Research Article

Karyotype Variations among Date Palm (*Phoenix dactylifera* L.) Cultivars of Sindh, Pakistan

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Abstract | Date palm (*Phoenix dactylifera* L.) is a monocot and dioecious plant species having uncertain diploidy levels because of scarcity of cytogenetic knowledge. Khairpur district in Sindh is known as the biodiversity center of date palm. Investigations were carried on the chromosome number and karyotype one of the seven indigenous commercial date palm varieties and four wild type date palm of Sindh province, Pakistan by using the traditional Fuelgen squash method. Results indicated that all seven commercial varieties and four wild types of date palm are diploid in nature and having 2n=36 chromosomes. First time detailed karyotype of these varieties have been developed which includes total chromosome length (TCL), relative length (RL) and centromeric index (CI). The chromosome complement consists of metacentric (m), sub-metacentric (sm), sub-telocentric (st) and telocentric (t) chromosomes and average length of chromosomes vary in all cultivars ranging from 0.99µm to 6.46µm. Results indicated that var. Wild03 have symmetric karyotype with 11m+4sm+3t. Wild03 was likely primitive karyotype as compared to var. Asul Khurmo which represented the advance karyotype by showing maximum number of telocentric chromosome.

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Introduction

Date palm (*Phoenix dactylifera* L.) is a major fruit crop belongs to genus *Phoenix* and *Arecaceae* (formerly Palmae) family. It is domesticated about 7000 years ago in or near Middle East (Flower et al., 2019). The family *Arecaceae* contains more than 200 genera and 2,280 species (Al-Antary et al., 2015; Al-Qurainy et al., 2018). Date palm is monocot and dioecious plant in nature (Barrow, 1998). The genus *Phoenix* is distributed from the Atlantic Islands throughout Mediterranean region, Africa, Middle East, and as far as Southern Asia and North-Western Pacific (Barrow, 1998; Henderson et al., 2006; Dransfield et al., 2008; Al-Antary et al., 2015). This is a green woody plant with long productive life cycle. It grows well under arid and semi-arid climatic conditions than other fruit crops (Lunde, 1978; Patankar et al., 2018; Alwahshi et al., 2019). It is the main fruit crop of Western Asia and North Africa situated between 24°N and 34°N (Zaid and Arias-Jimenez, 2002). Date palm is excellent among the fruits with a large number of cultivars throughout world (Fakir et al., 2018). Currently, date palm is

cultivated in more than 37 countries with almost 5000 varieties (Ali-Mohamed and Khamis, 2004; Akhtar et al., 2014). In Pakistan, more than 300 varieties have been reported and are being cultivated. All these varieties have wide range of genetic diversity regarding their morphological and biochemical fruit characteristics (Markhand et al., 2010).

Genome size of date palm is approximately 700Mb (Fakir et al., 2018). Chromosomal data of date palm is comparatively rarely published till the mid of 1980's (Johnson, 1985), however some progress was achieved later (Johnson and Brandham, 1997). However, some successful attempts were made in growing root tips with few members of *Arecaceae* family for chromosomal count. Very limited roots are produced by palm trees due to their slow growth. Cytogenetic studies in date palm are difficult because of small and numerous number of chromosomes (Johnson, 1985; Johnson and Brandham, 1997). Johnson and Raven (2001) reported 2n=4 and 2n=1262 chromosomes in Haplopappus and fungus *Penicillium* respectively.

Earlier, Nemec (1910) reported 2n=28 chromosomes in date palm. According to Beal (1937) the date palm had 2n=2x=36 chromosome number of same size and some variation was observed in mitotic metaphase in root tips of young seedlings germinated from seeds. Loutfi and Hadrami (2005) reported 2n=26 chromosomes in two tissue cultured Moroccan date palm cultivars. Karyotype analysis has been conducted for phylogenetic and genetic diversity in plants for more than hundred years and it is well-established by the method of Fukui and Kakeda (1994). Cytology is still considered an important technique for the characterization of plant species. The chromosomal data of species is more important to understand the similarities and differences on the basis of chromosome number, shape and size (Naruhashi and Iwatsubo, 1991). Generally, karyotype studies had been conducted for closely related varieties to know the changes in chromosomes shape during chromosomal evolution (Shan et al., 2003). Successful Karyotypic analysis studies were conducted on *Borago* (Selvi et al., 2006), *Secale* (Masoud and Ali-Jarrahei, 2008) and *Artemisia* (Naseri et al., 2009). Also successful studies were conducted at intraspecific level in cotton, *Agave* and *Thymus* (Sheidai et al., 2008; Guadalupe et al., 2008; Kalvandi et al., 2012).

