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Biochemical and molecular identification of ten plum (*Prunus salicina* L.) cultivars in Egypt based on their physiological traits, chloroplast and mitochondrial DNA markers

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Abstract:

Plum (*Prunus salicina* L.) includes more than 20 species with morphological variations, a wide genetic variety and nutrient qualities that are beneficial to human health. The properties of plum fruit cultivars that are influenced by their genetic profile can be used for effective evaluation. In order to identify chemical constituents and genetic relations among ten plum cultivars grown in Egypt, studies were carried out on the chlorophyll in leaves, some physiological characteristics of fruits, chloroplast (*cpDNA*) and mitochondrial DNA (*mtDNA*). Significant differences were observed in chlorophyll a, chlorophyll b and carotenoids in plum leaves, as well as total sugar, total soluble solids (TSS), pH and titratable acidity (TA) among plum cultivars. Five *cpDNA* regions (*trnL*, *trnL-trnF*, *trnH-psbA*, *matK* and *rbcL*) and two *mtDNA* regions (*nad4* and *nad9*) were amplified. Among the loci, the PCR success rate varied greatly; the locus *nad4* in the mitochondria had the greatest success rate 100 % followed by the chloroplast loci *rbcL* and *matK* 60%, then (*trnL*, *trnL-trnF*, *trnH-psbA* and *nad9*) with a success amplified rate of 50%. This study supports the applicability of the *cpDNA* and *mtDNA* markers to detect and identify the cytoplasmic variation in plum trees. The study will contribute to the knowledge about *Prunus salicina* cultivars and will be helpful in expanding the plum gene pool, which can be utilized in future plant breeding programs for the improvement of existing plum cultivars.

Keywords: Plum, Fruit quality, Genetic diversity, *cpDNA*, *mtDNA*.

1. Introduction

Plums (*P. domestica* and *P. salicina*) have been cultivated for over 2000 years all over the world. It has colorful fruits that can be eaten in both fresh and processed versions. The hexaploid European plum (*Prunus domestica* L.) and the diploid Japanese plum (*Prunus salicina* L.) have the most significant commercial varieties. Plums belonging to the subgenus *Prunophora* are considered important for *Prunus* evolution because they contain more than 20 species with wide range of morphological variations [1]. All irrigated regions of the world, including Egypt, are suitable for cultivating plums. In Egypt, the overall area under cultivation

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for plums (mainly European cultivars) recorded an increase from 1056 hectares in 2016 to 1373 hectares in 2020 and the whole annual production raised from 12187 tonnes in 2016 to 17864 tonnes in 2020. Egypt ranks 42nd in terms of plum output in 2020 [2].

Furthermore, cultivars that have been introduced and are known for their fruit quality primarily represent commercial production [3]. Most commercially important cultivars release little amounts of volatile scent compounds [4], so sweetness and sourness contribute most to plum flavors [5] and consumer acceptance [6]. Most genomic studies has increased to characterize and analyze genetic diversity and the preservation of fruit species germplasm resources using morphological traits and molecular markers [7, 8].

The nuclear DNA is typically utilized for evaluating the variation and relationships of different species. Early diversity analysis used RAPD markers [9] also, SSR markers produced in Japanese plum [10] and other *Prunus* species [11]. It is possible to estimate genetic relationships because Japanese plum cultivars exhibit significant levels of variety.

Additionally, there are two uniparental inherited cytoplasmic genomes called the chloroplast (*cp*) and mitochondrial (*mt*) genomes. As a result, they provide a potential supply of markers for evolutionary and ecological studies [12]. The genome of *Prunus* species was remarkably conserved throughout the evolutionary process, based on the genetic relationship among both chloroplast and mitochondrial DNA. These results are critical for assessing the diversity of chloroplast and mitochondrial genomes in different *Prunus* species and genotypes to give in-depth understanding of the organelle DNA [13].

The chloroplast genome is highly conserved throughout all plant species due to the absence of recombination. Several sequences will act as "repetitive" DNA in whole DNA preparations because the *cpDNA* in the cell has a high copy number and makes up between 10% and 15% of the total DNA [14]. Chloroplast DNA is commonly used for molecular identification today. For instance, three DNA barcodes, such as *matK*, *rbcL*, and the *trnL-trnF* intergenic spacer have been used to molecularly identify plants [15, 16, 17, 18, 19].

The ribulose bisphosphate carboxylase large chains and maturase K are respectively encoded by the *rbcL* and *matK* sequences, however the *trnL-trnF* intergenic spacer does not encode any protein with solely functions as a spacer across two genes, such as the *trnL* and *trnF* genes. The *trnL-trnF* intergenic spacer has a higher level of variation in plants than *matK* and *rbcL*, making it easier to distinguish between different plant species [15]. Characterization of the cytoplasm in plants depends on the assessment of chloroplast DNA (*cpDNA*) diversity.

Studies on *cpDNA* diversity are also essential for population genetics and phylogeographic analysis of rare, endemic and endangered varieties [20]. Additionally,

cpDNA's conserved structure and low nucleotide replacement rate are crucial for phylogenetic investigations [21]. In addition to being the main site of ATP generation in plants, mitochondria also play a significant role in nitrogen uptake, amino acid production, plant growth, yield, fertility and resistance to disease [12].

Compared to chloroplast DNA, mitochondrial DNA has undergone less evolutionary conservation throughout the plant kingdom. However, *mtDNA* gene sequences within a species only sometimes undergo point mutations. This is most likely caused by the fact that DNA recombination process exists in plant mitochondria, which enables mutant copy correction. In both angiosperms and gymnosperms, mitochondria primarily display maternal inheritance, while there are a few rare cases of paternal inheritance [22].

This DNA also behaves as 'repetitive' DNA component in total DNA preparations. Mitochondrial genes are a potential alternative because they typically do not duplicate and evolve generally faster than nuclear genomes [23, 24, 25]. In contrast, genes encoding for some conserved protein genes may be simple to align and protected enough to infer ancient phylogenetic relationships [23]. Nicotinamide adenine dinucleotide dehydrogenase (NADH) has been hypothesized to be the most conserved group of *Amoebozoa* mitochondrial genes [26].

The present study used ten plum cultivars grown in Egypt to be characterized based on their physiological traits and organelle genetics to evaluate biodiversity among the various cultivars. The study will contribute to the knowledge about the *Prunus salicina* cultivars and will be helpful in expanding the plum gene pool, which can be utilized in future plant breeding programs for the improvement of existing plum cultivars.

