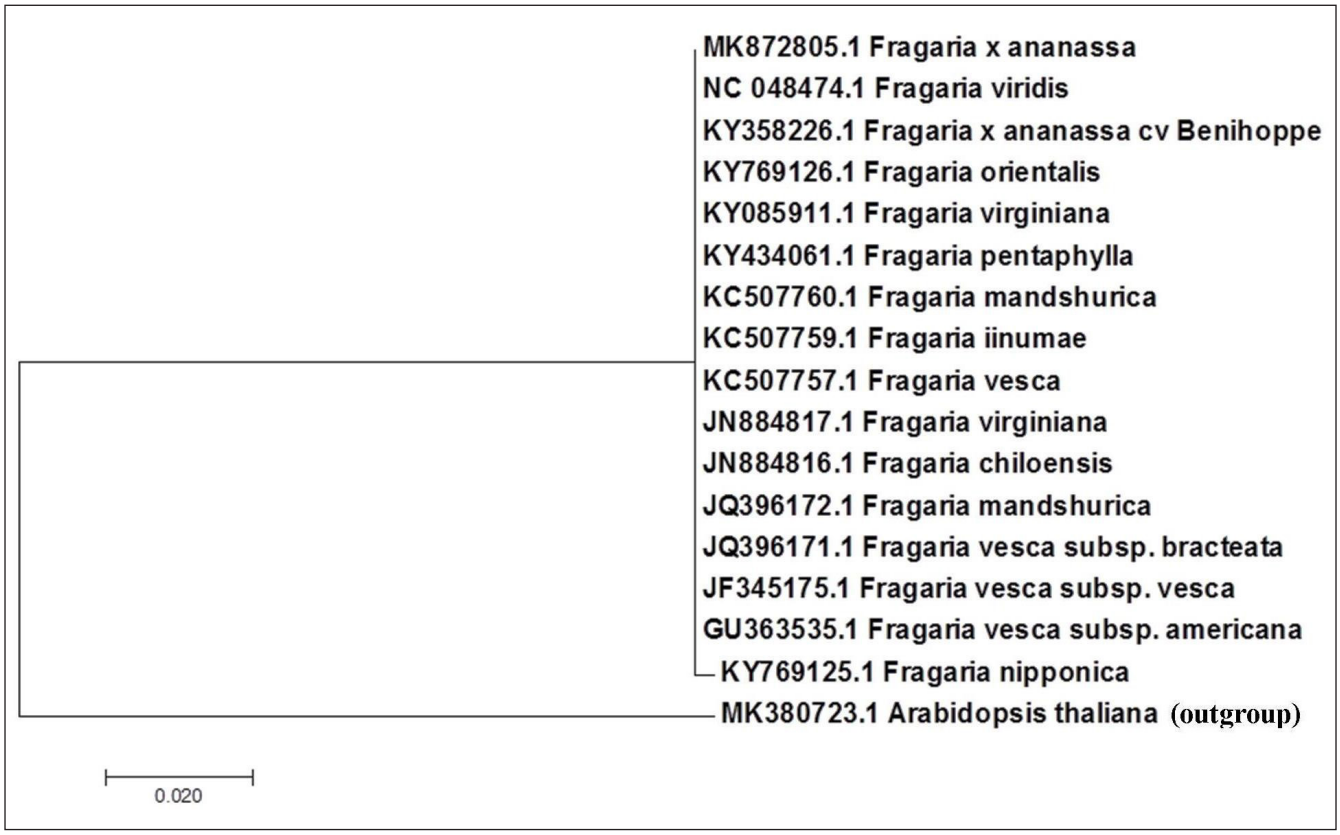

Figure 1: Phylogenetic tree of *Fragaria* species based on ccsA (cytochrome c heme attachment protein, for c-type cytochrome synthesis) nucleotide sequences constructed using maximum likelihood method in MEGA 7.0. The scale below the tree indicates the bootstrap value of divergence.

of tree node diversity. The phylogenetic analysis of Rosaceae family revealed that all the studied species were distributed into the eight main clusters (I-VIII). Among them, cluster-I and cluster-II mainly contained the species from *Fragaria* genus, cluster-I consisted maximum number of species from *Fragaria* genus *i.e*., 12 species including cultivated species of strawberry (*F. x ananassa*) cv. Chandler. While, the cluster-II contained only four members of diploid (2n=14) *Fragaria* species such as *F. mandshurica*, *F. iinumae*, *F. vesca* and *F. nipponica* indicates the divergence of other *Fragaria* species with different ploidy level from diploid species during evolution. Among all, Cluster-III was the largest cluster comprising maximum 17 species. Interestingly, cluster-III contained the species from the *Rose* genus only clearly indicating that *Rose* genus was evolved independently from *Fragaria* genus. However, four species of *Rose* genus were also grouped with *Alchemilla s*pecies as well as with model plants (*A. thaliana* and *S. lycopersicum*). Whereas, the smallest cluster-VI contained only three species such as *C. salesovianum*, *S. procumbens* and *S. retusa*. Likewise cluster-III, Cluster-IV comprises members from *Potentilla* genus only except *P. parvifolia* which alone belongs to the cluster-V with other species. Similarly, the molecular phylogenetic relationship between *Fragaria* species and Rosaceae family were established to study the evolutionary intraspecific and interspecific relationship by using both nuclear and chloroplast sequences (Potter *et al*., 2000; DiMeglio *et al*., 2014; Huang *et al*., 2019). Overall the phylogenetic analysis of Rosaceae family revealed that ccsA gene sequences can be used for diversity analysis and establishment of evolutionary relationship between Rosaceae family members.

