CHARACTERIZATION OF WILD JUTE SPECIES THROUGH MOLECULAR CYTOGENETICS

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Certificate

*This is to certify that the thesis entitled "**Characterization of wild jute species through molecular cytogenetics**" submitted by Mahin Afroz has been carried out under our combined supervision in the Department of Botany, University of Dhaka. This is further to certify that it is an original work and suitable for submission for the award of doctor of philosophy in Botany.*

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List of abbreviations

BJRI	:	Bangladesh Jute Research Institute
CTAB	:	Cetyl trimethyl ammonium bromide
PVP	:	Polyvinyl pyrrolidone
EDTA	:	Ethylene diamine tetra acetic acid
PCR	:	Polymerase Chain Reaction
RAPD	:	Random Amplified Polymorphic DNA
SSR	:	Simple Sequence Repeat
Kb	:	Kilo base
bp	:	Base pair
UPGMA		Unweighted Pair Group Method of Arithmetic Means
UV	:	Ultra violet
TE	:	Tris-EDTA
ddH_2O	:	Double distilled water
rpm	:	Revolution per minute
TAE	:	Tris-acetate-EDTA
m	:	Metacentric
sm	:	Sub-metacentric
ac	:	Acrocentric
t	:	Telocentric
CI	:	Centromeric Index
RL	:	Relative length
RTs	:	Root tips
CMA	:	Chromomycin A_3
DAPI	:	4´- 6 diamidino-2-phenylindole
GC	:	Guanine-Cytocine
AT	:	Adenine-Cytocine
m	:	Minute
s	:	Second

List of abbreviations

h	:	Hour
1N	:	1 Normal
MW	:	Molecular weight
g	:	Gram
mg	:	Milligram
ng		Nanogram
µ	:	Micron
µm	:	Micrometer
µM	:	Micro molar
µl	:	Micro liter
µg	:	Microgram
α	:	Alpha
β	:	Beta
γ	:	Gama
φ	:	Phi (small letter)
θ	:	Theta
λ	:	Lambda
δ	:	Delta
Ω	:	Omega
%	:	Percentage
°C	:	Degree centigrade
et al.	:	et alii (and others)
i.e.	:	id est (that is)
viz.	:	Videlicet, Namely
No.	:	Number
Fig(s)	:	Figure(s)
Vol.	:	Volume
W/V	:	Weight/Volume

Characterization of wild jute species through molecular cytogenetics

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Department of Botany

Abstract

Characterization of six wild species of *Corchorus* L *viz. C. brevicornatus*, *C. septentrionalis*, *C. urticifolius*, *C. hirtus*, *C. siliquosus* and *C. pinnatipartitus* was carried out cytogenetically following orcein, CMA-, DAPI- staining. Moreover, molecular characterization of these species was conducted using RAPD-, SSR-and barcode primers. This study has helped to understand the relationship as well as evolutionary trends among these wild species of jute. The interphase nuclei and prophase chromosomes of these species exibited variation in heterochromatin distribution using orcein-staining. Chromosomes with 2n = 2x = 14 were observed in case of *C. brevicornatus*, *C. septentrionalis* and *C. urticifolius* whereas *C. hirtus* and *C. siliquosus* were found to possess 2n = 4x = 28 somatic chromosome with basic chromosome number of x = 7. On the other hand, 2n = 78 chromosomes were observed in somatic cells of *C. pinnatipartitus* with different basic chromosome number except x = 7. This is the first report of the existence of 2n chromosome number for *C. pinnatipartitus*. These species also found to be different in respect of other karyotypic features including total length of 2n chromosome complements, number of satellites, range of relative length, centromeric index etc. The centromeric formula was 14m in *C. brevicornatus*, *C. septentrionalis* and *C. urticifolius*, while 28m in *C. hirtus* and *C. siliquosus* and 74m + 4sm in case of *C. pinnatipartitus*. A wide range of CMA-positive bands (4 - 23) and DAPI-positive bands (3 - 25) was found in the metaphase chromosomes of these six wild jute species. The number, location and distributions of GC- and AT-rich repeats are different for each species. DNA from six wild *Corchorus* species was investigated with 13 oligonucleotide primers, 8 microsatellite primer

pairs and 3 barcode primer pairs for RAPD, SSR and Phylogenetic analysis, respectively. The thirteen RAPD primers generated 69 loci with 100% polymorphism. *Corchorus septentrionalis* was distantly related from other 5 wild species and thus formed a separate cluster at highest genetic distance with *C. pinnatipartitus* (1.1431) whereas *C. hirtus* and *C. pinnatipartitus* were close to each other at lowest genetic distance 0.2638 in case of RAPD analysis. With SSR analysis, a total number of 27 loci were generated from eight primer pair combinations which resulted in 96.30% polymorphism for six wild *Corchorus* species. These results indicated diverse genetic variability among six wild *Corchorus* species. Moreover, unique RAPD and SSR bands were obtained through this investigation could be utilized as a useful tool for characterization of the studied six wild *Corchorus* species. With Maximum Likelihood analysis of combined (ITS, *MatK* and *rbcL*) data matrices, a well defined clade were constructed for six wild *Corchorus* species with strong bootstrap support (98%). *C. pinnatipartitus* and C. *siliquosus* were found to be distant from rest of the species with 98% and 100% bootstrap significance and make a different sub-clade. The phylogenetic tree generated from barcode primers made *C. pinnatipartitus* distinct from the rest of the studied *Corchorus* species and placed alone in a separate cluster that correlated with its phenotypic and cytogenetical features. Therefore, through this investigation wild species of *Corchorus* could be characterized authentically by cytogenetical as well as molecular analysis.

1. Introduction

Jute (*Corchorus* spp.) is the 2nd most important natural bast fiber next to cotton representing shrub, sub-shrub or annual and perennial herb that is widely distributed troughout the tropical and sub-tropical regions of Africa, America, Australia and Asia (Kundu 1951, Kubitzki and Bayer 2003, Benor 2011). The fiber of jute is generally known as "The Golden Fibre" as it is silky in nature and golden in color which were extracted by a natural microbial process known as retting (Mohiuddin *et al*. 2005, Tahir *et al*. 2011). Though it has diverse number of species, fiber is commercially collected from *C. capsularis* L. and *C. olitorius* L. These two species were selected and domesticated in course of time because of their fiber fitness and quality (Haque *et al*. 2007). For centuries jute contributes towards in economy, agriculture and industry of Bangladesh and eastern part of India. Although jute is produced by more than ten countries globally, 90% of its total world production of the jute fiber can be obtained from Indo-Bangladesh subcontinent. On the other hand, the fiber quality is poor in case of most of the wild jute species but they have different stress tolerant ability.

White jute (*C. capsularis*) is cultivated in low land and possesses flood, drought and saline resistant features. It is the main source of jute fiber with silky bright silver color. Another cultivated species Tossa jute (*C. olitorius*) is sensitive to water logging condition but have superior quality of fibre in terms of strength, length and texture (Haque *et al*. 2007). These fiber used for multiple purposes such as for making sacks, cotton bags, carpet, wool packs, pulp and paper, handicraft, yarn, automobile and twine. But, synthetic fiber replaces jute fiber for various reasons. As a result, possibilities of environmental pollution become higher. Such consequences already reported by many workers. Eunomia (2016) reported that every year about 12 million tones of plastics are thrown into the ocean. Later, it turned into micro-plastics and consumed by marine biodiversity

(Kelly *et al*. 2020). In 2018 UK government reported, every year 100000 marine mammals and turtles and 1 million sea birds are killed by marine plastic pollution. Thus non recycled plastic pollutants become a threat to ocean health, coastal tourism, human health and finally have a huge impact on climate change. With this concern UNO (2009) announce that year as "The International Year of Natural Fibers".

Therefore, alternative of plastic is a prime concern to avoid such causalities. In this regard 100% bio-degradable, non-abrasive, non-toxic and recyclable fibers of jute considered the most potential alternative of plastic fiber thus makes them eco-friendly (Edeerozey *et al*. 2007). This is the world's largest source of ligno-cellulosic bast fiber. The fiber of jute has the scope to combine with other fiber to give strength. Such attempts were successfully done by mixing jute and cotton fibre named "Jutton" by Bangladesh Jute Research Institute (BJRI). Polythene bags were banned in Bangladesh in 2002 because of environmental concern. An eco-friendly biodegradable alternative of polythene bags "Sonali Bag" were invented from jute fiber by Bangladesh Atomic Energy Commission (2018).

So, there is a huge opportunity for jute to utilize its fiber in various purposes. But there are lots of difficulties for production of jute. Ganges Delta Plain in Bangladesh *i. e.* Kushtia, Khulna, Jessore, Rajshahi and Pabna is the best place for jute production. According to World Atlas (2021), Bangladesh was the largest producer for jute but now held second position next to India with annual production estimated at 1.349 million tons. First of all, the cultivation area is declining thus pushed the crop in marginal lands. In addition, due to climate change agro-ecological condition become unfavorable for jute cultivation. As a result, the production is decreasing every year. To adjust with the present condition different stress tolerant and high yielding varieties of jute is more concern. In addition, both the cultivar is sensitive to various diseases such as stem

rot and root rot (Ahmed and Ahmed 1969). Moreover, the cultivated varieties posses narrow genetic diversity (Sobhan 1992). On the other hand, wild jute species have wide genetic variability as well as finest fiber quality (Palve *et al*. 2004, Mahaputra and Saha 2008). They have also stress tolerant ability such as drought resistant (*C. depressus*, *C. asplenifolius*, *C. cinerascens*, *C. erinoceus* and *C. erodiodes*), water logging resistant (*C. trilocularis* and *C. tridens*), biotic stress resistant (*C. junodii*, *C. pinnatipertitus*, *C. urticifolius*, *C. saxatilis*, and *C. gillettii*) and so on (Sinha *et al*. 2011). Some wild species also have high yield with quality fibers.

As a result, to improve the quality and quantity of jute fiber inter species improvement is more desirable. Therefore, wild species can be a good pedigree of variation and can be used as parent in breeding program during inter-specific hybridization to improve the genetic make-up for increasing the production and fiber quality. For this reason, International Jute Organization (IJO), Dhaka, Bangladesh took a project collaborated with other country *i. e.* India, China, Nepal, Thailand and Indonesia to enrich genetic resources of jute. Herbarium specimen, seeds as well as wild *Corchorus* germplasm was collected from natural habitats and deposited in Bangladesh Jute Research Institute's (BJRI) gene bank. According to BJRI, 4192 accessions germplasm of 17 *Corchorus* species were stored including two cultivated species (*C. capsularis* and *C. olitorius*). The rest were wild jute species *i. e. C. tridens, C. fascicularis, C. trilocularis, C. pseudo-olitorius, C. pseudo-capsularis, C. aestuans, C. hirtus, C. brevicornatus, C. siliquosus, C. pinnatipartitus, C. aesplenifolius, C. septentrionalis, C. urticifolius, C. baldaccii and C. depressus*. These germplasm were collected based on the plant morphology.