Al-Salih et al. (1987) reported 2n=32, 34, 36, and 64 chromosomes in date palm. The inconsistent and unpredictable chromosome number hindered the research results due to unavailability of soft roots at mitosis stage from adult palm trees (Al-Ani et al., 2010). Alzahrani (2016) also reported variation in chromosome number 2n=34, 36 of two date palm cultivars Khalas and Sheeshi. Evaluating the genetic diversity of date palm in the region by detailed analysis of chromosome morphology has a novel importance. However, a very limited work has been conducted on genetic diversity and no work has been reported regarding the karyological studies of this economical important plant being cultivated in Pakistan. The aim of this study was to elucidate inter-varietal relationship as well as evolution among elite varieties and wild type date palms grown in Sindh, Pakistan.

Materials and Methods

This study was carried out in Date Palm Research Institute (DPRI), Shah Abdul Latif University (SALU), Khairpur, Pakistan (GPS coordinates 27° 31' 47.8236" N and 68° 45' 29.3076" E) during 2015-2017 and from 2017 to 2018 in the Department of Plant Biology, University of Illinois, USA. Seeds of various date palm cultivars were collected from Sindh province during the fruit season in 2015 (Table 1). The four inedible fruit producing wild Date palm plants were selected from the vicinity of Khairpur and included in present study. These four wild types were assigned names as Wild01, Wild02, Wild03 and Wild 04. All the collected seeds were washed, dried and preserved in zipper bags until use. Later these seeds were used for root harvesting after geminating them in pots. The pots 6×6" were filled with growing medium having vermiculite and sand in 3:1 ratio and kept at 25 to 30°C. The pots were irrigated on alternate days to keep them moist. The primary roots were harvested when emerged 1.5 cm long. The secondary and tertiary roots were collected when these reached to 1.0 cm length (Figure 1, i, ii, iii, iv and v)

The harvested roots were washed with tap water then with deionized distilled water (ddH_2O) and pretreated with ice chilled water for two to four hours. After that roots were pretreated with 8-Hydroxiqunoline for two hours. The roots were quickly rinsed two times and washed two times with freshly prepared chilled ethanol and glacial acetic acid (3:1) to remove water contents. The roots were incubated in fixative for 24

to 48 hours at 4°C. After roots were stored in 70% ethanol at 4°C until use.

Table 1: *Seven commercial varieties and four wild type date palm of Sindh, Pakistan used for cytological investigations.*

Code	Name	Code	Name
a	Aseel	b	Asul Khurmo
c	Otakin	d	Kupro
e	Kashuwari	f	Karblain
g	Dedhi	h	Wild 01
i	Wild 02	j	Wild 03
k	Wild 04		

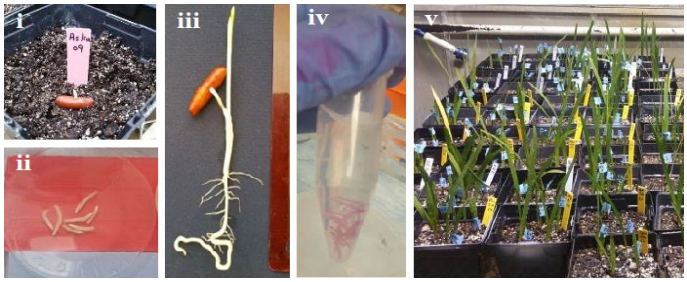

Figure 1: *(i) Seed germination; (ii, iii) harvested primary and secondary roots; (iv) pretreatment of roots; (v) date palm seedlings for cytological studies.*

The root tips were taken out from ethanol and hydrated with ddH_2O two times for three minutes each. Hydrolyzed with 5N HCL for 45 minutes then roots were transferred to pre-chilled ddH_2O. The roots were incubated in Fuelgen solution in dark at room temperature for two hours/until pink color observed in root tips. The roots were bleached with freshly prepared K-metabisulfite (10% K-metabisulfite +1N HCl + ddH_2O: 1:1:9). The root samples were washed with ddH_2O at room temperature and transferred to 1% acetocarmine for counter staining. The darkly stained root tip (apical meristem) of around 1mm was cut with sharp razor blade and temporary mounts were made in a drop of 45% acetic acid on acid clean slides. After putting cover slip (in a way to avoid air bubble trapping inside), it was tabbed softly then pressed with thumb up to nail white underneath folded filter paper to spread root tip material evenly having single cell layer then observed under Microscope.

Prepared slides were examined and Photomicrographs were taken under 100x oil imersion objective giving a total magnification of 1000x. Dividing cells and chromosomes per cell were recorded and photomicrographs were captured with Olympus B×61/B×62 Photo Microscope equipped with DP72 digital camera mounted on it, photomicrographs were taken with stage micrometer on the same magnification and later it was used for measurements of chromosomes with Imag J software by converting number of pixels to micrometer.