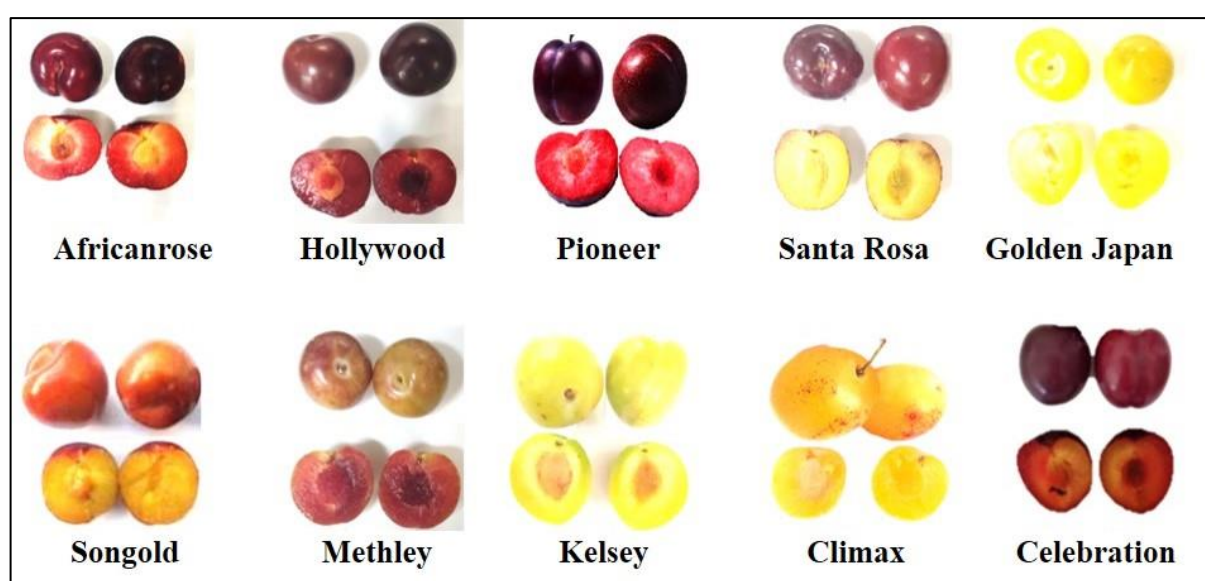
2. Materials and methods

2.1 Sample collection

Ten plum cultivars (*Prunus salicina* L.) cultivated in Egypt were collected from El-Kanater El-Khairia, Agriculture Research Center, Egypt and from private farms located in El-Menofia governorate and Alexandria Desert Road, Egypt (**Table 1, Fig. 1**). For fruit sampling, fruits of same size and same growth days for each cultivar that were free from any type of diseases, pests or mechanical damage were selected. **Figure 1** showed the variability in fruit characteristics of plum cultivars such as fruit shape, skin and flesh color.

Table (1): List of plum (*Prunus salicina* L.) cultivars analyzed.

No.	Accessions	Origin	No.	Accessions	Origin
1	<i>Africanrose</i>	ARC Infruitec, South Africa [27, 28].	2	<i>Hollywood</i>	Brooks of Modesto, California [29]
3	<i>Pioneer</i>	ARC Infruitec, South Africa [27, 30]	4	<i>Santa Rosa</i>	Burbank, USA [27, 31]
5	<i>Golden Japan</i>	Imported from Japan [27, 31]	6	<i>Songold</i>	ARC Infruitec, South Africa [27]
7	<i>Methley</i>	Burbank, USA [27, 31]	8	<i>Kelsey</i>	Imported from Japan [27]
9	<i>Climax</i>	Burbank, USA	10	<i>Celebration</i>	South Africa [[30, 32]

**Figure (1):** Variability in the phenotypic feature of ten plum cultivars' fruit showing fruit form, skin and flesh color.

2.2.1 Photosynthetic pigments

Using the **Lichtenthaler and Buschmann [33]** technique, the amounts of carotenoid and chlorophyll a and b were measured in fresh leaves. Fresh tissue was extracted in 80% ethanol. Using a spectrophotometer, the optical density of the solution was measured at 664, 649 and 470 nm for chlorophyll a, b and carotenoids.

2.2.2. Fruit quality analysis.

Total sugar concentration was estimated as the protocol of **Miller [34]**. Fruit samples (one gram) were extracted in five ml of 80% ethanol, followed by centrifugation at 2,000 g. Then 0.5 ml of 1% 3, 5-dinitrosalicylic acid solution was added to the collected supernatants, then mixture was incubated for five minutes in a boiling water bath. The mixture's absorbance was measured at 515 nm colorimetrically by V-630 spectrophotometer.

Total soluble solids (TSS) concentration, titratable acidity (TA) and pH value were evaluated by filtering the juice from a collective sample of three to five fruits. Digital pocket Refractometer (model PAL-3; Atago, Bellevue, WA) was used to determine their (TSS) concentrations.

Titrateable acidity (TA) was estimated by a manual burette titration of fruit juice using 5 mL of the prepared juice sample. The manually burette technique uses standard (0.1 N) sodium hydroxide and phenolphthalein as an indicator. Since malic acid (MA) is the main acid contained in plums, titratable acidity was reported as a percentage of MA [35]. The following equation was used to determine the percent MA:

$$\text{Percent acid} = (\text{milliliters NaOH}) \times (0.1 \text{ NaOH}) \times (0.067) \times (100) / \text{milliliters of fruit juice}$$

Ascorbic acid (vitamin c) was estimated by the technique of **Mau et al.** [36]. One gram of fruit flesh was grinded in 6% oxalic acid and then filtered. Ten ml of filtrate was mixed with 45ml of 3% oxalic acid solution. Ten ml of this filtrate was taken in 50 ml conical flasks and titrated with 2-6 dichlorophenol indophenol (DCPIP) dye and the amount of ascorbic acid was calculated:

$$\text{mg/100 gm sample} = (\text{milliliters DCPIP} \times 0.085 \times \text{Total volume (55)} \times 100) / (\text{wt.} \times \text{volume taken (10)})$$

2.3. Molecular analysis

Molecular studies were completed at the Laboratory of Biotechnology Research, Horticulture Research Institute, ARC, Egypt, throughout the period of 2019–2021.

2.3.1. DNA isolation

Young, fresh leaves of adult ten different plum cultivars trees are chosen, ground in liquid nitrogen then frozen at -20 °C for DNA extraction and amplification. Total genomic DNA was isolated from 100 mg of leaf tissues using cetyltrimethylammonium bromide (CTAB) method with some modifications [37]. The samples were examined using 1.8% agarose gel electrophoresis in 1X TBE (10 mM Tris-base, pH 8; 2.75 g/L boric acid; 1 mM EDTA, pH 8), stained with 0.5 mg/ml ethidium bromide, then seen under UV light. Using a 1 kb DNA ladder, the approximate size of the amplified fragments was calculated. The *mtDNA* was obtained using the methodology described by **Nunzia et al.** [38], with slight modifications according to **Said** [39] method. About 0.5 g was added to the homogenization buffer [100 mM Tris-HCl (pH 8.0), 1.5 M NaCl, 25 mM EDTA (pH 8.0), 0.2% BSA, and 56 mM β-mercaptoethanol] and centrifuged to pellet the mitochondria. To eliminate the nuclear DNA, resuspend the pellet in a cold DNase I buffer. Mitochondria were pelleted by centrifugation at

12,000 g for 15 min, then lysed in buffer containing 5% SDS, 50 mM Tris-HCl, pH 8 and 25 mM EDTA-HCl, pH 8. Add Proteinase K to a final concentration of 25 µg/ml and incubate at 37°C for 60 min with gentle shaking. After the addition of 0.1 volume of 2 M ammonium acetate, the nucleic acids were extracted with an equal volume of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and saturated phenol/chloroform (1:1). The supernatant was precipitated at -20°C for 1 h with 2 volumes of cold 100% ethanol and centrifuged at 15,000 g for 15 min, then washed and air dried. The *mtDNA* was suspended in TE buffer and stored at -20°C until further use. Samples were analyzed using 1.5% agarose gel electrophoreses in a 1X TBE and visualized under UV light after staining with 0.5 mg/ml ethidium bromide.