Many scientist reported different number of valid species such as 40 (Kundu *et al*. 1959), 50-60 (Jennifer 1990) and 100 (Willis 1973, Saunders 2001) for this genus. However, there are about 215 species, sub-species, varieties and forms reported

for *Corchorus* according to Global Biodiversity Information Facility (2008). The origin, dispersion route and domestication of wild jute have long history. Approximately 46 species including the cultivated two were reported from Africa thus Africa considered as the primary center of origin for jute (Edmonds 1990). The secondary center of origin for wild jute is Australia representing 53 species which includes 21 endemic and 2 cultivated species. Thirty wild taxa of jute are found in the USA of which 14 species are native of this continent (Sinha *et al.* 2011). In Asia, most of the wild species are distributed in Bangladesh, India, Thailand, Pakistan and Indonesia but none of these are native in this region. Most of the wild species were migrated from Africa (Sinha *et al.* 2011). The cultivated *C. capsularis* is supposed to be native in Indian Sub-continent but there are still controversies (Kundu 1951). However, Benor *et al*. (2012) suggested that the primary center of origin of two cultivated jute is Africa that later dispersed in Asia.

Different taxonomists placed this important genus in three different family of angiosperm such as Tiliaceace (Ahmed *et al.* 2009), Sparrmanniaceae (Heywood *et al.* 2007, Topdar *et al*. 2013, Choudhary *et al*. 2013) or sub-family Grewiodeae of Malvaceae (Alverson *et al.* 1999, Bayer *et al*. 1999, Barbara *et al*. 2003, Benor 2018). However, the confusion about the taxonomic rank of different species of *Corchorus* still exists. Most of the species of *Corchorus* posses 2n = 2x = 14 chromosomes (Islam and Qaium 1961, Arangzeb 1989, Alam and Rahman 2000, Saha *et al*. 2014, Olawuji *et al.* 2014). In addition, numerical chromosomal abberation for different *Corchorus* species were also reported such as hyper-euploidy (2n = 3x = 21, 2n = 4x = 28 in *C. olitorius* and 2n = 6x = 42 in wild species) and hypo- and hyper-aneuploidy (2n= 8, 16 in *C. capsularis*, 2n=15 in a hybrid of *C. capsularis* and *C. olitorius*, 2n=20-35 in *C. olitorius*) (Datta 1952, Rao and Datta 1953, Basak 1958, Patel and Datta 1960, Das 1972, Paria and Basak 1973, Kubitzki and Bayer 2003). The above reports indicate the

possibilities of hybridization between different species or genus belonging to Tiliaceae, Sparrmanniaceae and Malvaceae due to possessing similar genetic constituents. As most of the *Corchorus* species possess similar morphology, it is very difficult to identify them at vegetative stage. For meaningful breeding program for the improvement of cultivated *Corchorus* species finding wide variation within and between gene pool is first criteria. In this situation, karyotype analysis can be a good criterion for characterization of jute.

Cytological studies are particularly useful for characterization of various species and germplasm to get a clear idea about the evolutionary trends. Conventional karyotype analysis in addition with chromosome banding is being used for proper characterization and identification of different germplasms. Chromosome banding is performed with DNA base-specific flurochrome *i. e.* pyrimidine (GC-) and purine base (AT-) Chromomycin A_3 (CMA) and 4´- 6 Diamidino-2-phenyl Indole (DAPI), respectively are useful to identify single chromosome (Schweizer 1976, Alam and Kondo 1995, Akter and Alam 2005, Sultana and Alam 2007). Moreover, nature and distribution of heterochromatin as well as staining property of interphase nuclei and prophase chromosomes could be used as karyological tool because these showed unique characters (Tanaka 1971, Alam *et al.* 1996, Sultana and Alam 2016a).

For assessment of genetic diversity molecular markers become a prime tool (Bered *et al*. 2005). In this case, random amplification of polymorphic DNA (RAPD) and simple sequence repeats (SSR) analysis used by many workers in different plant species (He and Prakash 1997, Hopkins *et al.* 1999, Subrammanian *et al.* 2000, Raina *et al.* 2001, He *et al.* 2003, Qi *et al*. 2003a, Roy *et al*. 2006, Haque *et al*. 2007, Mir *et al*. 2008, Alam *et al.* 2012, Sultana and Alam 2016c). RAPD markers were used to observe genetic diversity and relatedness among different accessions, lines, variety of two cultivated jute species and SSR markers were used for

characterization, studying genetic diversity and cross- species transferability of cultivated jute species (Mir *et al*. 2008, Huq *et al*. 2009, Ghosh *et al*. 2014, Satya *et al*. 2016). Unfortunately, genetic diversity and evolutionary relationship of wild jute taxa are scary.

Molecular phylogenetic analysis become more reliable to solve taxonomic and identification problem as well as evolutionary relationship of plants. For this analysis, different coding and non-coding region of genome considered as primer called DNA barcode primers. However, DNA-based identification of higher organisms was reported earlier (Brown *et al*. 1999, Bucklin *et al*. 1999, Vincent *et al*. 2000, Kress *et al*. 2005, Ma *et al*. 2013, Yu *et al*. 2021). In case of non-coding region, internal transcribed spacer (ITS) gene (ribosomal DNA) are widely used due to having high copy number and high discrimination ability between species by showing adequate resolution even at lower taxonomic levels (Baldwin *et al.* 1995, Aguilar and Feliner 2003, Hamzeh and Dayanandan 2004, Li *et al*. 2011). Due to partial homogenization in parental sequences, ITS-based phylogeny cannot rule out the possibility because through segregation part of parental ITS sequence can eliminated (Sang *et al*. 1995, Wendel *et al*. 1995). In that case, chloroplast DNA (cpDNA) may be the alternate way to remove such chances (Sang *et al*. 1997). A protein-coding cpDNA *i. e.* the maturase K (*MatK*) gene can be used which posses a factor called transversions that helping in the angiosperm phylogeny reconstruction (Olmstead and Palmer 1994, Hilu and Liang 1997). Another protein-coding gene *i. e.* large subunit of the ribulose-bisphosphate carboxylase (*rbcL*) gene is an appropriate choice for inference of phylogenetic relationships at higher taxonomic levels (Les *et al*. 1991, Chase *et al*. 1993, Duvall *et al*. 1993). These two regions have good-quality sequences and ability to species discrimination at high levels (Burgess *et al.* 2011). Combined DNA barcodes region gives better result than single one. It also reported that all the barcode markers didn't show expected results (Roy *et al.* 2010). Many workers used these

two gene sequences for species identification, phylogenetic analysis as well as barcoding (Tamura *et al*. 2004, Manen *et al*. 2004, Starr *et al*. 2009, Asahina *et al*, 2010, Kuo *et al*. 2011). In case of *Corchorus*, different barcode region were analyzed to diversity analysis, make molecular phylogeny, revealed their evolution and dispersal routs (Brunken and Muellner 2012, Tonmay *et al*. 2015, Benor 2018). Moreover, *Corchorus* was placed under Malvaceae due to their monophyletic nature. Tanmoy *et al*. (2015) and Benor (2018) found the genus *Corchorus* as monophyletic. Therefore, the findings of present investigation will enrich the information about their genetic variability as well as will be helpful to characterize them.

Earlier, conventional karyotype and fluorescent banding analysis of two cultivated and six wild *Corchorus* species were characterized from Bangladesh which was available in BJRI (Alam and Rahman 2000, Khatun and Alam 2010, Alam *et al*. 2011, Khatun *et al.* 2011). In addition, characterization of various *Corchorus* species were carried out in abroad which is mostly confined in 2n chromosome count and conventional karyotype analysis (Saha *et al.* 2014, Olawuyi *et al.* 2014) In this study, six wild *Corchorus* species available in BJRI were analyzed those have unique features (Table 1).

Table 1. Stress tolerant ability of studied six wild *Corchorus* species given by Bangladesh Jute Research Institute (Personal Communication).

Species		**Features**
1.	*C. brevicornatus*	Cold tolerant
2.	*C. septentrionalis*	Unknown
3.	*C. urticifolius*	Drought and Shade tolerant
4.	*C. hirtus*	Tolerant to JMV and stem rot disease
5.	*C. siliquosus*	Tolerant to JMV, yellow mite and short day length
6.	*C. pinnatipartitus*	Drought tolerant

In the present investigation, to elucidate taxonomic status, characterization, find out genetic variability and phylogenetic relationship of six wild *Corchorus* species, conventional cytogenetical analysis, DNA base specific fluorochrome banding technique, molecular markers (RAPD and SSR) and molecular phylogeny with three barcode primer pairs will be used. The aims and objectives of the present investigation are given below.

i. Finding the heterochromatin distribution of interphase nuclei and prophase chromosomes with orcein as well as distribution of GC- and AT- rich repetitive region of these stages with CMA and DAPI stain, respectively.

ii. Conventional karyotype analysis following orcein staining.

iii. Analysis of CMA- and DAPI- banding pattern using these fluorochromes.

iv. Identification of marker chromosomes following orcein, CMA- and DAPI-staining.

v. Determination of genetic diversity and relationship among six wild *Corchorus* species through RAPD and SSR analysis.

vi. Elucidating molecular phylogeny among six wild *Corchorus* species with three barcode primers.

vii. Clarify the species status of morphologically different *Corchorus pinnatipartitus.*

viii. Authentic characterization of six wild *Corchorus* species with above cytogenetical and molecular approaches combindly.

2. Materials and Methods

2.1. Plant Materials

Six wild species of *Corchorus* were investigated in this study (Figs 1-6). The studied materials (seeds) were collected from Bangladesh Jute Research Institute (BJRI), Dhaka. Then these were grown and maintained in the Botanical Garden, Department of Botany, University of Dhaka. The detail and morphological features of six wild *Corchorus* species are listed in Tables 2 and 3, respectively. Photographs of flowers, mature pods and seeds of six wild species of *Corchorus* have been shown in Figures 7-12, 13-18 and 19-24, respectively.

Table 2. The six wild species of *Corchorus* used in the present study.

Species	Acc. No. (Provided by BJRI)	Country of origin
C. brevicornatus Vollesen	Acc. 3719	Kenya
C. septentrionalis Planch	Acc. 3122	USA
C. urticifolius Wight & Arn.	Acc. 3707	Kenya
C. hirtus L.	Acc. 1474	Australia
C. siliquosus L.	Acc. 1475	West Bengal, India
C. pinnatipartitus Wild	Acc. 4541	Thailand

Table 3. Morphological features of six wild *Corchorus* species.