Karyotypes were obtained keeping the criteria of chromosome number, chromosome length, position of centromere and arranged by Software Smart Type Karyotype, provided by Digital Scientific UK (http://dsuk.biz/DSUK/Home.html). Especially designed for the appearance and characteristics of chromosome size, numbers and form in metaphase plates. Paired and tint homologous chromosomes were arranged from longest to smallest.

The following numerical values of chromosomes were measured for each investigation:

- Total chromosome length (TCL) was calculated by measuring long arm (p) and short arm (q) in microns (µm).
- Average chromosome length (ACL) of haploid complement.
- Arm ratio was calculated by dividing long arm with short arm (L/S).
- Relative length (RL) was calculated by applying a formula: total chromosome length/average chromosome length.
- Centromeric index (CI) value or total frequency (TF %) was calculated in each observation following Huziwara (1962) by a formula which shows the proportion of short arm in chromosome.
- Finally, chromosomes were arranged according to their length in karyotype from larger to smaller and assigned them numbers.
- Sum of haploid set of chromosomes was also calculated. Chromosomes were classified according to Hussain (2005).

According to Eroğlu (2015) new classification model for karyotype symmetry/asymmetry was followed to determine the karyotype symmetry. In this model a perfect symmetrical karyotype is characterized by completely metacentric chromosomes. In contrast, an asymmetric karyotype consists of a complete set of telocentric chromosomes. The formula includes chromosomal type and centromeric position. The chromosome types were determined according to nomenclature recommended by Levan et al. (1964). The general formula is described with the use of different chromosome types.