Table (2): The *cpDNA*, *mtDNA* primer sequences and annealing temperature (T_a °C) used for PCR-amplification for ten plum cultivars.

Marker	Primers	Sequence 5'-3'	T (°C)	Size Range (bp)	References
Chloroplast	C	CGAAATCGGTAGACGCTACG	56	607	[40]
	D	GGGGATAGAGGGACTTGAAC			
	E	GGTTCAAGTCCCTCTATCCC	56	407-429	[40]
	F	ATTTGAACTGGTGACACGAG			
	<i>trnH-psbA</i> F	CGCGCATGGTGGATTCAACAATCC	54	370-483	[41]
	<i>trnH-psbA</i> R	GTTATGCATGAACGTAATGCTC			
	<i>rbcL</i> F	ATTACTTGAATGCGACTGCG	54	551-662	[41]
	<i>rbcL</i> R	GCCAAACATGAATACCACCT			
	<i>matK</i> F	CGTACAGTCCATCTGGAAATCTTGGTTC	60	835	[42]
	<i>matK</i> R	ACCCAGTCCATCTGGAAATCTTGGTTC			
Mitochondria	<i>nad4</i> F	CAGTGGGTTGGTCTGGTATG'	60	117	[43]
	<i>nad4</i> R	TCATATGGGCTACTGAGGAG'			
	<i>nad9</i> F	AGTAAGTCTATTTCCATCAGCCG'	56	140	[43]
	<i>nad9</i> R	CACTCAGAGGAAGGTCTTTTCG			

2.3.2. Polymerase Chain Reaction (PCR)

The PCR amplification reactions 25 µl contained 20 ng template DNA, 1X PCR buffer, 2.5 mM MgCl₂, 1 µM of each primer (**Table 2**), 200 M dNTP mix and 1.0 U of Taq DNA polymerase (Promega Corporation). However, PCR amplifications were carried out using the Techni (TC-512) PCR thermo-cycle, which consisted of an Initial denaturation at 95°C for 4 minutes, followed by 35 cycles of amplification with denaturation at 94°C for 1 minute,

annealing at 54–60°C for 2 minutes, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes. The amplified products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.5 g/ml) using 1X TBE buffer at 100 V for an hour. The amplified products were synthesized using *cpDNA* and *mtDNA* primers. The gel documentation device was used to visualize and photograph the DNA fragments under UV illumination.

2.4. Statistical analysis

The statistical analysis was performed among the different cultivars using statistical package for the social sciences (SPSS) for Windows ver. 20.0. Means and standard deviations were calculated for the studied parameters. The differences among different cultivars were treated statistically using one way analysis of variance (ANOVA-1) and the means were compared using Duncan test [44]. The distance matrix was shown as a phonogram using the unweighted Pair Group Method with Arithmetic Averages (UPGMA), employing the SAHN (Sequential, Agglomerative, Hierarchical and Nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 (Applied Biostatistics) program [45].

3. Results and discussion

3.1. Biochemical analysis

3.1.1. Photosynthetic pigment content:

The variation in photosynthetic pigment content in plum cultivars' leaves was shown in (Table 3). The contents of chlorophyll a, b and carotenoids were significantly differ in plum cultivar leaves, with a range from 0.620 in Africanrose to 0.175 in Pioneer for chlorophyll a, 0.134 in Africanrose to 0.048 in Kelsey for chlorophyll b and from 0.017 in Celebration to 0.002 in Pioneer for carotenoids content. Total chlorophyll was significantly different among cultivars where the higher content was found in Africanrose but the lower was found in Celebration, Pioneer and Kelsey respectively. Plant pigments have a great importance in both plant and human biology. . In plants, pigments play important roles in photoprotection (carotenoids), photosynthesis (chlorophylls) and defense against several abiotic stresses (anthocyanins) [46]. The breakdown of the chloroplast composition and the destruction of the protein complexes of pigment may have contributed to the decline in photosynthetic pigments [47]. The total chlorophyll content (Chla+Chlb) in the plum leaves was varied to different rootstock and different seasons [48]. **Murtić *et al.* [49]** investigated that these differences in chlorophyll content in leaves between cultivars can occur for several different reasons, one of these reasons is the effect of genetic potential of a particular cultivar.

Table (3):- Mean performance of photosynthetic pigments of ten plum cultivars studied.

Cultivars	Chlorophyll a	Chlorophyll b	Total chl.	Carotenoids
<i>Africanrose</i>	0.620±0.030a	0.134±0.006a	0.75±0.04a	0.011±0.004bc
<i>Hollywood</i>	0.310±0.020ef	0.084±0.004b	0.39±0.02e	0.005±0.002de
<i>Pioneer</i>	0.175±0.005g	0.059±0.004cd	0.23±0.01g	0.002±0.001e
<i>Santa Rosa</i>	0.550±0.050b	0.120±0.010a	0.67±0.06b	0.008±0.003cd
<i>Golden Japan</i>	0.410±0.020d	0.096±0.006b	0.51±0.03d	0.007±0.002cd
<i>Songold</i>	0.280±0.030f	0.053±0.003cd	0.33±0.03f	0.006±0.002de
<i>Methley</i>	0.325±0.005e	0.067±0.007c	0.39±0.01e	0.008±0.002cd
<i>Kelsey</i>	0.320±0.020ef	0.048±0.003d	0.37±0.02ef	0.011±0.001bc
<i>Climax</i>	0.455±0.005c	0.085±0.005b	0.54±0.01cd	0.013±0.003ab
<i>Celebration</i>	0.460±0.010c	0.090±0.020b	0.55±0.03c	0.017±0.002a
F-Value	95.079***	34.226***	148.72***	9.976***

Means ±SD, n=3 Means with the same letters in the same column are not significant different (Duncan's test).

***: $P < 0.001$

3.1.2 Fruit quality analysis.

3.1.2.1. Total soluble Sugar

Total soluble sugar content is thought to be a key indicator of fruit quality [50]. The sugar content of plum varieties (**Fig. 2A**) varied significantly, ranging from 33.56 % in Golden Japan cultivar to 13.67 % in Kelsey cultivar, respectively. The hierarchy was as follows: Golden Japan > Santa Rosa > Hollywood > Pioneer > Celebration > Climax > Methley > Songold > Africanrose > Kelsey. Many mature fruits, like plums, have an abundance of soluble sugars in their flesh and the amount of sugar to organic acid in a fruit's flesh greatly influences how it tastes [51]. The sugar content of plums can alter significantly as they develop because they are climacteric fruits as reported by Minas *et al.* [52]. Fruit quality depends on sugars since they immediately affect tasting, which influences customer acceptability [53]. The primary sugars found in the flesh of ripe plums are in soluble form [54, 55]. However, there is a lot of diversity amongst plum cultivars in terms of the total amount of soluble carbohydrates per gram of fresh weight of the flesh [56, 57]. Rezica *et al.* [58] reported that sugar content ranges from 10.19 % to 11.89 % in cultivars, which is less than the values that were observed. The amount also exceeds that which was previously reported by Dugalic *et al.* [59] in plum fruits as 11.1% to 16.7%. Fruit ripening causes complex carbohydrates to break down into sugars, decreases fruit hardness, changes in colour and titratable acidity while increasing flavour and scent [60]. The mechanisms of sugar buildup and the level of sugar metabolism enzyme activity in different cultivars can be attributed to regulated hormonal responses [61].