Species / Features	*C. brevicornatus*	*C. septentrionalis*	*C. urticifolius*	*C. hirtus*	*C. siliquosus*	*C. pinnatipartitus*
Stem	A sub-erect, bushy annual herb about 10-40 cm tall.	A sub-erect, spreading annual herb about 10- 40 cm tall.	An annual, erect herb growing up to 2 m.	A sub-erect, bushy annual herb about 10- 20 cm tall.	An erect, spreading annual herb which is about 1 m tall.	Perennial herb with a woody root stock, growing up to 1.5 m.
Leaf	Ovate to elliptic. Both surfaces are glabrous.	Ovate to elliptic. Both surfaces are glabrous.	Leaves oblong to lanceolate. Cuspidate at the apex, sparsely hairy.	Ovate to elliptic. Both surfaces are glabrous.	Ovate to elliptic. Both the surface is smooth.	Lanceolate to linear lanceolate or narrowly oblong, sparsely hairy.
Flower	Small flowers yellow, reddish brown at the base, papillonaceous.	Small flowers yellow, reddish brown at the base, papillonaceous.	Flowers yellow, reddish brown at the base, papillonaceous.	Small flowers yellow, reddish brown at the base, papillonaceous.	Flowers yellow in colour.	Pinkish purple in color.
Pod	Capsule in shape, reddish green, beaked, 5-loculed, hairy.	Capsule in shape, beaked, 5-loculed, hairy.	Cylindrical, slightly narrowed towards acuminate apex with distinct ridges.	Capsule in shape, reddish green beaked, 5-loculed, hairy.	Capsule in shape, smooth, and no ridges is found.	Red round capsules with distinct ridges.
Seed	Very small in size, ovate, wrinkle.	Very small in size, ovate, wrinkle.	Seeds many, angular, wrinkle.	Very small in size, ovate, wrinkle.	Square in shape, wrinkle.	Angular, bigger than rest five spp.

2.2. Methods

A. Cytogenetical technique

Preparation of reagents

i. 0.002 M 8-hydroxyquinoline (Pre-fixative)

8-hydroxyquinoline (0.29 gm) was dissolved in distilled water (1 L) by constant stirring and kept at room temperature.

ii. 45% acetic acid (Fixative)

Glacial acetic acid (45 ml) and distilled water (55 ml) was mixed properly to make a dissolve solution.

iii. 70% alcohol (Preservative)

Absolute alcohol (70 ml) was dissolved in distilled water (30 ml).

iv. 1N HCl (Hydrolyzing agent)

HCl (36.5 ml) pour in distilled water (1000 ml) and stored in room temperature.

v. Hydrolyzing solution

For making hydrolyzing solution, 1N HCl and 45% acetic acid as 2:1 ratio was used.

vi. Buffer-1 (B_1)

For making McIlvaine's buffer (pH 7.0), $Na_2HPO_4.12H_2O$ (58.99) gm and citric acid (3.71 gm) were dissolved in distilled water (1 L) and then autoclaved for 10 m. This mixture was kept at 4 ^oC for future use.

vii. Buffer-2 (B_2)

For making McIlvaine's buffer (pH 7.0), $Na_2HPO_4.12H_2O$ (58.99) gm and citric acid (3.71 gm) were dissolved in distilled water (1 L). After

autoclaving for 10 min, $MgSO_4$ (2.46 gm) was added to it and kept at 4 ºC for future use.

viii. 1% Aceto-orcein

Orcein (Sigma) (1 gm) was dissolved in glacial acetic acid (45 ml) in a shaking chamber (60 ºC) for 20 h and for making final volume 100 ml, few ml of distilled water was added. Finally it was filtered and stored in room temperature.

ix. CMA (Chromomycin A_3) (0.1 mg/ml)

Chromomycin A_3 (Sigma) (1 mg) was thoroughly mixed with B_2 (10 ml) until dissolvation and kept this at -20 ºC for further utilization.

x. DAPI (4´- 6 Diamidino-2-Phenylindole) (0.01 mg/ml)

4´, 6-Diamidino-2-phenylindole (DAPI) (Carl Roth) (1 mg) was thoroughly dissolved in B_1 (100 ml) by stirring and kept at –20 ºC for future use.

Procedure for studying mitotic chromosomes

(i) Collection of root-tips (RTs)

Plants were grown in the garden of the Department of Botany, University of Dhaka. Roots with clean root tips of 5 to 7 days old seedling were cut up to 0.5 cm.

(ii) Pre-treatment

After removing surface water by soaking on filter paper, the excised RTs kept in 0.002 M 8-hydroxyquinoline at 6-10 ºC for 1 h and 10 m.

(iii) Fixation

The pretreated roots are fixed in 45% acetic acid for 15 m at 4 ºC.

(iv) Orcein-stained slide preparation

For preparation of orcein staining slide, the pretreated root was kept in the hydrolyzing solution for 45 s at 65 ºC in water bath. After hydrolysis the excess solution of RTs was removed by soaking on filter paper and then proceeds on oil free clean slide. After that, meristematic region of RTs was finely cut with a blade and add one drop of 1% aceto-orcein. In this stage the slide was hold for 3 h in acetic acid chamber and then RTs were ruptured by tapping the materials mildly with tooth pick which was covered with oil free and cleaned cover slip. Then the materials were pressed with thumbs to make sure the homogenous distribution of cells over the slide. To make the whole process successful cover slip should not displace in any step. Nikon (Eclipse 100) microscope was used for slide observation.

(v) Air-dried slide preparation for fluorescent staining (CMA and DAPI)

After hydrolyzing and cutting the meristematic region of the pretreated roots, one drop of 45% acetic acid added and covered by a cover slip. With a tooth pick the materials were tapped and pressed by thumb. Later the cover slip separated from the slide very carefully. Finally these were dried in the air for 12 to 24 h.

(vi) CMA-staining

For the process of CMA-staining, Alam and Kondo's (1995) methods were used with some moderation. First of all, the prepared air dried slides were immersed for 25 m in B_1. The slides were then immersed in a cup-linger containing B_2 for 15 m. Then one drop of the fluorochrome (chromomycin A_3) was put on the slides with materials and a clean cover slip was put down on it. The slide was kept in humid chamber for 30 m. To remove the excess fluorochrome, the slides were washed with distilled water until the cover slip separation. The slides were immersed again in B_2 and B_1, respectively for 10 m in each case. Finally for observation, the slides were prepared by a drop of glycerol (50%) and kept at 4 ºC for 12 to 24 h. With the help of blue violet

(BV) filter cassette of Nikon (Eclipse 50i) fluorescent microscope the prepared slides were observed.

(vii) DAPI-staining

Method proposed by Alam and Kondo (1995) was followed after slight modifications for DAPI-staining. The air dried slide was submerged for 25 m in B_1. The slides were then immersed in cup-linger containing DAPI solution. After that, the slides again kept on the B_1 for 10 m. For observation, one drop of glycerol (50%) were placed on the materials and covered by a cover glass. Then these were kept at 4 ºC for 12 to 24 h. Finally, the slides were observed using ultra violet (UV) filter cassette of Nikon (Eclipse 50i) fluorescent microscope.

Assesment of Centromeric type, relative length and centromeric index

(i) Centromeric type

According to Levan *et al.* 1964, centromeric types were depends on l/s arm ratio (l = length of long arm and s = length of short arm) which are mentioned below:

Chromosome type (ct)	l/s ratio
Metacentric (m)	1.00- 1.50
Sub-metacentric (sm)	1.51- 3.00
Acrocentric (ac)	3.01- 7.00
Telocentric (t)	above 7.00

(ii) Relative length (RL) of chromosomes

For the calculation of relative length of chromosomes the following formula was used.

$$RL = \frac{\text{length of a particular chromosome}}{\text{total length of the diploid complements}}$$

(iii) Centromeric index (CI)

The centromeric index was calculated and represented as per cent (%) with the following formula:

$$CI = \frac{\text{length of short arm}}{\text{total length of that chromosome}} \times 100$$

(iv) Assessment of karyotype asymmetry

For karyotype asymmetry assessment, several parameters were used. These were conducted according to different scientist's references such as total form percent ("TF %") by Huziwara 1962, karyotype asymmetry index ("AsK%") by Arano 1963, chromosome size resemblance index ("Rec%") and karyotype symmetry index ("Syi%") by Greilhuber and Speta 1976, intrachromosomal asymmetry index ("A_1") and interchromosomal asymmetry index ("A_2") by Romero Zarco 1986, dispersion index ("DI") of Lavania and Srivastava 1992, degree of karyotype asymmetry ("A") by Watanabe *et al*. 1999, asymmetry index ("AI"), coefficient of variation of the centromeric index ("CV_{CI}") and coefficient of variation of the chromosome length ("CV_{CL}") by Paszko 2006 and mean centromeric asymmetry ("M_{CA}") by Peruzzi and Erog˘lu 2013 .

Photography and Magnifications

The photographs were taken by using 8 mega pixels canon power shoot A720 model with the magnification of 7× at auto mode. For measuring the magnification, at first the magnification was calculated by multiplying the magnification of objective (100×), tube length (1.25×) and camera lens (7×). From print the final magnification was calculated.

B. RAPD and SSR analysis

For RAPD and SSR analysis the following methods were conducted.

Sample collection and reagents preparation for DNA isolation

For DNA isolation young and actively growing fresh leaves were used for six wild *Corchorus* species. With the sterilized scissors, leaves were cut into pieces after washing with both distilled water and ethanol. Before DNA isolation different stock and working solutions were prepared which are mentioned below:

(i) 1M Tris-HCl stock solution (100 ml)

For making the stock solution, Trizma base (12.14 g) were dissolved in distilled water (75 ml). Concentrated HCl were added with it in fume hood until the pH value become 8. Finally it was made 100 ml by adding distilled water. Then it was sterilized by autoclaving and stored at 4 ºC.

(ii) 0.5 M EDTA stock solution (100 ml)

EDTA (18.61 g) was dissolved in distilled water (75 ml) by continuous stirring with magnetic stirrer. To dissolve and adjust the pH at 8.0 pellets of NaOH was used. Final volume 100 ml was made by adding distilled water. The solution was sterilized by autoclaving and stored at 4 ºC.

(iii) 5 M NaCl stock solution (100 ml)

For stock solution preparation, sodium chloride (29.22 g) was added (not at once) to distilled water (75 ml). The total volume of the solution was adjusted to 100 ml with distilled water. To ensure all NaCl was dissolved, the solution heated for 20 s in oven and stirring by magnetic stirrer. It was sterilized by autoclaving and then stored at 4 ºC.

(iv) β-Mercaptoethanol

14.4 M solution of β-Mercaptoethanol (Merck, Germany) was picked up which had to store at room temperature.

(v) Ribonuclease-A stock solution

RNase-A (10 mg, Merck, Germany) was dissolved in deionized distilled water (1 ml) and stored in -20 ºC.

(vi) Tris-HCl saturated phenol

At 65 ºC crystal phenol was kept in water bath until its melting. After that equal volume of Tris-HCl combined with the melted phenol. With the help of magnetic stirrer it was mixed for 10 m and after that kept for settle down. Then two layers were visible where upper layer was colorless and lower layer was colorful. The upper layer was removed by a dropper and repeated for six times. After saturation, the phenol became the half of the initial volume.