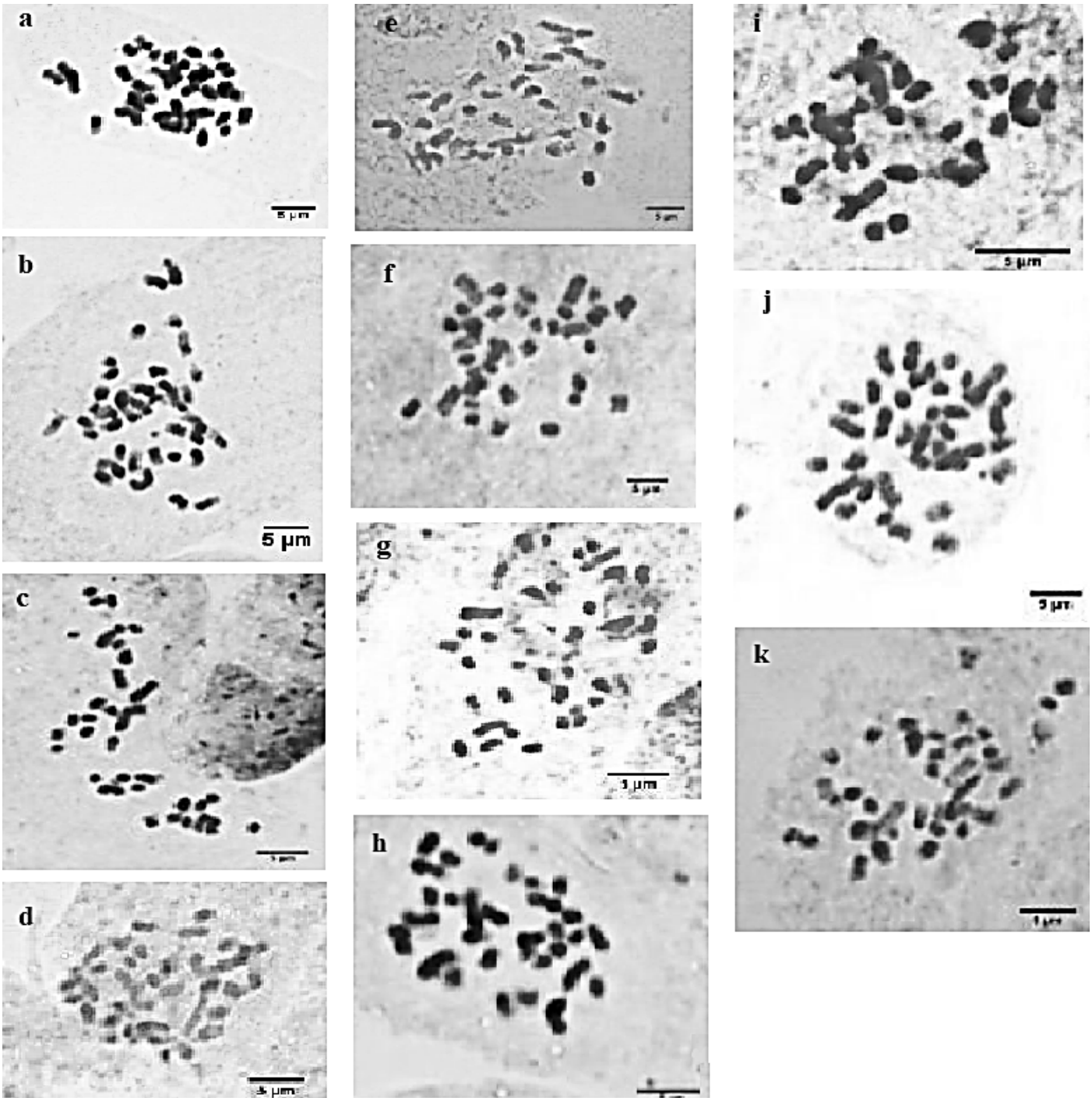

Figure 2: *Mitotic metaphase photomicrographs of six date palm cultivars and two wild type date palms:* **a:** *Aseel;* **b:** *Asul Khurmo;* **c:** *Otakin;* **d:** *Kupro;* **e:** *Kashuwari;* **f:** *Karblain;* **g:** *Dedhi;* **h:** *Wild01;* **i:** *Wild02;* **j:** *Wild03 and* **k:** *Wild04.*

S/As= $(1 \times m) + (2 \times sm) + (3 \times st) + (4 \times t)/ 2n$. In these equations m = metacentric chromosome number; sm = sub-metacentric chromosome number; st= sub-telocentric chromosome number; t= telocentric chromosome number and 2n= diploid chromosome number. Resultant value 1.0-2.0 is considered as symmetric, 2.1-3.0 is between symmetric and asymmetric and 3.1-4.0 is asymmetric karyotype.

Means were calculated using the obtained data from five well spread metaphases. ANOVA was calculated using software SPSS version 20.

Results and Discussion

In this study the karyotype variation of seven elite commercial varieties and four wild type date palms were investigated (Table 1). Generally, date palm chromosomes are recalcitrant in nature, very sticky and exceptionally small in size. The somatic chromosome number determined in seven varieties and four wild type date palm is 2n=36 (Table 2, Figure 3 and 4) confirmed previous reports in date palm cv. Khadrawi by Solimann and Al-Mayah (1978); in cvs. Barhi, Nabut Seif and Succary by Aly and Bacha (2000) and in cvs.