Significant differences in sugar concentration among cultivars may have been caused by genetic or phenotypic characteristics, as well as maturity/ripening stage [62]. In general, cultivars with high total sugar content were chosen for further breeding to provide customers with plums of superior nutritional value [63].

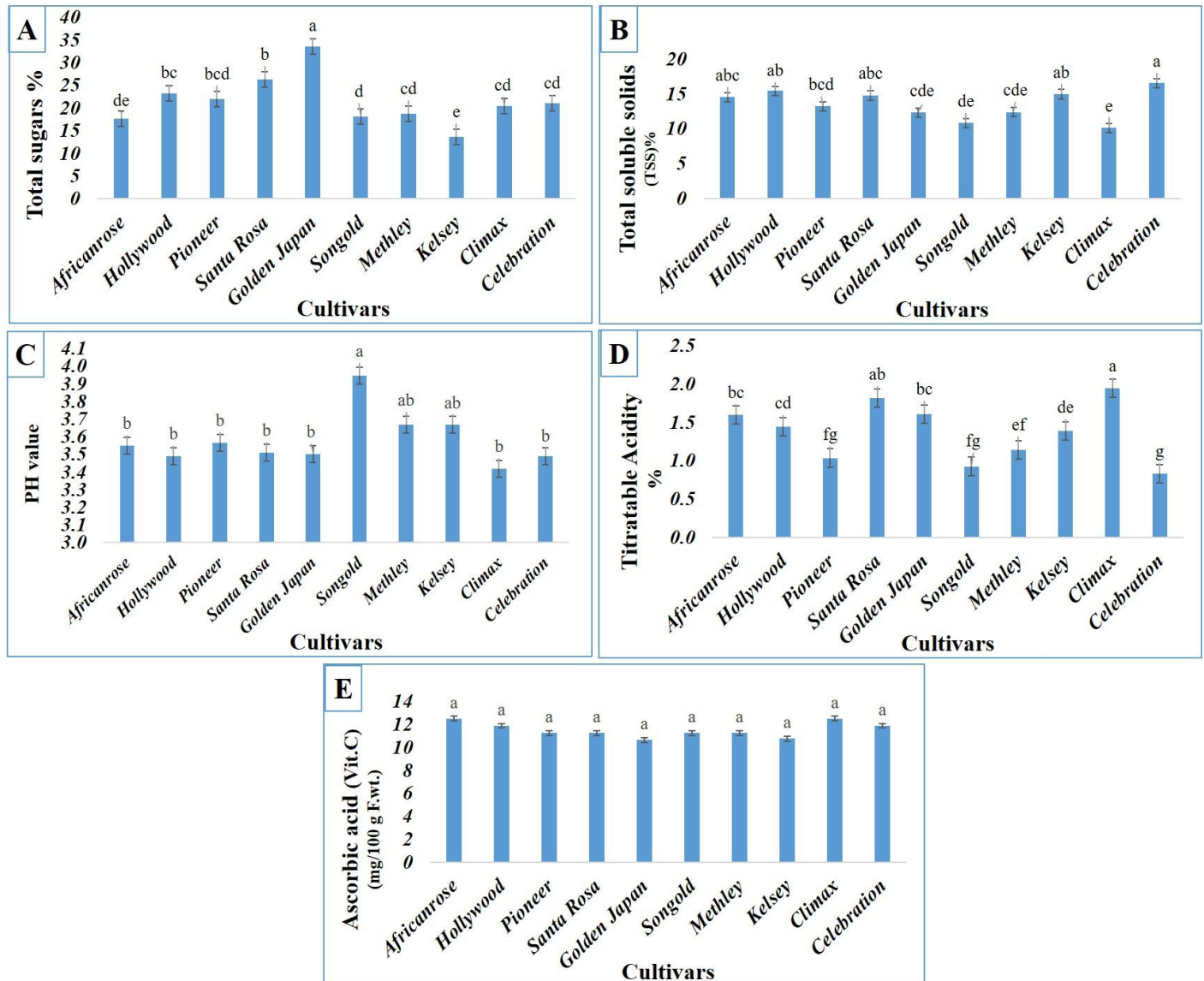


Figure (2): Mean performance of fruit quality characteristics of ten plum cultivars; **A-** Total sugars%, **B-** Total soluble solids%, **C-** pH value, **D-** Titratable acidity% and **E-** Ascorbic acid (Vit.C) (mg100/ g f.wt)

3.1.2.2. Total soluble solids (TSS)

Total soluble solids content of different plum cultivars are showed in (**Fig. 2B**). Celebration cultivar showed the greatest TSS value (16.53 %), while the lowest value (10.13 %) was seen in Climax cultivar. The order of the hierarchy of total soluble solid was Celebration> Hollywood> Kelsey> Santa Rosa> Africanrose> Pioneer> Methley> Golden Japan> Songold> Climax. Numerous researches have revealed the genotypic heterogeneity in

the total soluble solids concentration in various cultivars (**Patel et al.** [64] on cherry; **Kaur et al.** [65] on guava). The variance may result from the morphological and genetic makeup of some cultivars, which may have required food consumption and increased carbohydrate absorption into the fruit, leading to the production of larger fruits with greater TSS [66]. The present result was in agreement with **Qiu et al.** [67] who reported that TSS ranged from 9.63% to 14.30% in different plum cultivars and **Zeulov et al.** [68] who found a range of 12.5% to 24.5% in different plum cultivars with different rootstocks. The values reported in this work were quite similar to those reported by **Hassan et al.** [28] in different plum cultivars. The previous reports of **Kumar et al.** [69] observed higher TSS content (21.1%) in different plum cultivars. Where, **Saridas et al.** [61] stated that TSS varied between plum cultivars but declined with advanced maturation.

3.1.2.3. Titratable acidity (TA) and pH

Total acid content (TA) was significantly varied between plum cultivars (**Fig. 2D**). Generally, it ranged from 0.83 to 1.94 where Climax, Santa Rosa and Africanrose cultivar have higher amount of (TA) but Celebration, Songold and Pioneer cultivars have lower amounts of TA, respectively. All cultivars were found to have slightly high pH values (**Fig. 2C**). Regardless of the cultivar, no significant difference between all cultivars which ranged from 3.42 to 3.95. The genotypic variability in TA concentration in diverse plum cultivars, which is a hereditary characteristic of each variety, has also been demonstrated by numerous researchers, **Gündüz and Saraçoğlu** [70] stated a significant difference in TA between accessions and cultivars of plum (*Prunus cerasifera*). **Zeulová et al.** [68] reported that TA ranged from 0.53 to 2.37 in different plum cultivars with different rootstocks. The outcomes are consistent with earlier observations by **Kumar et al.** [69], who studied a cultivar of plum and found a reduced TA content (0.69%). Higher acidity was found in a variety, which may be related to the variety's genetics and environmental factors that favor an increase in acidity content [69]. With the low TA level, all cultivars were found to have slightly high pH values (**Fig. 2C**). Regardless of the cultivar, there was no significant difference between all cultivars which ranged from 3.42 to 3.95. These results in agreement with those obtained by **Kitzberger et al.** [71] who find that TA ranged from 0.62 to 2.47 % and pH ranged from 2.83 to 4.02.