(vii) Phenol: Chloroform: Isoamyl alcohol (25: 24: 1)

25 ml of Phenol, 24 ml of Chloroform and 1 ml of Isoamyl alcohol were mixed by vortex for making 50 ml solution. It was stored at stored at 4 ºC. It should be shaken well before using.

(viii) 70% Ethanol

Double distilled water (30 ml) was added in absolute ethanol (70 ml).

(ix) Stock solution of TE (Tris-HCl EDTA) buffer pH 8.0

1 M Tris-HCl (1 ml) was added to 0.5 M EDTA (200 µl). The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4 ºC.

(x) Sodium acetate (3 M) pH 5.2 (100 ml)

Sodium acetate (40.824 g) was mixed with of double distilled water (ddH_2O) (70 ml) and then adjusted the final volume to 100 ml with ddH_2O. It was sterilized by autoclaving.

(xi) Extraction buffer

The amounts of chemicals are given below for making 100 ml extraction buffer (Table 4).

Table 4. Volume of solutions to prepare extraction buffer.

Chemicals	Molecular weight	Stock con.	Working con.	Working volume
				100 ml
CTAB	---	---	2%	2 g
NaCl	58.44	5 M	1.4 M	28 ml
EDTA (pH 8)	372.24	0.5 M	20 mM	4 ml
Trisbase (pH 8)	121.1	1.0 M	100 mM	10 ml
β-Mercaptoethanol	---	14.4 M	100 mM	700 µl

1 M Tris HCl (10 ml), 5 M NaCl (28 ml) and 0.5 M EDTA (4 ml) were mixed in conical flask (250 ml) followed by autoclaving. After that, CTAB (2 g) was taken into the mixture and stirred by magnetic stirrer. Then mixed with β-mercaptoethanol (700 µl) under fume hood before use. The pH of mixture adjusted to 5 with HCl. The final volume make up to 100 ml by adding distilled water.

DNA isolation procedure

In this experiment, the DNA isolation was done by Doyle and Doyle 1987 procedure with some modification. The protocol was as follows:

(i) Required equipments

1. Plant materials (leaf)
2. Beaker
3. Water de-ionizer
4. Water bath
5. Autoclave machine
6. Conical flasks
7. Mortar and pestle
8. Gloves
9. Water distillation plant
10. Eppendorf tubes
11. Micropipettes and nuclease free micropipette tips
12. Ice machine
13. Digital balance
14. Refrigerator
15. pH meter
16. Centrifuge machine with 10,000-14,000 rpm
17. Fume hood

(ii) Required chemicals

1. Tris base
2. 100% ethanol
3. CTAB (Cetyl Trimethyl Ammonium Bromide)
4. PVP (Polyvinyl pyrrolidone)
5. β-Mercaptoethanol
6. Isopropanol
7. Sodium acetate
8. Isoamyl alchohol
9. Chloroform
10. EDTA (Ethylenediaminetetraacetic acid)
11. Phenol

12. NaCl (Sodium chloride)
13. 70% ethanol
14. RNase-A

(iii) Procedure

1. Freshly harvested leaf (200 mg) was macerated with the help of mortar-pestle using above extraction buffer (1ml) for each sample. These were placed in a 2 ml eppendorf tubes and vortexed for 5-10 s for proper mixing and then incubated those on the preheated water bath at 65 ºC for 1 h. In the interval of 10 m the eppendorf were inverted carefully. After that, the tubes were taken from the water bath and cooled down at room temperature.

2. The samples were centrifuged at 13,000 rpm for 10 m at room temperature to remove non soluble debris. The supernatant was transferred to fresh tubes.

3. Now add 700µl of Phenol: Chloroform: Isoamyl alcohol solution (25:24:1) in the materials and carefully mixed by continuous inverting for 5 m. Lids should be placed tightly in this step. Then centrifuge the tubes at 13,000 rpm for 10 m. This step repeated for 2 times.

4. After completing the above process, add 0.1 vol. of 3 M sodium acetate (pH 5.2) and 0.6 vol. of 100% chilled Isopropanol which was taken into another fresh tubes. The solution was shaken slowly. In this step, DNA became visible as whitish fiber in the solution.
5. After that, pellet the DNA precipitate by centrifuge the tubes at 13,000 rpm for 10 m. The upper layer of the solution was discarded carefully by using adjustable micropipette.

6. For washing the pellet 1 ml 70% ethanol and 100% ethanol was added for two times. Then it was centrifuge for 10 minutes (4 ºC) at 10000 rpm and supernatant was discarded. Pellets were dried by putting the tubes on a

paper towel for about 1 h at inverted position. Pellets should neither contain residual ethanol, nor allow for too dry.

7. The dried DNA was dissolved in 100 µl of TE buffer and treated with 2 µl RNase-A for 30 m at 37 ºC and store at -20 ºC for future use.

Qualification and quantification of DNA

Proper concentration of genomic DNA is needed to get desirable bands. It is necessary to optimize the amount of DNA for reproducibility and strong signal in PCR assay. Excessive genomic DNA may show smears bands on gel and reproducible bands may not occur due to low DNA (Williams *et al*. 1993). In this study, estimation of DNA concentration was done through agarose gel electrophoresis by comparing the DNA with the standard ladder DNA or by estimating the absorbance of DNA by spectrophotometer at 260 nm wave lengths.

Measurement of DNA concentration and quality by agarose gel electrophoresis

The list of required equipments and chemicals were given below.

1. Gloves
2. Pipette and tips
3. Gel casting tray and combs
4. A horizontal electrophoresis chamber and power supply
5. Gel documentation system (Cleaver Scientific Ltd. UK)
6. Agarose
7. 6× sample loading buffer
8. Electrophoresis buffer (TAE)
9. 10 mg/ml ethidium bromide
10. DNA ladder (1 kb and 100 bp)

(i) Stock solutions preparation

(a) TAE buffer (50×)

Trizma base (242 g) dissolved in sterile de-ionized distilled water (700 ml) and then added glacial acetic acid (57 ml) and 0.5 EDTA (100 ml) with this solution. The pH of the solution was adjusted by mixing concentrated HCl to pH 8.3. The final volume of the solution was adjusted to 1000 ml.

(b) Loading dye (10×)

For making 10 ml of 10× loading dye, 40 mg of Bromophenol blue (*i.e*., the final concentration was 0.4%), 40 mg of Xylene cyanol FF (*i.e*., the final concentration was 0.4%) and 5 ml of 98% glycerol (*i.e*., the final concentration was 50%) were added to 4 ml of sterile de-ionized distilled water and mixed properly. The final volume was adjusted to 10 ml with sterile de-ionized distilled water and stored at 4 ºC.

(ii) 1% Agarose gel preparation

Agarose powder (1g) was added in 1× TAE buffer (100 ml) in conical flask (250 ml). The agarose was melted in microwave oven for several short intervals until the solution became clear. Add ethidium bromide (10 µl) when the agarose solution was cooled enough to hold comfortably with bare hand and mixed well by gentle shaking.

(iii) Comb set-up

The ends of the gel casting tray was sealed or fitted with casting dams and the combs placed in the gel casting tray. The melted agarose solution was poured into the casting tray and let it to cool until solid. The comb was removed carefully from the solidified gel. The casting dams or gates were removed from the edges of the gel tray carefully so that the gel did not slide off the tray.

(iv) DNA sample preparation

6× loading dye (1 μl) was placed on a piece of parafilm by a micropipette. DNA (10 μl) sample (after thawing from frozen stock) was added to it and mixed 2-4 times with the adjustable micropipette.

(v) Electrophoresis

During electrophoresis, DNA would run from cathode *i. e.* negative end to anode *i. e.* positive end so the gel was placed in that manner and covered with 1× TAE buffer. Then loaded the prepared samples slowly that will go to the bottom of the wells. 4 μl of standard DNA ladder (1 kb) used as marker was also loaded in one well. Electrophoresis was carried out at 50 volts and 100 mA for 45 m. When the first dye (bromophenol blue) had reached about three-fourths of the gel length, the electrophoresis was completed and stopped.

(vi) Documentation of the DNA sample

After electrophoresis, DNA bands were observed on UV-transilluminator and photographed by a gel documentation system (Cleaver Scientific Ltd. UK) (Fig. 109).

Quantification and qualification of DNA by spectrophotometer

By spectrophotometer DNA concentration and purity was also measured. A260/A280 ratio 1.8 to 2.0 indicates good quality DNA. Higher and lower that range suggested presence of RNA and protein with DNA, respectively. According to Sambrook *et al.* (1989), the DNA concentration was calculated (1 O. D.) at 260 nm corresponds to 50 μl/ml of double standard DNA.

(i) Set-up the spectrophotometer

At first, UV-lamp of spectrophotometer (Analylikjena, Specord 50, Germany) was turned on to warm up and then set at 260 nm wave length. For standardization absorbance reading was adjusted to zero by a cuvette placed on chamber containing 2 ml sterile distilled water.

(ii) Preparation of the DNA samples for spectrophotometry

DNA (2 µl) and sterile distilled water (1998 µl) was mixed with micropipette in 2 ml cuvette for each sample. The absorbance value was taken at 260 nm and 280 nm wave length of spectrophotometer. The absorbance reading for each sample was recorded in the same way after washing the used cuvette with sterile distilled water. The ratios of spectrophotometric absorption readings at 260 nm of different samples for quality determination of DNA are shown in the Table 5.

(iii) Calculation of DNA concentration

Before PCR, DNA concentrations were calculated according to the following formula:

$$\text{DNA concentration} = \text{Dilution factor} \times \text{Conversion factor}$$

$$= A260 \times \frac{\text{Volume of distilled water (µl)}}{\text{Amount of DNA sample (µl)}} \times 50$$

$$= \text{(ng/µl)}$$

$$= \text{(µg/ml)[since 1 µg} = 10^{-3}\text{ng i.e., µg/ml = ng/µl]}$$

A260 = spectrophotometric absorbance reading at 260 nm of DNA sample.

Dilution factor = the ratio of distilled water (µl) to amount of DNA sample (µl).

Conversion factor 50 = the 50 µg/ml of DNA contained in a solution which gives the spectrophotometric absorbance reading at 260 nm equal to 1.

Note: The calculated DNA concentration expressed in µg/ml gave a fraction. To avoid fraction it was converted into ng/µl (1µg = 10^{-3} ng) and therefore, multiplied with 1000.

Table 5. Spectrophotometric readings at 260 nm wave length and estimated concentration of DNA of six wild *Corchorus* species.

Materials	Absorbance reading at 260 nm	Concentration of DNA (ng/µl)
C. hirtus	1.87	463.3
C. siliquosus	1.87	463.3
C. septentrionalis	1.82	383.6
C. brevicornatus	1.90	679.0
C. urticifolius	1.96	259.1
C. pinnatipartitus	1.77	588.0

Amplification of DNA by polymerase chain reaction (PCR) using RAPD and SSR primers

To perform the amplification of target DNA, RAPD and SSR primer sequences were mixed with genomic DNA in the presence of a thermo stable DNA polymerase with suitable buffer and subjected to temperature cycling conditions typical for PCR.