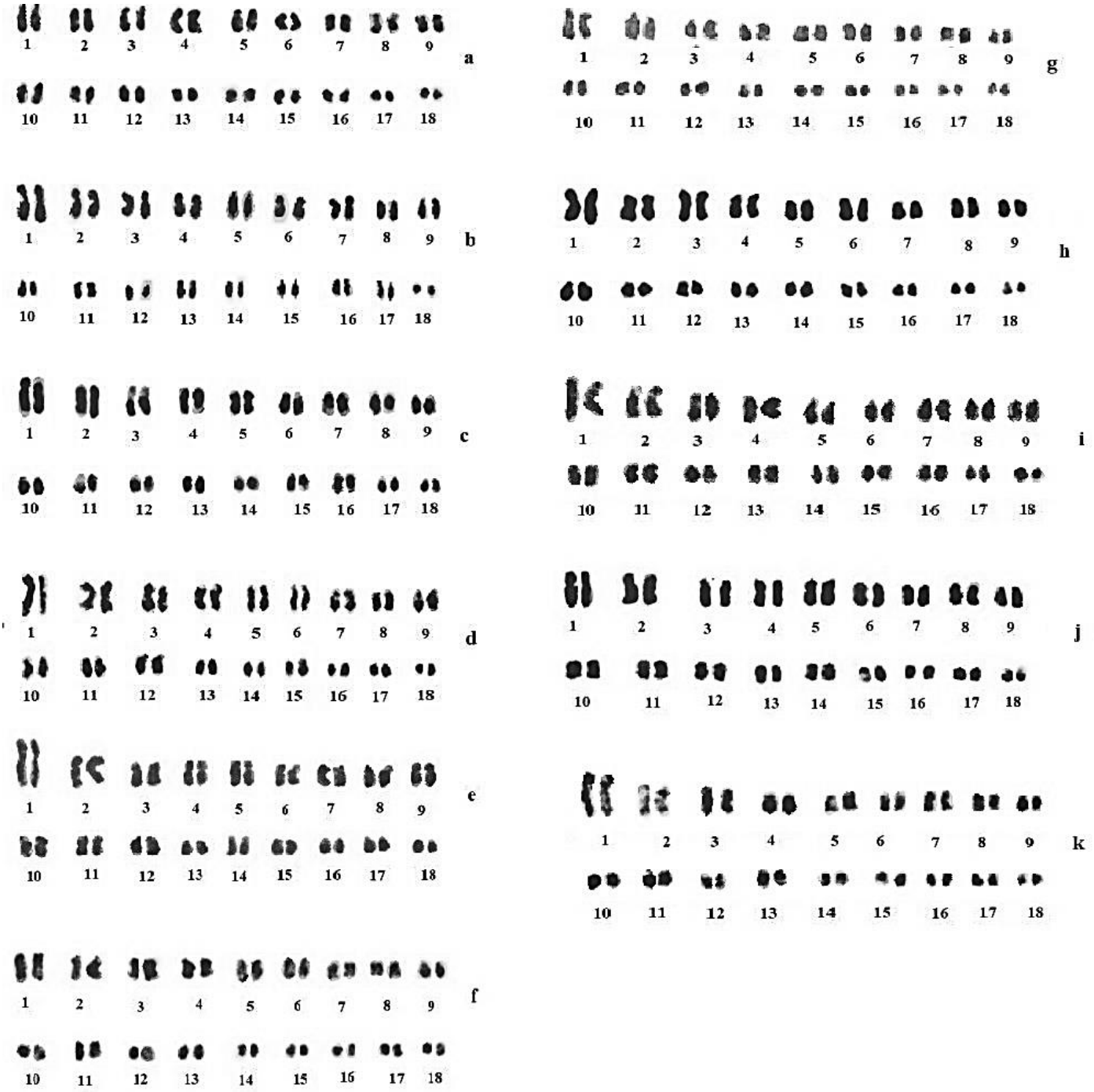

Figure 3: *A detailed Karyograms of six date palm cultivars and four wild type date palms:* **a:** *Aseel;* **b:** *Asul Khurmo;* **c:** *Otakin;* **d:** *Kupro;* **e:** *Kashuwari;* **f:** *Karblain;* **g:** *Dedhi;* **h:** *Wild01;* **i:** *Wild02;* **j:** *Wild03 and* **k:** *Wild04.*

Khalas, Sukkary, Sheeshi, Shibeebi and Sillije by Alzahrani (2016). On the contrary, some of the previous studies reported different number of chromosome pairs such as 14, 16, 17, 18, and 32 in cv. Lilwi (Al-Salih and Al-Rawi, 1987); in cvs. Sayer and Khsab ranging from 2n=32, 34, 36 (Al-Salih et al., 1987) and in two tissue culture derived Moroccan cultivars having 2n=26 (Loutfi and Chlyah, 1998). This variation in somatic chromosome numbers from the present results of seven varieties and four wild type date palms having 2n=36 could be because of stable genome which did not show fusion or duplication of whole chromosome pair or possibly because of difference in origin of date palm varieties.

Data recorded on total chromosome length, longest and smallest chromosomes, arm ratio, relative length and centromeric index of seven varieties and four wild type date palm examined in this study is presented in Table 2 which showed the karyotype variation among chromosomal morphology of different date palm varieties of Sindh, Pakistan. Mitotic metaphase chromosomes were generally found small as presented in Figure 2 a to k.