3.1.2.4. Ascorbic acid (vitamin C)

In this study, Africanrose and Climax fruit had the highest ascorbic acid (AA) content (12.50 mg/100 g FW), while Golden Japan fruit had the lowest (10.63 mg/100 g FW) (**Fig. 2E**) but there is no significant difference between ascorbic acid content for plum cultivars. One of the most vital substances for the human body is ascorbic acid, also known as vitamin C. Compounds containing antioxidant properties, such as vitamin C which is an essential fruit

component in terms of nutrition [72]. Ascorbic acid was present in significant levels in the majority of the fruits [73]. The results disagreed with YU *et al.* [74] who found that the ascorbic acid levels in the 13 selected cultivars varied significantly, demonstrating that plum cultivars cultivated in subtropical climates have a higher ascorbic acid content than those grown in other regions and Gündoğdu *et al.* [75] who found a significant variance in the amount of ascorbic acid in different apricot cultivars. In several crops, such the Japanese plum [76] and the apricot [77], an increase in ascorbic acid was noted as the fruit ripened.

3.2. Molecular analysis

3.2.1. Amplification of chloroplast DNA (*cpDNA*)

Polymorphic coding and noncoding regions of the chloroplast DNA were investigated in ten cultivars of *Prunus salicina* cultivated in Egypt. Primarily, *cpDNA* was amplified using five sets of universal primers. The noncoding *trnL-trnF* region was successfully amplified in ten plum cultivars. Two primer combinations were utilized separately since there was sequencing difficulty in the entire *trnL-trnF* region. The (C+D) primers amplified the *trnL* intron, whereas (E+F) primers amplified the partial *trnL* gene and *trnL-trnF* regions. (Fig. 3).

The PCR amplification of the (C+D) was successfully obtained for (Santa Rosa, Songold, Methley, Kelsey and Climax) cultivars, but it was not successful for (Africanrose, Hollywood, Pioneer, Golden Japan and Celebration) cultivars. The amplified *trnL* gene's length was 607 bp. Further, the partial *trnL* gene and *trnL-trnF* regions, were amplified by PCR using the (E+F) primer and were successfully obtained for cultivars (Golden Japan, Methley, Kelsey, Climax and Celebration). Meanwhile, amplification was not successful for (Africanrose, Hollywood, Pioneer, Santa Rosa and Songold) cultivars. The length of the amplified fragment was ranged from 407-429 bp. The present PCR tests revealed that some of the study plum cultivars had success amplifying the non-coding *trnL-trnF* region, whereas other cultivars had less success. It has been observed that specific intron deletions have occurred in other chloroplast genes [78, 79]. There is already evidence of group I and group II intron loss in plants, and mammals [80, 81]. Intron loss may also result from in-frame intron deletion and simple genomic deletion [82]. The *trnH-psbA* intergenic region was successfully amplified by PCR for (Africanrose, Hollywood, Kelsey, Climax and Celebration) cultivars. On the other hand, the amplification was not successful for (Pioneer, Santa Rosa, Golden Japan, Songold and Methley) plum cultivars. The samples contained a variety of PCR products, ranging from 370-483 bp. (Fig. 3). Nucleotide alterations as single-nucleotide substitutions may be the cause of the failure of PCR amplification of the *trnH-psbA* intergenic region for some cultivars [83]. Amplification of *matK* coding region was successful in all plum cultivars except (Pioneer, Methley, Kelsey and Climax) at length of 835 bp, where amplification of *rbcL* coding region

was successful in all cultivars except (Hollywood, Pioneer, Songold and Methley) cultivars ranged from 551-662 bp length (**Fig. 3**).

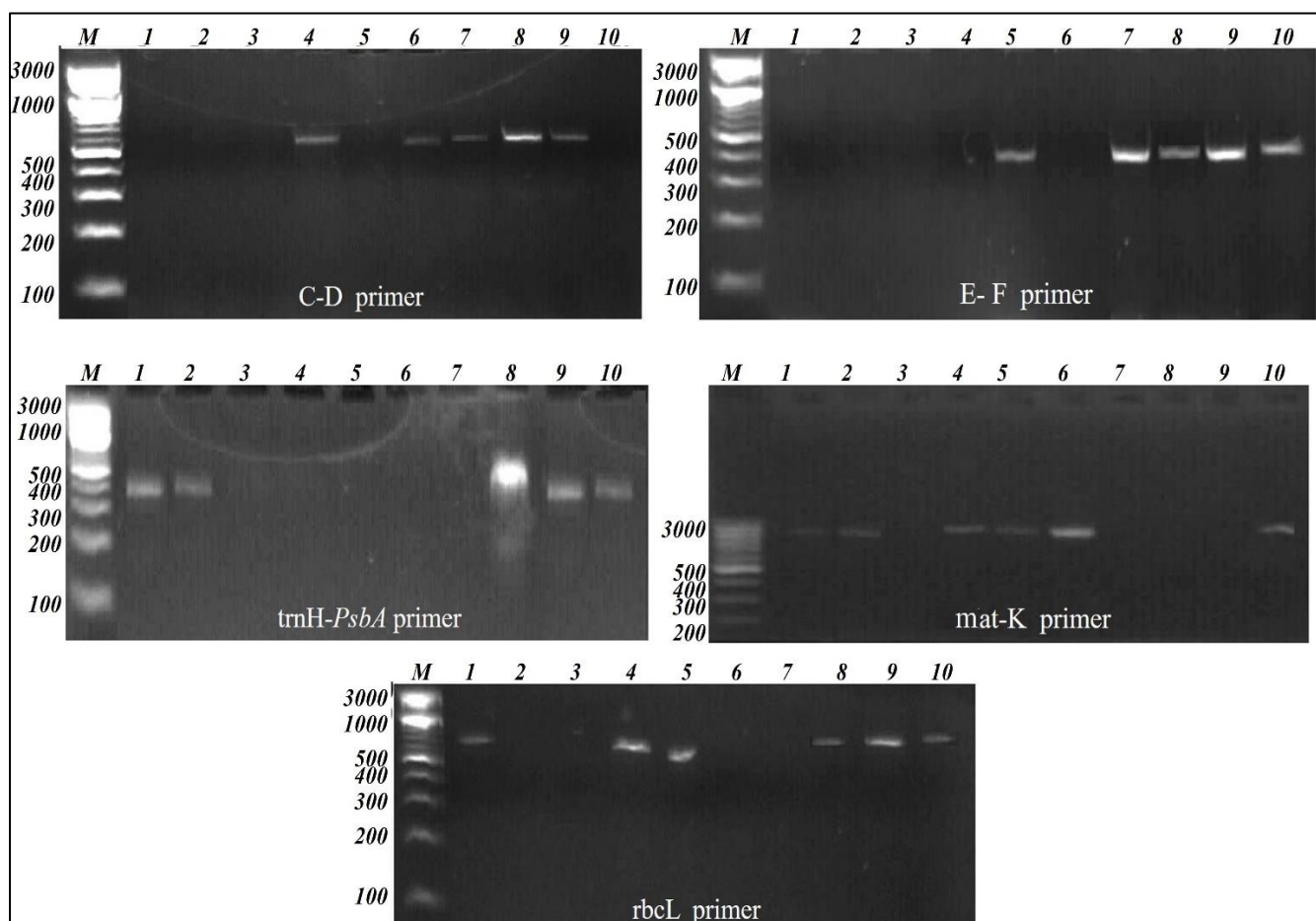


Figure (3): Agarose gel showing PCR product of chloroplast noncoding *trnL*- *trnF* region (C-D) and (E-F) primers, *trnH-psbA* intergenic region and coding *matK*, *rbcL*, regions for ten plum cultivars. Lan1: Africanrose, 2: Hollywood, 3: Pioneer, 4: Santa Rosa, 5: Golden Japan, 6: Songold, 7: Methley, 8: Kelsey, 9: Climax, 10: Celebration.