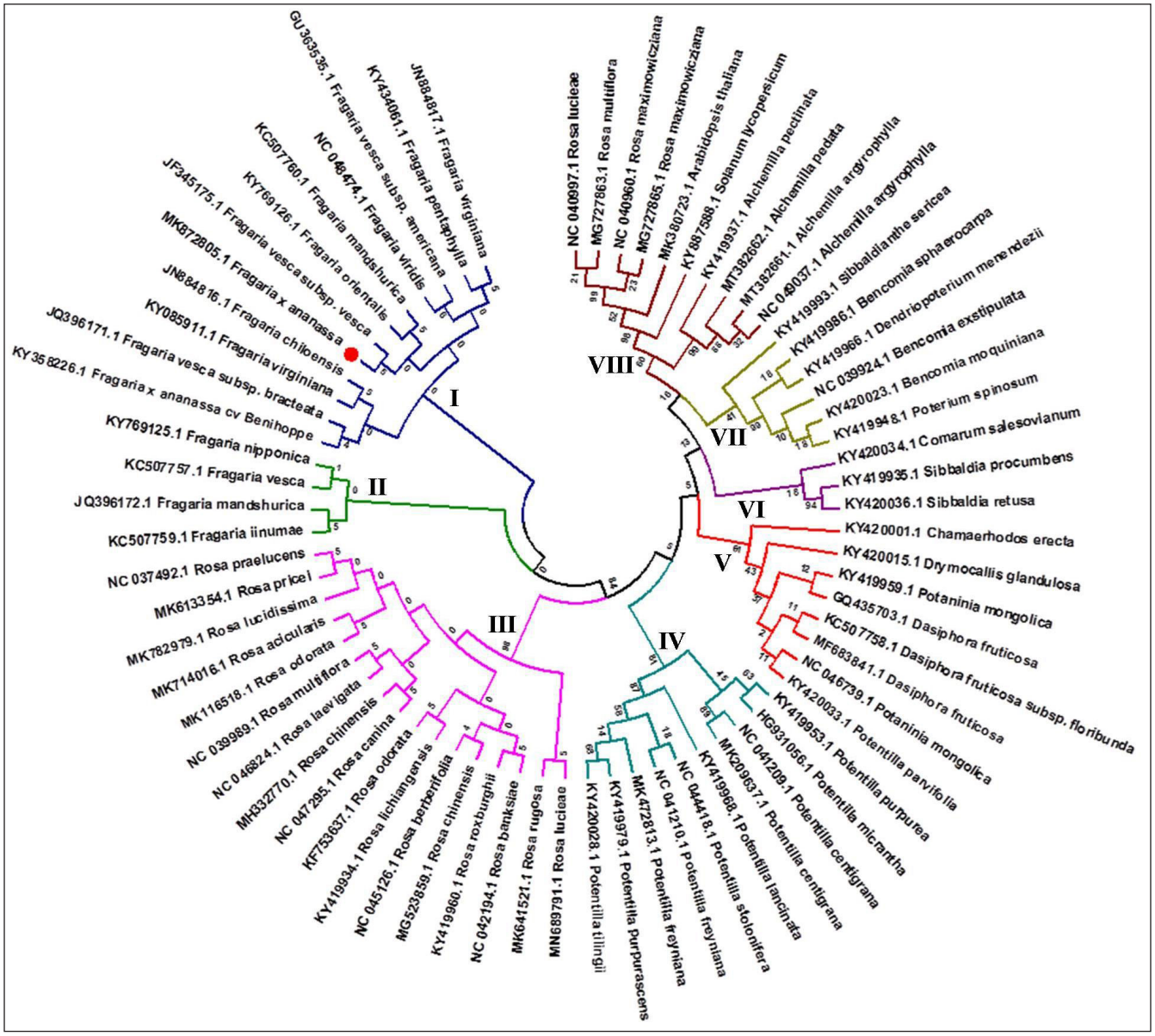

Figure 2: Phylogenetic tree of Rosaceae family species closely related to Fragaria genus based on ccsA (cytochrome c heme attachment protein, for c-type cytochrome synthesis) nucleotide sequences constructed using neighbor joining method in MEGA 7.0. The cultivated strawberry (*F. x ananassa*) cv. Chandler marked in red in the phylogenetic tree. Black bold fond on tree node indicates bootstrap values in percent.

4. Conclusion

In conclusion, in the light of the findings of the present study, ccsA (cytochrome c heme attachment protein, for c-type cytochrome synthesis) sequence of cultivated strawberry (*F. x ananassa*) cv. Chandler had no genetic variation and hence it is not informative for phylogenetic resolution between *Frgaria* species. However, the ccsA sequences can be used for intra genus phylogenetic evolutionary study of Rosaceae family. Hence, further study is needed and it must involve the sequences of (nrDNA ITS) and other non-coding cpDNA for reliable resolution evolutionary history of *Fragaria* species, particularly how the genetic variation in genes involved in development of different ploidy level from common ancestors during evolution.

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