Preparation of DNA samples for PCR (25 ng/µl)

For making a standard concentration *i. e.* 25 ng/µl of DNA the following formula was used.

$S_1 \times V_1 = S_2 \times V_2$

$V_1 = S_2 \times V_2 / S_1$

Where,

S_1= stock DNA concentration (ng/µl)

V_1= volume require (µl)

S_2= working DNA concentration (ng/µl)

V_2= working volume of DNA solution (µl)

2 µl of stock DNA and required amount of TE buffer were mixed in 1.5 ml eppendorf tube for each sample (Table 6).

Table 6. Preparation of working DNA solution (25 ng/µl) for PCR reaction.

Materials	Working Solution (25 ng/µl) for PCR	
	TE buffer/ de-ionized water (µl) required	DNA (µl) required
C. hirtus	16	2
C. siliquosus	16	2
C. septentrionalis	13	2
C. brevicornatus	25	2
C. urticifolius	08	2
C. pinnatipartitus	21	2

Primer test

Twenty five (25) and twelve (12) primers were used for RAPD and SSR amplification, respectively. After amplification 13 for RAPD and 8 for SSR primers showed quality banding patterns with sufficient variability. The details of the four primers were given in Tables 7 and 8.

Table 7. Features of thirteen arbitrary RAPD primers used in the present study.

Sl. No.	Primer code	Sequence (5′—3′)	Annealing Temp. (°C)	GC-content (%)
1	OPA-18	AGG TGA CCG T	32	60.00
2	OPC-10	TGT CTG GGT G	32	60.00
3	OPC-13	AAG CCT CGT C	32	60.00
4	OPC-14	TGC GTG CTT G	32	60.00
5	OPC-16	CAC ACT CCA G	32	60.00
6	OPC-26	CAC GTT ATC GCA	36	50.00
7	OPC-96	ACC AAG AAA GGG	36	50.00
8	OPD-20	AAG ACC CTA CGA	36	50.00
9	OPD-69	CGC TCC AAA TCA	36	50.00
10	OPF-22	AAG ATC AAA GAC	32	33.33
11	OPG-3	GAG CCC TCC A	34	70.00
12	OPG-6	GTG CCT AAC C	32	60.00
13	OPG-9	CTG ACG TCA C	32	60.00

Table 8. Features of eight SSR primers used in the present study.

Primer code	Sequence (5′—3′)	Annealing Temp. (°C)	GC content (%)
AW-584539	F-TTGATGGGCAATACATGTCG	55.2	45
	R- GTTGAAGGAAGGTGGTGGTG	57.7	55
CEDG-30	F- TGAGGGAATGGGAGAGAGGC	59.7	60
	R- TCCGCAGATAGAGGCTCACG	60.5	60
CEDG-56	F- TTCCATCTATAGGGGAAGGG	56.8	50
	R- GCTATGATGGAAGAGGGCAT	59.9	50
HK-7	F- AATGATTATGAACCATAGTGGTACA	59.2	32
	R- TTATCACAAAGTAGCAGACTAACA	58.4	37.5
HK-12	F- CGCTCGCCTAAGTGAAGGCA	55.1	60
	R- ATAAAATACAAGGGACACTT	61.9	30
HK-16	F- TGGAACCTGAGCATCTCTCCAGA	64.7	52.2
	R- CTTTTTCTTGTTCAGGCACCTGA	61.1	43.5
HK-18	F- GCTGTTGTCTCTCTATTGGTGA	60.3	45.5
	R- TTCCACGCTCCTTGTTGCCA	60.5	55
HK-30	F- GAGTGATTAGAGGGCAGCCA	57.0	55
	R- TGCAACAAAAGTATCCAAATC	58.2	35

Preparation of primers

The supplied primers were diluted to 100 µM by adding PCR water. The amount of water was added recommended by the company which was written on the primer tubes. All primers were diluted to 10 times *i.e.* 10 µM to make working solution for use.

Preparation of PCR reaction mixture/ PCR Cocktail

The following components were used to prepare PCR cocktail (Tables 9 and 10). The total volume of PCR cocktail was 20 µl per sample.

Table 9. Component of PCR cocktail (7 reactions) for RAPD primers.

Sl. No.	Reagents	Amount per sample	Total
1	Master mixture	10.0 µl	70.0 µl
2	PCR water	8.5 µl	59.5 µl
3	Primer (10 µM)	0.5 µl	3.5 µl
6	Template DNA (25 ng/µl)	1.0 µl	---
	Total	**20.0 µl**	

Table 10. Component of PCR cocktail (7 reactions) for SSR markers.

Sl. No.	Reagents	Amount per sample	Total
1	Master mixture	10.0 µl	70.0 µl
2	PCR water	8.0 µl	56.0 µl
3	Primer forward (10 µM)	0.5 µl	3.5 µl
4	Primer reverse (10 µM)	0.5 µl	3.5 µl
6	Template DNA (25 ng/µl)	1.0 µl	---
	Total	**20.0 µl**	

During the experiment, Master mixture, primers and DNA sample were thawed from frozen stocks. These were vortexed to mix well and kept on ice. At first, 1.0 µl of template DNA were pipetted in 0.5ml PCR tubes which compatible with thermocycler. Then 20 µl of the above pre-mixture was added to PCR tubes to test each DNA sample. After that lid the PCR tubes carefully and placed in thermocycler following started cycling immediately.

PCR amplification

PCR amplification was done in an oil-free thermal cycler (2720 model of Applied biosystems, USA). The optimum amplification cycle has been presented in Tables 11 and 12.

Table 11. Arbitrary primer-PCR profile.

Denaturation/Extention/Annealing		Temperature(ºC)	Time
	Initial denaturation	95	5m
35 cycles	Denaturation at	95	45s
	Annealing at	32	30s
	Extension at	72	3m
	Final extension at	72	7m

Table 12. SSR marker-PCR profile.

Denaturation/Extention/Annealing		Temperature (°C)	Time
	Initial denaturation	94	2m
32 cycles	Denaturation at	94	1m
	Annealing at	55	30s
	Extension at	65	2m
	Final extension at	65	5m

After completion of cycling programme, the reactions were held at 4 °C.

Electrophoresis of the amplified products (RAPD and SSR) and documentation

The amplified products were resolved by electrophoresis on 1.5% agarose gel for RAPD and SSR. The gel was prepared using 1.5g agarose for RAPD and SSR with 8 µl (8 mg/ml) ethidium bromide and 100 ml 1× TAE buffer at 50 volts and 100 mA for 1.0 h. 1 kb DNA and 100 bp ladder was electrophoresed alongside the RAPD and SSR product as marker. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system for both the markers.

Data analysis for RAPD and SSR marker

The size of bands and polymorphism were analyzed from the gel electrophoresis photographs of PCR products. In this case, 1 considered the presence of bands and 0 indicates bands absence. Single data matrixes in each case were prepared from all primers of RAPD and SSR markers. Useful and reliable software "POPGENE 32" (Version 1.32) were used for conducting Nei's (1972) genetic distance (D) and constructing UPGMA dendrogram among six wild *Corchorus* species.

C. Molecular phylogeny with barcode marker analysis

Molecular phylogeny with barcode marker analysis was performed in Key Laboratory of Biodiversity and Biogeography of Chinese Academy of Sciences, Kunming Institute of Botany, Kunming, China.

Molecular phylogeny with Bar-code marker analysis has been presented below:

Isolation of genomic DNA

Silica gel dried leaves of six wild *Corchorus* spp. were used for genomic DNA isolation and then Doyle and Doyle (1987) methods were followed with some modifications.

1. Dithio-theritol (2 µl) was added in 4×CTAB (1 ml) which was preheated in water bath at 65 ºC.

2. Then midribs of samples were removed and homogenous macerate was prepared with the help of mortar-pestle using above buffer (1ml) and sand in each sample. These were placed in a 1.5ml eppendorf and incubated on the heated block at 65 ºC for 1 h. In the interval of 10 m the eppendorf were inverted carefully.

3. The samples were centrifuged at 10,000 rpm for 10 m at room temperature to remove non soluble debris. The supernatant was transferred to fresh tubes.

4. Now 500µl of chloroform: isoamyl alcohol (24:1) was added in the materials and mixed carefully by continuous inverting for 5 m. Lids should be placed tightly in this step. Then centrifuge the tubes at 10,000 rpm for 5 m. This step repeated for 2 times.

5. In this step the supernatant (upper layer) transferred to a clean 1.5ml eppendorf and add ice chilled isopropanol (70% of sample volume) and kept in freeze for 30 m.

6. After that, pellet the DNA precipitate by centrifuge the tubes at 10,000 rpm for 8 m.

7. The pellet was washed two times with 70% ethanol (1ml) and 100% ethanol (1 ml) and then centrifuged for 10 minutes (4 ºC) at 8000 rpm and discarded the supernatant. Pellet was dried in vacuum centrifuge for 5 minutes at 50 ºC

8. Finally, TE buffer (pH8.0) (50-100µl) and RNase (2 µl) was added into the tubes to dissolve the pellet. This was stored at -20 ºC for future utilization.

DNA qualification

1. Loading dye (1 µl) and sample DNA (4 µl) was taken and then centrifuged for few sec in 2700 rpm.

2. 4S green plus nucleic acid stain (1 µl) was added in 1% Agarose gel (50 ml).

3. The prepared samples were loaded slowly and the separation process was monitored by the migration of the dye on the gel.

4. Then the gel was placed in gel documentation after complete the electrophoresis for detecting DNA bands.

5. The electrophoregram of DNA samples of six wild *Corchorus* spp. are shown in figure 109.

Amplification of DNA by polymerase chain reaction (PCR) using ITS, *MatK* and *rbcL* primers

To amplification of target DNA, bar code primer was mixed with genomic DNA in thermo stable DNA polymerase with suitable buffer and subjected to temperature cycling conditions typical for PCR.

Dilution of DNA samples

DNA was diluted by adding distilled water (20 µl) in sample (10 µl) for performing PCR reaction.

Primer test

Three barcode primers ITS, *MatK* and *rbcL* were used to make molecular phylogeny. The details of the primer are given below (Table 13):

Table 13. Details of three barcode primers used in present investigation.

DNA type	Region Name	Primer pairs	Sequence 5′-3′
rDNA	Inter-transcribed Spacer (ITS)	ITS5 ITS4	GCAAGTAAAAGTCGTAACAAGG CCTTATCATTTAGAGGAAGGAG
cpDNA (Coding)	Maturase K (*MatK)*	3F_KIM 1R_KIM	CGTACAGTACTTTTGTGTTTACGAG ACCCAGTCCATCTGGAAATCTTGGTTC
cpDNA (Coding)	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*)	1F 724R	ATGTCACCACAAACAGAAAC TCGCATGTACCTGCAGTAGC

Preparation of primers

The supplied primers were diluted to 100 µM. To make 5 µl of each primer, primers (10 µl) were mixed with distilled water (190 µl).