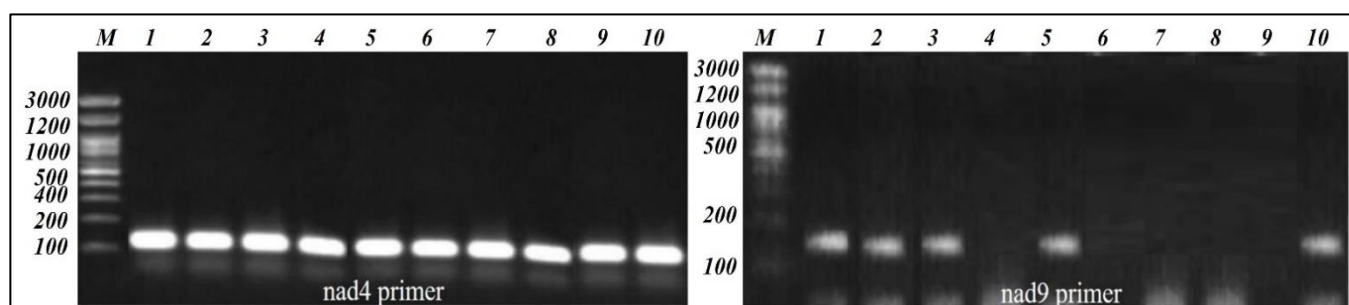


Figure (4): Agarose gel showing PCR product of mitochondrial coding *nad4* and *nad9* regions for ten plum cultivars. Lan1: Africanrose, 2: Hollywood, 3: Pioneer, 4: Santa Rosa, 5: Golden Japan, 6: Songold, 7: Methley, 8: Kelsey, 9: Climax, 10: Celebration.

3.2.2. Amplification of Mitochondrial DNA (*mtDNA*)

Amplification of *nad4* coding region was successful in all plum cultivars at range length of 117 bp, whereas amplification of *nad9* coding region was successful in all cultivars except (Santa Rosa, Songold, Methley, Kelsey and Climax) plum cultivars at a range length of 140 bp (**Fig. 4**).

All ten plum cultivars' DNA extracts were used in the PCR process. Among the loci, the PCR success rate varied greatly; the locus *nad4* in the mitochondria had the greatest success rate, at 100%. The following-best PCR success rate was demonstrated in chloroplast loci *rbcL* and *matK* at (60%) and success amplified rate at (50%) with other loci (*trn L*, *trnF*, *trnH*- *psbA* and *nad9*) as dictated in (**Fig. 5**).

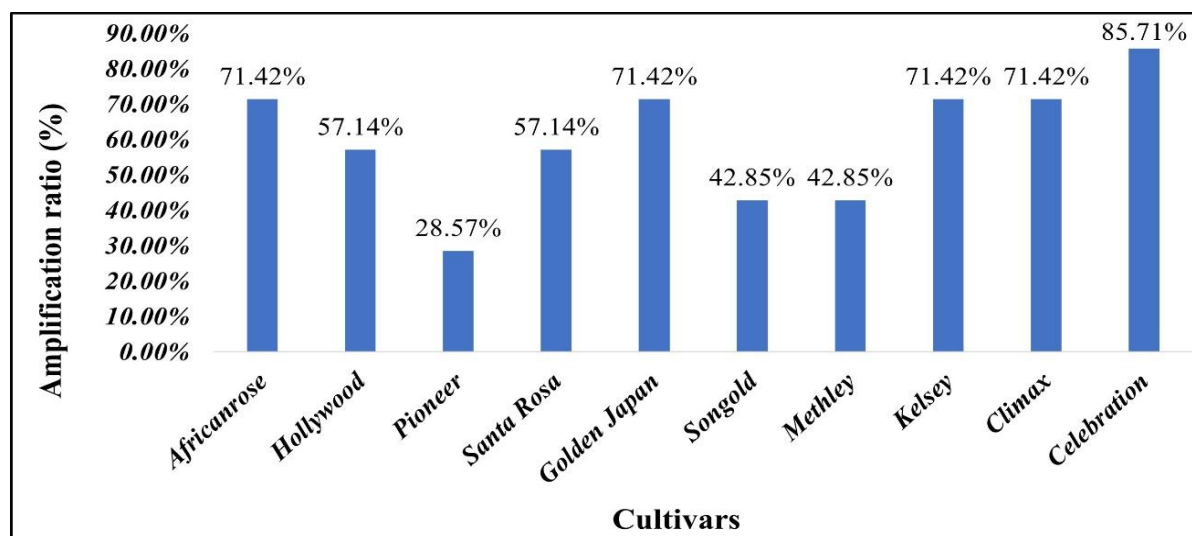


Figure (5): Cultivar amplification ratio against *cpDNA* and *mtDNA* primers.

Mitochondria are abundant in cells so most taxa inherit via a maternal line of descent, *mtDNA* is a great and helpful marker for rebuilding the systematic evaluation and phylogenetics of species since evolution happens at a relatively faster rate [84]. Numerous studies using various *mtDNA* genes have investigated the genetic variance of plum cultivars, such as **Mustapha et al.** [85], which exhibit little intra-specific sequencing variation. In the current study, we compared the genetic variations in the *mtDNA*, *nad4* and *nad9* genes in the current study. The *nad9* gene has higher genetic variation than the *nad4* gene, indicating that it is more variable and valuable for researching genetic diversity in the *Prunus salicina* L species. Our results showed the importance of chloroplast markers in detecting polymorphisms because *mtDNA* shows less variation than *cpDNA*. These results are in agreement with those obtained for Tunisian plum cultivars [85]. **Palmer** [86] hypothesized that the slower rate of nucleotide substitutions in *mtDNA* relative to *cpDNA* may be the cause of the low variety of *mtDNA*. The fact that the evolutionary changes in *mtDNA* differ from those in *cpDNA* shows

that the mitochondrial genome is conservative, and that the development of mitochondrial organelle genomes proceeds slowly. This strongly suggests that the chloroplast genome is highly diverse. The regulated presence or absence of a few restriction sequences is definitely consistent with insertions or deletions [13].

In addition to an investigation of genetic diversity through the classification of variations, molecular systematic information offers a richer understanding of genetic structure [87]. The genetic variability of *Prunus* species has been investigated in earlier studies utilizing RAPD [88], SSR technique [89], ISSR [90], *cpDNA* (*trnL-trnF*, *atpB-rbcL* and *rps16-trnQ*) regions [91], internal transcribed spacer (*ITS*) region [92] and *cpDNA trnL-F* region [93]. Moon *et al.* [94] show that polymorphisms in chloroplast DNA (*cpDNA*) have better resolution levels than nuclear markers, making it a good alternative for comparative genomic information. Since non-coding regions of *cpDNA* are less restricted in their functionality than coding regions, it has been hypothesized that they will display higher variability levels in phylogenetic studies [95]. The *trnL-F* region of chloroplast DNA can be used to provide responses to questions about relationships among related genera and species [96]. Similar studies utilized the genomes and populations of important plant species, including olive (*Olea europaea*) [97], rice (*Oryza sativa*) [98] and apple (*Malus* L.) genotypes [99].