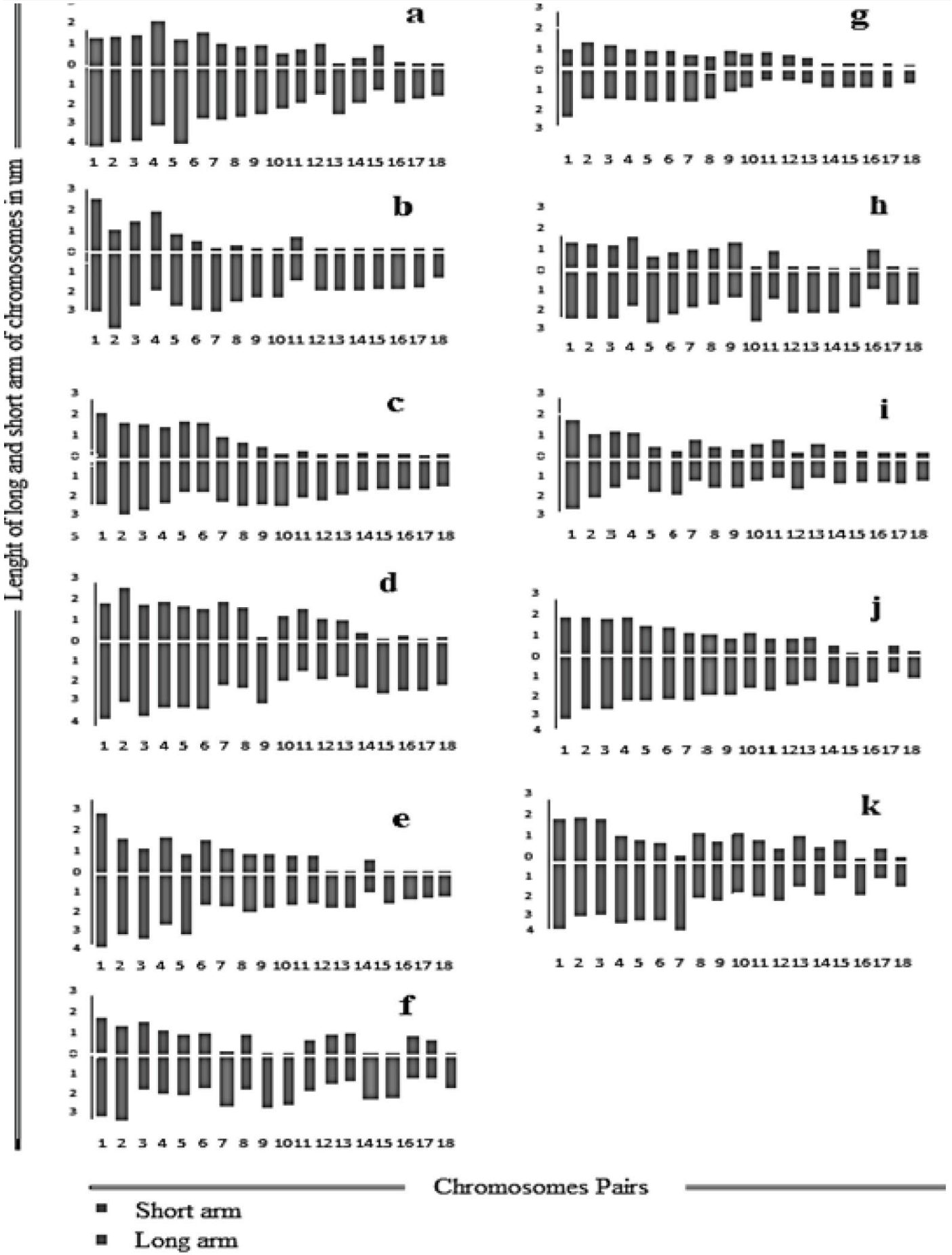

Figure 4: *Ideograms of different varieties and wild date type palm: showing long arm, short arm and centromere position; length in µm (y-axis), haploid (n) number of chromosome (x-axis).* **a:** *Aseel;* **b:** *Asul Khurmo;* **c:** *Otakin;* **d:** *Kupro;* **e:** *Kashuwari;* **f:** *Karblain;* **g:** *Dedhi* **h:** *Wild01;* **i:** *Wild02;* **j:** *Wild03 and* **k:** *Wild04. (Alphabetic codes have been given in materials and methods section).*

Table 2: *Karyotype variation in chromosomal morphology of different date palm cultivars of Khairpur, Pakistan: a: Aseel; b: Asul Khurmo; c: Otakin; d: Kupro; e: Kashuwari; f: Karblain; g: Dedhi; h: Wild01; i: Wild02; j: Wild03 and k: Wild04.*

Chromosome length in µm							
Code	Cultivars Name	2n	LCL	SCL	TCL	Mean Arm Ratio	Mean CI
a	Aseel	36	3.75±0.6 bcde	1.16 ± 0.4	43.11 ± 1.8	2.30 ± 0.4	29.95
b	Asul Khurmo	36	4.69 ±0.5 bcde	1.26 ± 0.5	43.66 ± 1.2	4.09 ± 0.6	19.64
c	Otakin	36	3.53±0.7 cde	1.23 ± 0.6	39.76 ± 1.5	2.45 ± 0.6	25.98
d	Kupro	36	4.83±0.7 a	2.04 ± 0.3	58.78 ± 1.8	2.38 ± 0.5	27.86
e	Kashuwari	36	6.46±0.4 abc	1.32 ± 0.7	50.69 ± 1.2	2.21 ± 0.3	32.44
f	Karblain	36	4.00±0.3 bcde	1.45 + 0.2	41.39 ± 0.6	2.37 ± 0.5	27.97
g	Dedhi	36	3.60±0.5 de	0.99 ± 0.3	35.12 ± 1.2	2.33 ± 0.4	25.38
h	Wild 01	36	2.79 ±0.4 de	1.37 ± 0.4	36.52 ± 1.5	2.78 ± 0.5	26.39
i	Wild 02	36	3.56±0.3 e	1.16 ± 0.3	31.00 ± 0.9	2.49 ± 0.5	26.24
j	Wild 03	36	5.10±0.3 ab	1.32 ± 0.4	53.13 ± 1.2	1.73 ± 0.4	33.57
k	Wild 04	36	5.45±0.5 abcd	1.32 ± 0.4	48.06 ± 1.3	2.33 ± 0.3	26.93

Note: *2n: Diploid set of somatic Chromosomes; LCL: Longest Chromosome length; SCL: Smallest chromosome length; TCL: Sum of total chromosome length of haploid compliment; L/S: Sum of Long arm/Sum of short arm of chromosome; CI: Centromeric Index.*

Table 3: *Symmetry and asymmetry values of date palm cultivars of Sindh, Pakistan: A: Aseel; b: Asul Khurmo; c: Otakin; d: Kupro; e: Kashuwari; f: Karblain; g: Dedhi; h: Wild01; i: Wild02; j: Wild03 and k: Wild04.*