Preparation of PCR reaction mixture

The following components were used to prepare PCR cocktail (Table 14). The total volume of PCR cocktail was 20 µl per sample.

Table 14. Component of PCR mixture (20 reactions) for Bar-Code primers.

Sl. No.	Reagents	Amount per sample	Total
1	Master mixture	10.0 µl	200.0 µl
2	PCR water	8.5 µl	170.0 µl
3	Primer forward (10 µM)	0.5 µl	10.0 µl
4	Primer reverse (10 µM)	0.5 µl	10.0 µl
6	Template DNA (25 ng/µl)	1.0 µl	---
	Total	**20.0 µl**	

During the experiment, Master mixture, primers and DNA sample were thawed from frozen stocks. These were vortexed to mix well and kept on ice. At first, 1.0 µl of template DNA were pipetted in 0.5ml PCR tubes which compatible with thermocycler. Then the above pre-mixture (20 µl) was added to PCR tubes to test each DNA sample. After that lid the PCR tubes carefully and placed in thermocycler following started cycling immediately.

PCR amplification

PCR amplification was done in an oil free thermal cycler (Gene Amp PCR system 9700, Perkin Elmer, Foster City, USA). In this study general PCR profile were used. The optimum amplification cycle has been presented in Table 15.

Table 15. General-PCR profile.

Denaturation/Extention/Annealing		Temperature (°C)	Time
	Initial denaturation	94	2m
32 cycles	Denaturation at	94	1m
	Annealing at	48	10s
	Extension at	65	2m
	Final extension at	65	5m

After completion of cycling programme, the reactions were held at 4 °C.

Electrophoresis of the amplified products and documentation

The amplified products were resolved by electrophoresis on 1.0% agarose gel. 4S green plus nucleic acid stain (1 µl), 1% Agarose gel (1 gm) and 1× TAE buffer (100 ml) was mixed well for gel preparation at 50 volts and 100 mA for 1.0 h. Then 100 bp ladder was electrophoresed alongside the product as marker. DNA bands were observed on UV-transilluminator and photographed

by a gel documentation system. After getting single sharp band this PCR product further purified for sequencing.

DNA sequencing

The PCR product showing single sharp band were considered for further sequencing. The details were given below.

1. Purification of PCR product

   To purification of PCR product enzyme (Exosap IT) (2 µl) was added in sample (5 µl) and centrifuged for 2000 rpm for 5-6 sec. Then it was kept in PCR machine for 15 m in 37 ºC (1st cycle) and for 15 m in 80 ºC (2nd cycle). In this step other cycle of PCR profile was deleted.

2. Preparation of sample for sequencing

   All chemical listed in Table 16 except sample were mixed in two separate tubes for forward and reverse primer. Here for safety of reaction, some more mixture should be taken. Then it was centrifuged for few sec.

Table 16. Components of sequencing mixture (for each sample).

Sl. No.	Reagents	Amount
1	Sequencing buffer (5× sequencing buffer from Applied Biosystems)	1µl
2	PCR water	3.75µl
3	Sequence mixture (Big Dye ® Terminator V 1.1 and V. 3.1.5× sequencing buffer)	0.3µl
4	Primer	0.5 µl
6	Sample DNA	0.8 µl
	Total	**6.35 µl**

3. Sequencing mixture (6µl) and purified PCR product (0.8 µl) was mixed and centrifuged for few sec in 2000 rpm for each sample.

4. Then the mixture was placed for PCR at sequencing programme. The PCR profile is given below (Table 17):

Table 17. PCR profile for sequencing programme.

Denaturation/Extention/Annealing		Temperature (ºC)	Time
	Initial denaturation	94	30s
32 cycles	Denaturation at	96	10s
	Annealing at	50	0.05s
	Extension at	60	3m

After completion of cycling programme, the reactions were held at 4 ºC.

5. After sequencing reaction (20:1) 3M Na-acetate (20µl) and Alcohol (70%) were added.
6. Then it was centrifuged for few sec in 2000 rpm and kept it in -20 ºC freezer for more than 30 m.
7. Then it was centrifuged for 10 min in 12 ºC and 3700 rpm.
8. After centrifuge sample is inverted in tissue. Then it was centrifuged for 3 m in 12 ºC and 560 rpm to soak unwanted materials.
9. 70% alcohol (150 µl) was added and centrifuged in 3700 rpm for 10 m at 12 ºC.
10. Then it was inverted in tissue paper to soak alcohol and again inverted in new tissue paper and then centrifuged for 3 m in 12 ºC at 560 rpm.
11. Then it was kept in PCR machine at 80 ºC to remove all alcohol. Here tube cap and PCR should be opened.
12. Then HiDiTM formamide (10 µl) was added and centrifuged for few second.
13. It was kept in centrifuge for 2 min at 95 ºC. Here temperature of the 2nd cycle should be similar.
14. Then it was covered in ice immediately for 2 min and kept it in freeze and then placed in automatic sequencer (ABI 3730×1 DNA Sequencer, Applied Biosystems, Foster City, USA).

Data Analysis

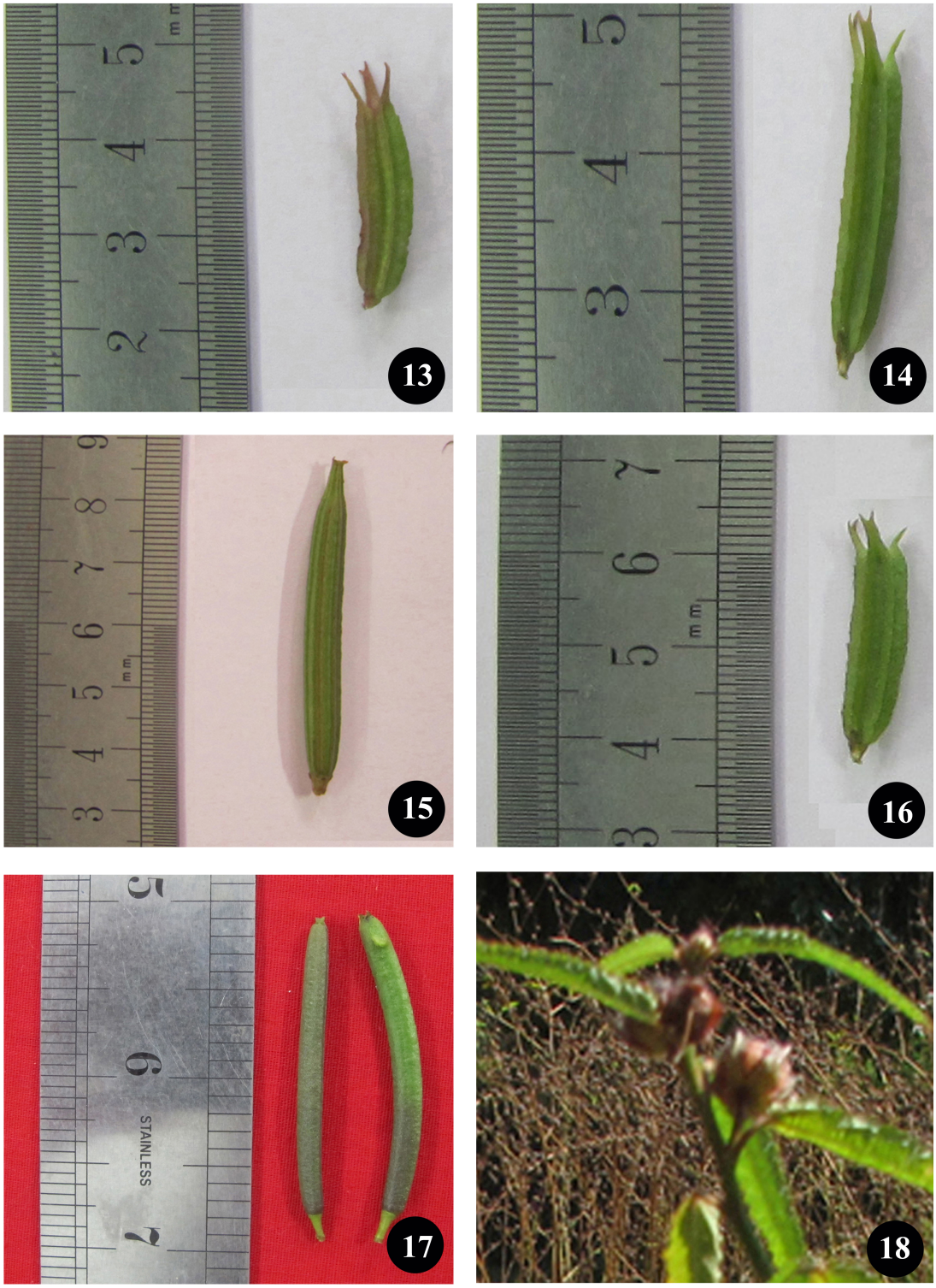
1. Firstly file was renamed in Excel sheet.
2. Then folder was renamed.
3. Then the sequences were checked and edited by using sequencher 4.1.4 software and saved as fasta file.
4. Final multiple alignments of the sequences were constructed using CLUSTAL X in MEGA X software.
5. Maximum Likelihood (ML) method was applied for phylogenetic analysis following the Tamura-Nei model (bootstrap test and 1000 replicates) using MEGA X software (Tamura and Nei 1993, Kumar *et al*. 2018).