3.2.3. Phylogenetic analysis

The genetic variability of *Prunus* genotypes was investigated using chloroplast and mitochondrial markers. Phylogenetic relationships between ten plum cultivars were determined based on chemical characters using UPGMA computer program. Phylogenetic tree construction based on chemical characters classified the ten plum cultivars into two main clusters (**Fig. 6**). The first cluster included Golden Japan cultivar whereas the second cluster was separated into two sub-clusters; the first sub-cluster included Climax cultivar. The second sub-cluster was separated into two groups; one of them contained only Kelsey cultivar but the other group was divided into two major groups; one of them included Celebration and Africanrose cultivars while the other major group was divided into two minor groups; one of them included Santa Rosa cultivar where the other included Pioneer, Hollywood, Methley and Songold cultivars. The greatest distance was recorded between golden Japan and Songold cultivars, while the lowest one was between Songold and Methley. In addition, Climax, Kelsey, Celebration, Africanrose, Santa Rosa, Pioneer and Hollywood cultivars were located between them, respectively.

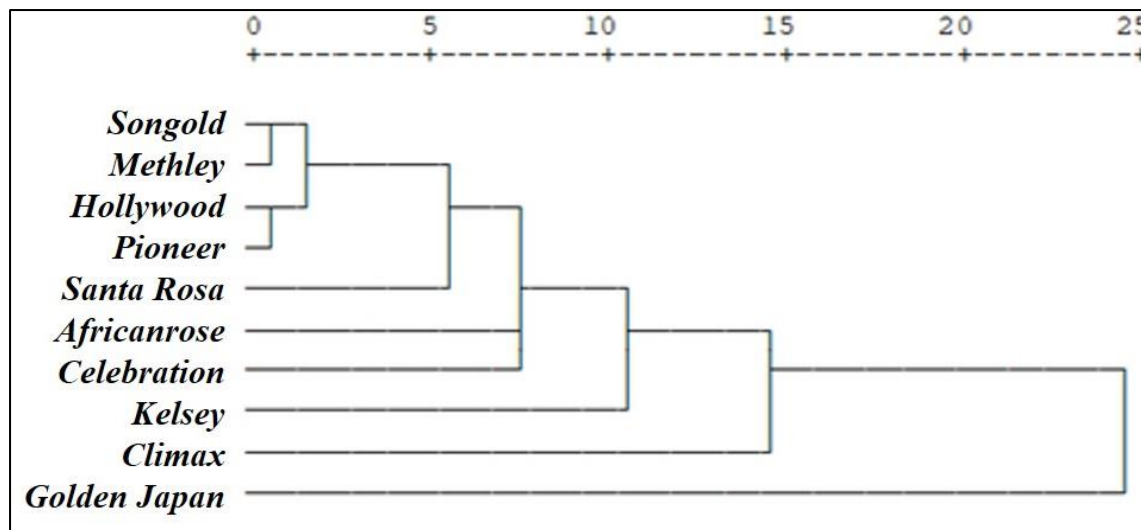


Figure (6): Phylogenetic tree performed from the ten plum cultivars tested using UPGMA computer program based on Biochemical analysis of fruit.

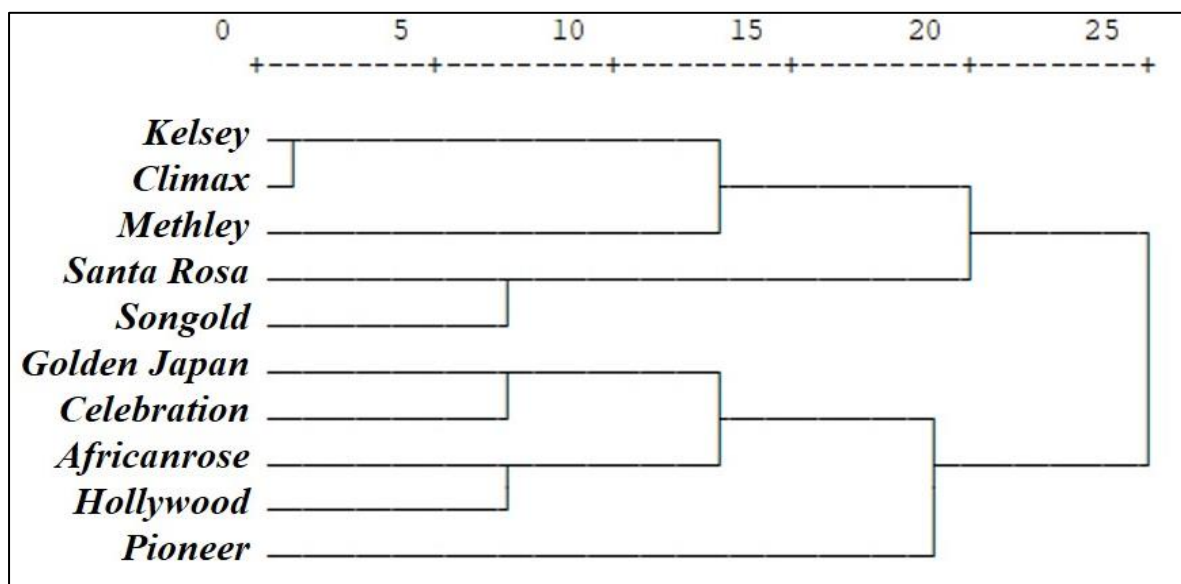


Figure (7): Phylogenetic tree performed from the ten plum cultivars tested using UPGMA computer program based on *cpDNA* and *mtDNA* primers analysis.

The genetic distances determined between the accessions ranged from 0.00 to 1.00; the Kelsey and Climax cultivars had the lowest distance, indicating that they have the most genetic similarities. A greater distance was observed between the Africanrose and the Methley cultivars, reflecting their significant differences. Phylogenetic tree construction based on chloroplast and mitochondrial markers classified the ten plum cultivars into two main clusters (**Fig. 7**). The first cluster was separated into two sub-clusters, one of them included Pioneer cultivar meanwhile the other sub-cluster was divided into two groups. The first group contained Golden Japan and Celebration, while the second group contained Hollywood and Africanrose cultivars. Two sub-clusters were produced from the second cluster; the first sub-cluster included Santa Rosa and Songgold cultivars and the second sub-cluster was separated into two

groups; one of them contained only Methley cultivar but the other group included Kelsey and Climax cultivars. The current findings were in agreement with **Mustapha et al.** [85] who used a phylogenetic tree to distinguish 23 cultivars of Tunisian plums in two major clusters. The first one included *Prunus* ‘Golden Japan’. The second cluster included *Prunus* cultivar Santa Rosa. Meanwhile **Guerrero et al.** [27] distinguished Africanrose, Kelsey, Santa Rosa and Methley in different groups.

Based on *cpDNA* and *mtDNA* primers polymorphisms, a proximity matrix was developed by UPGMA computer program and the analysis was based on the number of bands that were different between any given pair of cultivars (**Table 4**). The percentage of dissimilarity between the studied cultivars revealed that, the maximum value is 1.00 observed between Africanrose and Methley cultivars, whereas the minimum value is 0.00 observed between Kelsey and Climax cultivars. Organelle genomes, *cpDNA* and *mtDNA*, have been used for a long time as a source to explore the evolutionary relationships of species, mainly due to their uniparental inheritance pattern and rapidly changing gene content [100].