Karyotype Symmetry/Asymmetry				
Code	Cultivars Name	Karyotype	S/A Value	Karyotype Symmetry/Asymmetry
A	Aseel	4m + 7sm + 3st + 4t	2.33	Between Symmetric and Asymmetric
B	Asul Khurmo	4m + 2sm + 4st + 8t	2.88	Between Symmetric and Asymmetric
C	Otakin	6m + 1sm + 8st + 3t	2.44	Between Symmetric and Asymmetric
D	Kupro	5m + 7sm + 1st + 5t	2.33	Between Symmetric and Asymmetric
E	Kashuwari	6m + 5sm + 2st + 5t	2.38	Between Symmetric and Asymmetric
F	Karblain	8m + 5sm + 5t	2.12	Between Symmetric and Asymmetric
G	Dedhi	5m + 6sm + 4st + 3t	2.27	Between Symmetric and Asymmetric
H	Wild 01	4m + 5sm + 1st + 8t	2.72	Between Symmetric and Asymmetric
I	Wild 02	7m + 2sm + 2st + 7t	2.50	Between Symmetric and Asymmetric
J	Wild 03	11 + 4sm + 3t	1.72	Symmetric
K	Wild 04	6m + 6sm + 2st + 4t	2.22	Between Symmetric and Asymmetric

Although, few chromosome pairs posed some difficulties during analysis due to their very small size which affected both centromeric position and arm ratio.

It was consistently noted in seven varieties and four wild type date palm that chromosome pairs from number one to five were relatively longer, six to twelve were median and rest were smaller (Figure 3 and 4). Total length of the haploid complement ranges from 58.78 ± 1.8µm (Kupro) to 31.00 ± 0.9µm (Wild02). The longest to smallest chromosome values indicated the significant length variations within the complement, where chromosome number 1 the longest chromosome in the complement was found in var. Kashuwari (6.46±0.4µm) and smallest in var. Wild01 (2.79 ±0.4µm), whereas chromosome number 18 the smallest chromosome in whole set was found longer in var. Kupro (2.04 ± 0.3µm) and smaller in var. Dedhi (0.99 ± 0.3µm). The similar findings have been reported in cv. Nebut Seif in which longest chromosome was observed 6.31µm (Aly and Bacha, 2000) which is similar to var. Kashuwari (6.46 µm) whereas smallest in Succary 2.41µm (Aly and Bacha, 2000) which is similar to var. Kupro (2.04 µm) and Ashkar 0.75µm (Al-Salih and Al-Rawi, 1987) which is similar to var. Dedhi (0.99µm). Highest average arm ratio was recorded in var. Asul Khurmo (4.09 ± 0.6) and lowest in Wild03 (1.73 ± 0.4). The highest centromeric index percentage was observed in Wild03 (33.57) and lowest in Asul Khurmo (19.64)

which shows the proportion of short arm in whole chromosome length and the centromeric position of individual chromosome number.

The results depicted in Table 3 and Figure 3 and 4 showed differences in centromeric position of chromosomes which is determined type of chromosome like metacentric, sub-metacentric, sub-telocentric or telocentric. Karyotype formula of var. Wild03 showed 4m + 11m + 4sm + 3t chromosomes. Karyotype formula reported in var. Sheeshi is 8m + 4sm+ 2st + 4t by Alzahrani (2016). Out of seven varieties and four wild type date palms under this study only Wild03 has symmetric karyotype because of value less than 2.00 showing maximum number of median and sub-median chromosomes. These results are in agreement with cv. Sheeshi (Alzahrani, 2016) as per formula of Eroğlu (2015). The remaining cultivars and wild type date palm under this study have between symmetric and asymmetric Karyotype with value more than 2.00 and showed tendency towards more sub-telocentric and telocentric chromosome (Table 3 and Figure 5). These results are in agreement with the results reported by Aly and Bacha (2000) in cvs. Barhi and Nebut saif.