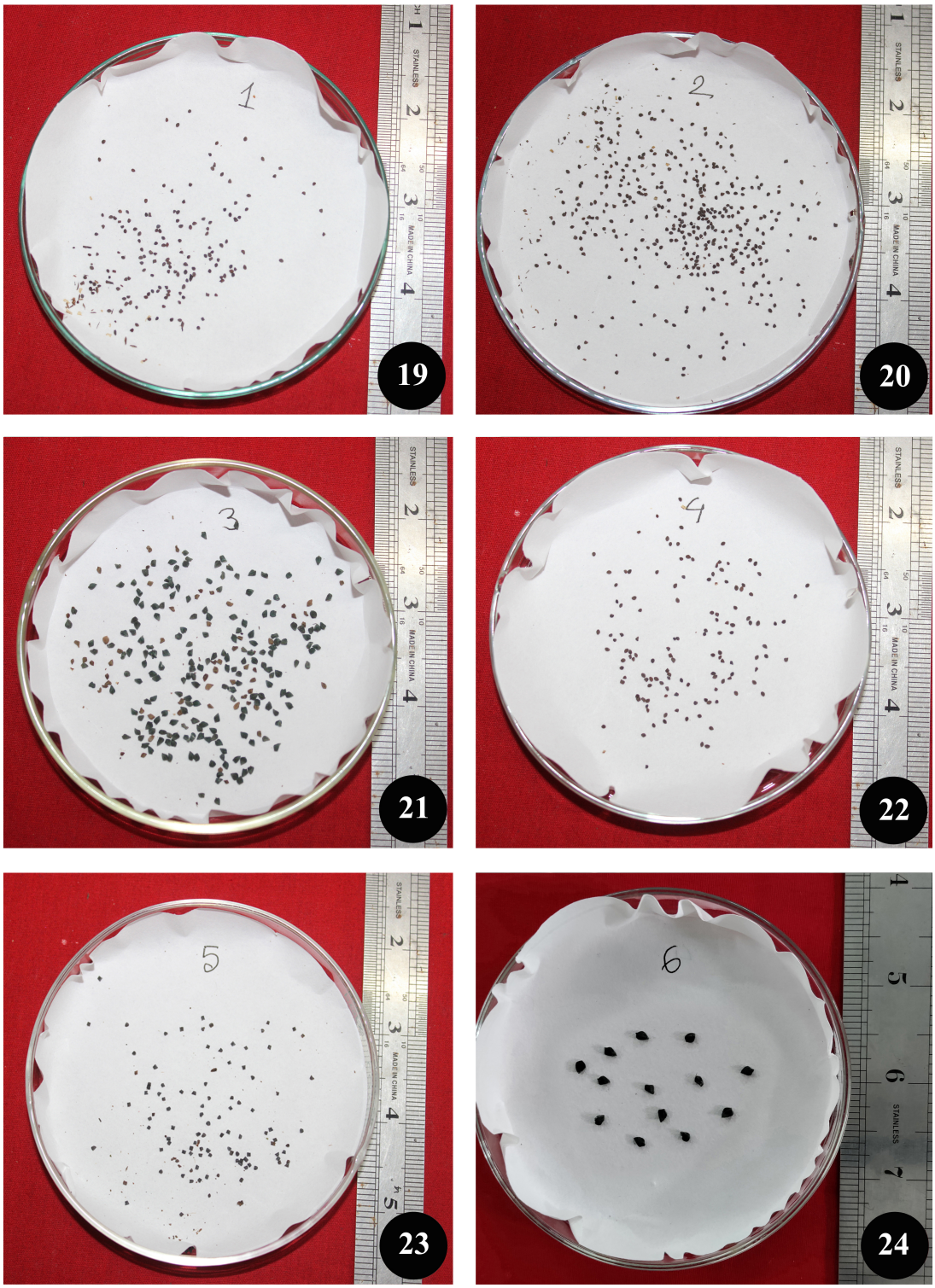
Figs 1-6. Plant morphology of six wild *Corchorus* L. species. 1. *C. brevicornatus*, 2. *C. septentrionalis*, 3. *C. urticifolius* (Leaves in inset), 4. *C. hirtus*, 5. *C. siliquosus* and 6. *C. pinnatipartitus* (Leaf in inset).



Figs 7-12. Flowers of six wild *Corchorus* L. species. 7. *C. brevicornatus*, 8. *C. septentrionalis*, 9. *C. urticifolius*, 10. *C. hirtus*, 11. *C. siliquosus* and 12. *C. pinnatipartitus*.



Figs 13-18. Pods of six wild *Corchorus* L. species. 13. *C. brevicornatus*, 14. *C. septentrionalis*, 15. *C. urticifolius*, 16. *C. hirtus*, 17. *C. siliquosus* and 18. *C. pinnatipartitus*.



Figs 19-24. Seeds of six wild *Corchorus* L. species. 19. *C. brevicornatus*, 20. *C. septentrionalis*, 21. *C. urticifolius*, 22. *C. hirtus*, 23. *C. siliquosus* and 24. *C. pinnatipartitus*.

3. RESULTS

3.1. Appropriate season obtaining maximum number of dividing cells from the root-tips

Maximum number of dividing cells (about eighty per cent) were found in the root tip cells (RTCs) studied during the month of June to August, although few dividing cells were observed throughout the year. The numbers of dividing cells were found to be very poor when the temperature was either very low or very high.

3.2. Appropriate time in obtaining maximum number of dividing cells from the root tips

Root tips of *Corchorus* species were collected regularly at 9.00, 9.30, 10.00, 10.30, 11.00 and 11.30 a.m. It was found that 9.00 to 10.00 a.m. was the optimum time period in obtaining maximum number of dividing cells (about eighty per cent).

3.3. Appropriate chemical for pretreatment

Different chemicals like 8-hydroxyquinoline, colchicine and para dichloro benzene (PDB) were tried for pretreatment of dividing cells to find out proper contraction and in achieving intact morphology of chromosomes. It was found that only in cold condition (6-10ºC) 8-hydroxyquinoline solution (0.002 M) for 1 h and 10 min gave the best result.

3.4. Karyotypes and Idiograms

On the basis of overall length and centromeric position, somatic chromosomes of *C. brevicornatus*, *C. septentrionalis* and *C. urticifolius* could be assembled in 7 pairs (Figs 85-87 and 97-99, Tables 18-20). On the other hand, *C. hirtus* and *C. siliquosus* could be placed in 14 pairs (Figs 88-89 and 100-101, Tables 21-22). However, the somatic chromosomes of *C. pinnatipartitus* could be

tallied in 39 pairs (Figs 90 and 102, Table 23). From the obtained data, idiograms were prepared supplementing the karyotypes (Figs 97- 102).

3.5. Interphase nuclei

Classical cytological analysis with orcein-staining and fluorescent banding technique with CMA and DAPI were used to find out the staining property of interphase nuclei of six wild *Corchorus* species. For every staining, at least 50 interphase nuclei were observed for each species.

3.5.1. Orcein staining

The six wild *Corchorus* spp. were found to possess a prominent nucleolous in the interphase nuclei following orcein staining except *C. hirtus* (Figs. 25-27, 29-30, arrow). Heterochromatins were homogenously distributed in the interphase nuclei of *C. brevicornatus*, *C. septentrionalis* and *C. urticifolius* (Figs 25-27). On the other hand, *C. hirtus*, *C. siliquosus* and *C. pinnatipartitus* showed a number of darkly stained small scatterdly arranged heterochromatic regions in the nucleus (Figs 28-30).

3.5.2. CMA-staining

Two bright and prominent CMA-bands were observed in the interphase nuclei of *C. septentrionalis* and *C. hirtus* (Figs 32 and 34). In contrast, few small dot like CMA-bands were scatterdly distributed throughout the nucleus of *C. brevicornatus*, *C. urticifolius* and *C. siliquosus* (Figs 31, 33 and 35). On the other hand, *C. pinnatipartitus* was found to possess numerous dot like CMA-bands (Fig. 36).

3.5.3. DAPI-staining

Few faintly stained DAPI-bands were found in the interphase nuclei of *C. brevicornatus* (Fig. 37). Two bright DAPI-bands were found in *C. septentrionalis* (Fig. 38) whereas 12-15 DAPI-bands were present in *C. urticifolius, C. hirtus* and *C. siliquosus* (Figs 39-41). On the other hand, *C.*

pinnatipartitus was found to possess numerous dot like DAPI- bands (Fig. 42). A non-staining region was observed in the interphase nuclei of *C. brevicornatus*, *C. septentrionalis* and *C. pinnatipartitus* (Figs 37, 38 and 42).

3.6. Prophase chromosomes

To find out the staining property of prophase chromosomes of six wild *Corchorus* species, fluorescent banding technique with CMA and DAPI were used. For every staining method, at least 50 prophase chromosomes were observed for each species.

3.6.1. Orcein staining

A prominent nucleolus was observed in every prophase stage in six wild *Corchorus* spp (Figs 43-48). In *C. brevicornatus* and *C. hirtus* most of the prophase chromosomes were stained homogeneously along the entire length (Figs 43 and 46). The prophase chromosomes of *C. siliquosus* and *C. pinnatipartitus* stained only at the interitial regions (Figs 47 and 48). On the other hand, the prophase chromosomes of *C. septentrionalis* and *C. urticifolius* were darker in one end and gradually faint to other end (Figs 44 and 45).

3.6.2. CMA-staining

In prophase chromosomes, two prominent and bright CMA-positive bands were found in *C. septentrionalis* and *C. urticifolius* (Figs 50 and 51). In addition, few bright bands were observed in that two species (Figs 50 and 51). On the other hand, several CMA-positive bands were found at different location of the prophase chromosomes in *C. pinnatipartitus* (Fig. 54). In *C. brevicornatus, C. hirtus* and *C. siliquosus* few CMA- bands were present at different regions of the prophase chromosome (Figs 49, 52 and 53).

3.6.3. DAPI-staining

No prominent DAPI-positive band was observed in the prophase chromosomes in *C. brevicornatus* and *C. hirtus* (Figs 55 and 58). A number of DAPI-positive

bands were found to be scattered among the prophase chromosomes of *C. septentrionalis*, *C. urticifolius*, *C. hirtus* and *C. siliquosus* (Figs 56, 57 and 59). In contrast, numerous DAPI fluoresced bands were scatterdly distributed in the prophase chromosomes of *C. pinnatipartitus* (Fig. 60). A non staining region was observed in *C. brevicornatus* and *C. septentrionalis* (Figs 55 and 56).

3.7. Pro-metaphase chromosomes

CMA- and DAPI-staining were used to study the pro-metaphase chromosome of six wild *Corchorus* species (Figs 61-72). For every staining, at least 40 pro-metaphase stages were observed for each species.

3.7.1. CMA-staining

In pro-metaphase chromosomes, two prominent, bright and round CMA-positive satellites were found in *C. brevicornatus*, *C. septentrionalis*, *C. urticifolius*, *C. hirtus* and *C. siliquosus* (Figs 61-65, arrows). On the other hand, *C. pinnatipartitus* showed six prominent and bright CMA-positive satellites (Fig. 66).

3.7.2. DAPI-staining

In pro-metaphase chromosomes, two prominent satellites were observed after DAPI-staining in *C. septentrionalis* and *C. hirtus* (Figs 68 and 70, arrows). On the other hand, six prominent and bright DAPI-positive satellites were found in *C. pinnatipartitus* (Fig. 72). After DAPI- banding *C. brevicornatus*, *C. urticifolius* and *C. siliquosus* showed no satellite (Figs 67, 69 and 71).

3.8. Metaphase chromosomes

For every staining, at least 50 metaphase cells were observed for each species.

3.8.1. Orcein staining

For each species three clear metaphase plates were considered for chromosome measurement (Figs 73-78). Total length, arm ratio, centromeric index, relative

length and centromeric type of six wild *Corchorus* species are presented in Tables 18-23.