Table (4): Proximity matrix among the ten plum cultivars tested based on *cpDNA* and *mtDNA* primers analysis.

Cultivars	<i>Africanrose</i>	<i>Hollywood</i>	<i>Pioneer</i>	<i>Santa Rosa</i>	<i>Golden Japan</i>	<i>Songold</i>	<i>Methley</i>	<i>Kelsey</i>	<i>Climax</i>	<i>Celebration</i>
<i>Africanrose</i>	0.00									
<i>Hollywood</i>	0.167	0.00								
<i>Pioneer</i>	0.500	0.333	0.00							
<i>Santa Rosa</i>	0.500	0.667	0.667	0.00						
<i>Golden Japan</i>	0.333	0.500	0.500	0.500	0.00					
<i>Songold</i>	0.667	0.500	0.500	0.167	0.667	0.00				
<i>Methley</i>	1.00	0.833	0.500	0.500	0.667	0.333	0.00			
<i>Kelsey</i>	0.667	0.833	0.833	0.500	0.667	0.667	0.333	0.00		
<i>Climax</i>	0.667	0.833	0.833	0.500	0.667	0.667	0.333	0.00	0.00	
<i>Celebration</i>	0.167	0.333	0.667	0.667	0.167	0.833	0.833	0.500	0.500	0.00

It is evident that the clustering based on polymorphisms in the *cpDNA* and *mtDNA* primers differs from that based on biochemical markers. Whereas the morphology originated from both parents and influenced by environment, the chloroplast and mitochondria were mainly inherited by the female parent [101]. Chloroplast maternal inheritance can be used to monitor interspecific introgression events, although it may result in incorrect species relationships [102]. Some *cpDNA* sequences have evolved slowly and their significance in determining phylogenetic relationships among a few angiosperm species that are closely related, notably *Prunus*, has been reviewed by **Small et al.** [103] and **Shaw et al.** [104, 105]. Therefore, the biochemical marker clusters found in ten plum cultivars using *cpDNA* and *mtDNA* clustering analysis did not match those found in mango by **Kostermans and Bompard** [106]. The possibility of using the entire chloroplast genome sequences to comprehend structural and functional evolution and resolve phylogenetic relationships at various taxonomic levels has been documented in different reports [107, 108, 109].

Chloroplast DNA evolves at a slow rate, which helps scientists better understand the relationships between various species. Because this organelle can be transferred through interspecific hybridization, chloroplast-based phylogenies are constrained. Studying species relationships becomes challenging because *Prunus* has been known to naturally produce interspecific hybrids, which makes it difficult to identify species relationships using *cpDNA*-based phylogenies [110]. Up to 15 different diploid *Prunus* species have hybridized with the Japanese plum (*Prunus salicina* L.). Due to this high amount of introgression, the current crop of cultivars exhibits a wide range of traits and agronomic behaviours [27]. Identification of chloroplast regions that would give the most characters for low-level molecular phylogenetic studies was the main goal of the study by **Shaw et al.** [105]. The genetic diversity was similar to that of earlier researches such as [111, 112, 11, 113]. This might be a result of the extensive analysis of breeding programs' enhanced selections of modern cultivars, which reveals a bottleneck role played by breeding system techniques. Because of their interspecific origin, establishing genetic relationships among Japanese plum-type accessions is quite difficult, although the parentage lines of commercial cultivars can provide support. However, certain of the ancestors who are frequently employed in breeding operations don't have available genomes [114]. Next-generation sequencing technologies (NGS), high-density SNP-based genotyping, and the utilization of chloroplast markers (*cpDNA*) [115, 116], may provide further information about the degree of hybrid Japanese plum diversity and the reconstruction of each cultivar's pedigree.

The maintenance of the indigenous *Prunus* germplasm utilized in early plum breeding could contribute to preserving and enhancing the genetic variety in cultivars of Japanese plum-like trees. Unfortunately, breeders can only currently choose from a small assortment of this material [117]. The recognition of the genetic diversity among Japanese plum-type accessions can help breeders choose parents in a more informed manner, preserve biodiversity through the conservation of germplasm and identify genotype-phenotype association patterns that can be used by agricultural producers and in genetic research.

5. Conclusion

The current study characterized ten plum cultivars growing in Egypt based on physiological traits and organelle genetics to evaluate biodiversity among the cultivars. The present finding supports the applicability of *cpDNA* and *mtDNA* for plum cultivar identification and phylogenetic analysis. The study will contribute to knowledge about *Prunus salicina* cultivars and will be helpful in broadening the plum gene pool, which can be utilized in future plant breeding programs for the improvement of existing plum cultivars.

Declarations

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الملخص العربي

التعريف البيوكيميائي و الجزيئي لعشرة أصناف من البرقوق (*Prunus salicina* L.) في مصر استناداً إلى سماتها الفسيولوجية، وعلامات الحمض النووي للبلاستيدات الخضراء و للميتوكوندريا.

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يضم البرقوق (*Prunus salicina* L.) أكثر من ٢٠ نوعاً تتباين في الشكل الظاهري والتكوين الجيني وكذلك الخصائص الغذائية المفيدة لصحة الإنسان. ويمكن استخدام خصائص أصناف فاكهة البرقوق والتي تتأثر بملفها الوراثي لأغراض التقييم الفعال. وبهدف تحديد المكونات الكيميائية والعلاقات الوراثية بين عشرة أصناف من البرقوق المنزرعة في مصر، أجريت دراسات علي الكلوروفيل في الأوراق وبعض الخصائص الفسيولوجية للفواكه والحمض النووي لكل من البلاستيدات الخضراء (*cpDNA*) والميتوكوندريا (*mtDNA*).

أظهرت النتائج اختلافات كبيرة في كل من الكلوروفيل (أ)، والكلوروفيل (ب)، والكاروتينات في الأوراق، وكذلك محتوى الثمار من السكريات الكلية، والمواد الصلبة الذائبة الكلية (TSS)، ودرجة الحموضة بين أصناف البرقوق.

تم مضاعفة خمس مناطق من *cpDNA* (*trnL-trnF* و *trnL-trnF-psbA* و *trnH-psbA* و *matK* و *rbcL*) ومنطقتان من *mtDNA* (*nad4* و *nad9*). من بين هذه المواقع، تباين معدل نجاح PCR تبايناً كبيراً؛ وحقق الموقع *Nad4* في الميتوكوندريا أكبر معدل نجاح بنسبة ١٠٠٪ يليه موقع البلاستيدات الخضراء *rbcL* و *matK* بنسبة ٦٠٪، ثم (*trnL-trnF* و *trnH-psbA* و *nad9*) بمعدل نجاح بنسبة ٥٠٪.

تدعم هذه الدراسة إمكانية تطبيق علامات الحمض النووي لكلا من البلاستيدات الخضراء و الميتوكوندريا (*cpDNA* و *mtDNA*) لاكتشاف وتحديد التباين السيتوبلازمي في أشجار البرقوق. و ستسهم الدراسة في المعلومات المتعلقة بأصناف البرقوق *Prunus salicina* L. وكذلك التوسع في تجميع جينات البرقوق، والتي يمكن استخدامها في برامج تربية النباتات في المستقبل من أجل تحسين الأصناف الحالية.