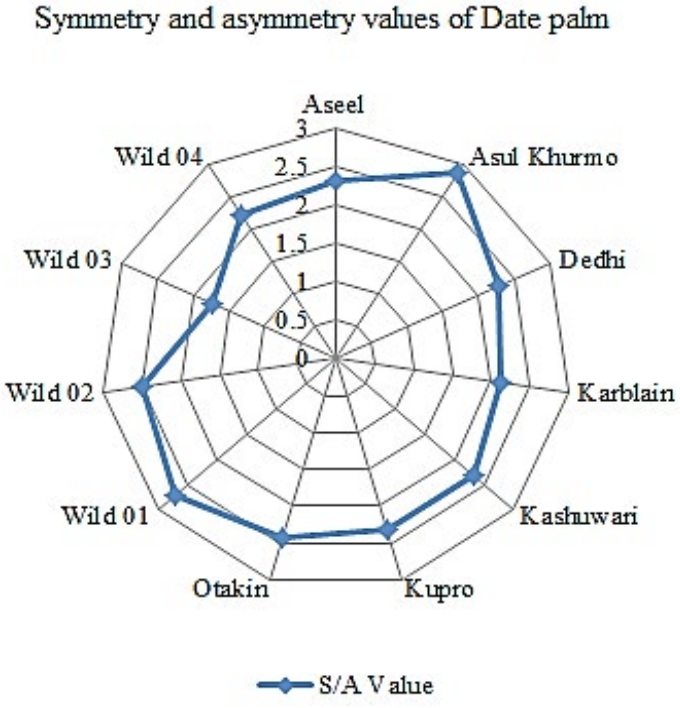

Figure 5: *Symmetry of* **a:** *Aseel;* **b:** *Asul Khurmo;* **c:** *Otakin;* **d:** *Kupro;* **e:** *Kashuwari;* **f:** *Karblain;* **g:** *Dedhi;* **h:** *Wild01;* **i:** *Wild02;* **j:** *Wild03 and* **k:** *Wild04.*

Symmetrical and asymmetrical analysis not recommended generally where three half of chromosomes arise 1µm or less than 1µm because centromere position is difficult to determine (Stebbins, 1971). However, in this study symmetry of the date palm karyotypes have been calculated where six pairs of chromosomes are around 1µm or less. Karyotype is a primitive feature whereas increasing chromosomal asymmetry occurs because of the shift in centromere position from the median to sub-median to the sub-terminal or terminal due to unequal translocations (Stebbins, 1971). Asymmetrical karyotype is characterized by mainly median and sub-median chromosomes of approximately equal size as in Wild03 is likely primitive karyotype showed trend towards their origin as compared to var. Asul Khurmo which represented the advance karyotype by showing maximum number of telocentric chromosome (Figure 5). The changes to an asymmetric karyotype can occur by shifts in centromere position towards the telomere due to rearrangements in both heterochromatin and euchromatin during evolution and size variation among different chromosomes and different karyotype formula could be due to these rearrangements (Peruzzi et al., 2009). Differential amplification of heterochromatic regions or even in the hybridization between species with different chromosome sizes. All these events increase the interchromosomal asymmetry by increasing the morphological discontinuities between chromosomes in a karyotype (de la Herrán et al., 2001).

It has been suggested by Stebbins (1971) that the lowest CI value showed the advanced karyotype therefore, variety Asul Khurmo is more evolved (asymmetrical) as compared to the Wild03. Highest CI value showed in primitive type (symmetrical) to seven varieties and four wild type date palms had comparable chromosome size, karyotype symmetry and chromosome type exhibit close relationship which indicated their probable origin from a common ancestor. However, some recognizable differences have been noticed among these varieties, thus the karyotype analysis revealed minute structural alterations in chromosomes associated with total chromosome length and centromere deviation from the median to telocentric, has played an important role in establishment of new cultivars. It has been suggested by Kuterekar and Wanjari (1983) that varietal demarcation is a result of changes in heterochromatic part as well as repetitive sequences in the genome. The variation among chromosome size indicated that ample rearrangements in chromatin has occurred as reported by Tayyar et al. (1996) and

Galasso et al. (1996) that there were rearrangements both in heterochromatin and euchromatin during evolution in plants.

Conclusions and Recommendations

A detailed karyotypic study was carried out on seven commercial date palm varieties and four wild type plants grown in Sindh, Pakistan. The study has confirmed total number of chromosome pairs is 2n=36 but all the studied varieties including wild types showed variation in terms of chromosome size and structure. The symmetric karyotype was found only in one variety whereas rest of the varieties were between symmetric and asymmetric karyotype. The results presented here will be useful in further cytogenetic analyses and the ongoing genome sequencing. The present study is first ever of its kind because no one has conducted karyotypic studies on date palm varieties in Pakistan before this. More cytogenetic research is needed to understand the cytogenetic history of this commercially important plant because of scarcity of literature regarding chromosomal nature of date palm and most of the cytological studies were done long ago in the other parts of world who have different date palm varieties than this region. Hence, the current study has presented diagnostic features of the date palm karyotype in detail for the first time as recent addition in the field.

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Novelty Statement

The detailed karyotypes of commercial cultivars and wild type date palm is presented for the first time which includes variation in chromosomes and evolutionary pattern. Furthermore, protocol is optimized for the date palm chromosomes to be studied, which is considered one of the most recalcitrant materials.

Author's Contribution

Tahira Jatt performed experimental work and wrote the manuscript. Ghulam Sarwar Markhand and Ray Meng designed experiments, Rayburn helped in experimental work, Mushtaq Ali helped in data analysis and Ameer Ahmed Mirbahar helped in writing and revision of manuscript.

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