Corchorus brevicornatus was found to possess 2n = 14 chromosomes (Figs 73a-73c). Total length of diploid chromosome complements in this species was 50.87 µm. Range of relative length of individual chromosome was 0.06 to 0.10. Individual chromosome length ranged from 2.49 ± 0.46 to 5.29 ± 0.20 µm. The centromeric formula was recorded 14m for this species (Figs 85 and 97, Tables 18 and 24). Two pair of satellites (0.92 µm) was observed on short arm of each member of chromosome pair IV and VI (Figs 85 and 97).

Somatic chromosome number (2n = 14) was observed in *C. septentrionalis* (Figs 74a-74c). Total length of diploid chromosome complements 40.35 µm was recorded for this species. This length was the lowest among six wild *Corchorus* species. Relative length of individual chromosome ranged from 0.05 to 0.08 whereas individual chromosomal length ranged from 2.11 ± 0.24 to 3.43 ± 0.12 µm. The centromeric formula of this species was 14m (Figs 86 and 98, Tables 19 and 24). A pair of satellite or secondary constriction (0.46 µm) was found on short arm in pair IV of this species after orcein staining (Figs 86 and 98).

2n = 14 chromosomes was found in case of *C. urticifolius* (Figs 75a-75c). The total length of 2n chromosome complements was 51.27 µm. The relative length of the individual chromosomes was ranged from 0.06 to 0.09. The individual chromosome length ranged from 2.80 ± 0.29 to 4.58 ± 0.69 µm. The centromeric formula of this species was 14m (Figs 87 and 99, Tables 20 and 24). Two pair of satellites (0.81 µm) was observed on short arm of each member of chromosome pair IV and VI (Figs 87 and 99).

In *C. hirtus*, total chromosome number was 2n = 28 (Figs 76a-76c). Total length of diploid chromosome complements was 74.19 µm. Range of relative length of individual chromosome was 0.03 to 0.04. Individual chromosome length ranged from 1.92 ± 0.29 to 3.43 ± 0.45 µm. The centromeric formula of

this species was 28m (Figs 88 and 100, Tables 21 and 24). A pair of satellite or secondary constriction (0.86 µm) was found on short arm in pair IV of this species after orcein staining (Figs 88 and 100).

Corchorus siliquosus was found to possess 2n = 28 chromosomes (Figs 77a-77c). Total length of diploid chromosome complements was 67.22 µm. Range of relative length of individual chromosome was 0.02 to 0.05. Individual chromosome length ranged from 1.65 ± 0.18 to 3.39 ± 0.26 µm. The centromeric formula of this species was 28m (Figs 89 and 101, Tables 22 and 24). A pair of satellite or secondary constriction (0.46 µm) was found on short arm in pair VI of this species after orcein staining (Figs 89 and 101).

Somatic chromosome number (2n = 78) was observed in *C. pinnatipartitus* (Figs 78a-78c). Total length of diploid chromosome complements 220.28 µm was recorded for this species. This length was the highest among six wild *Corchorus* species. Relative length of individual chromosome ranged from 0.01 to 0.02 whereas individual chromosomal length ranging from 1.96 ± 0.12 to 4.37 ± 0.48 µm. The centromeric formula of this species was 74m + 4sm (Figs 90 and 102, Tables 23 and 24). No satellite was observed in this species after orcein staining.

3.8.2. Orcein staining karyotype asymmetry assessment

Karyotype asymmetry assessment was carried out both manually and by using KaryoType software (Tables 24 and 25). According to KaryoType software analysis, CV_{CI} value ranged from 3.21 (*C. hirtus*) – 5.87 (*C. pinnatipartitus*). *Corchorus septentrionalis* had lowest value in respect of CV_{CL} and AsK% and highest for TF% and Syi%. A slight variation observed in all species respect of A_1 and A_2 value which ranged from 0.06-0.18 and 0.15-0.23, respectively. In case of both above value was highest in *C. brevicornatus* and lowest in *C. septentrionalis*. To determine the karyotype asymmetry, dispersion index (DI) was computed. DI value was highest in *C. brevicornatus* (9.79) and lowest in *C. urticifolius* (7.13). It also represents that, higher DI value correspond to

more asymmetric karyotype while lower value correspond to symmetric karyotype. AI value was observed highest in *C. pinnatipartitus* (1.13) and lowest in *C. septentrionalis* (0.50). AI value was >1.0 in *C. brevicornatus* and *C. pinnatipartitus* and <1.0 in *C. septentrionalis, C. urticifolius, C. hirtus and C. siliquosus.* 1A type karyotype was observed in *C. septentrionalis, C. urticifolius, C. hirtus* and 1B in *C. brevicornatus, C. siliquosus* and *C. pinnatipartitus* (Table 25).

3.8.3. CMA-staining

The number and total length of CMA-positive band and percentage of CMA-positive banded regions was measured for each species by considering three clear CMA-stained mitotic metaphase plate (Figs 79a-84a, Table 26).

A total of eight CMA-positive bands were found in different chromosomes of *C. brevicornatus*. Both the member of chromosome pair 1 and 4 were found to possess CMA-positive band on whole short arm. On the other hand, both homologue of pairs 5 and 6 were entirely fluoresced with CMA. In addition, a pair of satellite was also observed on short arm of both members of pair 6 (Figs 79a, 91a and 103). The total length of CMA-banded region was 5.64 µm which occupied about 11.09% of the total chromatin length. The CMA-positive karyotypic formula was $4\alpha + 4\gamma + 6\delta$ in case of this species (Table 26).

In *C. septentrinalis*, four CMA- positive banded chromosomes were observed. Both the member of chromosome pair 1 was entirely fluoresced whereas pair 4 had band in short arm after CMA- staining (Figs 80a, 92a and 104). The total length of CMA-banded region was 3.22 µm which covered about 7.98% of the total chromatin length. The CMA-banded karyotypic formula of this species was $2\alpha + 2\gamma + 10\delta$ (Table 26).

Eight CMA-positive bands were found in *C. urticifolius*. Both the members of pair 3 and 7 showed terminal CMA-positive band on short arm. On the other hand, both the member of pairs 1 and 5 had band on whole short arm with

centromere. In addition, two pair of satellite was observed on short arm in both the member of chromosome pair 4 and 6 (Figs 81a, 93a and 105). The total length of CMA-banded region was 6.79 µm which occupied about 13.24% of the total chromatin length. The CMA-positive karyotype formula of this species was 4γ + 4λ + 6δ (Table 26).

In *C. hirtus*, nine CMA-positive bands were found. Six centromeric CMA-positive band was observed in both the member of pair 8, 9 and 10. In addition, three terminal bands on short arm were found in both the member of chromosome pair 12 and a homologue member of pair 2 (Figs 82a, 94a and 106). The total length of CMA-banded region was 7.13 µm which covered about 9.61% of the total chromatin length. The CMA-positive karyotypic formula of this species was 3γ + 6ϕ + 19δ (Table 26).

In *C. siliquosus*, four CMA-positive bands were found at two terminal region of both the member of chromosome pair 7. In addition, both the member of chromosome pair 4 had terminal bands on short arm (Figs 83a, 95a and 107). The total length of CMA-banded region was 2.99 µm, which covered about 4.45% of the total chromatin length. The CMA-positive karyotypic formula of this species was 2γ + 2β + 24δ (Table 26).

In total 23 CMA-positive bands were found in *C. pinnatipartitus*. Both the member of chromosome pair 2 had band in long arm. On the other hand, three centromeric band was observed in both the member of pairs 4 and a homologue member of pair 23. Both the member of chromosome pair 8, 16, 18, 22 and 38 and a homologue member of pair 5 aand 39 had terminal bands on short arm. Both the members of pairs 10 and 26 were entirely fluoresced with CMA. However, pair 20 showed band in the terminal position of long arm. Six satellites were observed in both the member of chromosome pair 11, 20 and 36 (Figs 84a, 96a and 108). The total length of CMA-banded region 27.72 µm which occupied about 12.58% of the total chromatin length. The CMA-positive

karyotype formula of this species was 4α + 12γ + 2φ + 2 Ω + 3ϕ + 55δ (Table 26).

3.8.4. DAPI-staining

Three clear DAPI-stained mitotic metaphase plate for each species were considered for counting the number of DAPI-band, total length of DAPI-positive band and percentage of DAPI-positive banded regions (Figs 79b-84b, Table 27).

In *C. brevicornatus*, three entirely fluoresced DAPI-positive chromosomes were found in both the members of pair 4 and a member of pair 5 (Figs 79b, 91b and 103). The total length of DAPI-banded region was 3.22 µm which occupied about 6.33% of the total chromatin length. The DAPI-positive karyotypic formula of this species was 3α + 11δ (Table 27).

Six DAPI-bands were found in *C. septentrionalis*. Both the members of pair 1 and 3 were found to possess entirely fluoresced DAPI-positive chromosomes. On the other hand, pair 4 had DAPI-positive bands on short arm (Figs 80b, 92b and 104). The total length of DAPI-banded region was 3.68 µm which covered about 9.12% of the total chromatin length. The DAPI-positive karyotypic formula of this species was 4α + 2γ + 8δ (Table 27).

In *C. urticifolius*, six DAPI-positive bands were found. Four DAPI-positive bands were found in short arm of both the members of pairs 4 and 5. On the other hand, pair of centromeric DAPI-positive bands were found in both member of pair 1 (Figs 81b, 93b and 105). The total length of DAPI-banded region was 4.60 µm which covered about 8.97% of the total chromatin length. The DAPI-positive karyotypic formula of this species was 2ϕ + 4γ + 8δ (Table 27).

In *C. hirtus*, six DAPI-positive bands were observed. Both the member of chromosome pair 7 was entirely fluoresced DAPI-positive bands. On the other

hand, both the member of pair 3 and 10 showed terminal DAPI-positive bands on short arm (Figs 82b, 94b and 106). Two pair of satellites were observed on short arm of both member of pair 4 and 14. The total length of DAPI-banded region was 11.5 µm which occupied about 15.50% of the total chromatin length. The DAPI-positive karyotypic formula of this species was 2α + 4γ + 22δ (Table 27).

Three DAPI-positive bands were found in *C. siliquosus*. A centromeric DAPI-positive band was observed in a member of chromosome pair 1. On the other hand, a member of pair 6 had centromeric band where as its homologue member was entirely fluoresced after DAPI- staining (Figs 83b, 95b and 107). The total length of DAPI-banded region was 2.19 µm which occupied about 3.26% of the total chromatin length. The DAPI-positive karyotypic formula of this species was 1α + 2ϕ + 25δ (Table 27).

A total number of 25 DAPI-positive bands were found in *C. pinnatipartitus*. Both the members of pair 5, 7 and 21 had band on long arm. Twelve terminal bands were observed in the short arm of both the member of pair 6, 13, 15, 22, 29 and 33. However, both the members of pair 36 and one member of pair 9 were entirely fluoresced with DAPI. On the other hand, both member of pair 14 and 30 had terminal band on both short arm and long arm. Six satellites were observed on short arm in both the member of chromosome pair 11, 20 and 36 (Figs 84b, 96b and 108). The total length of DAPI-banded region 38.64 µm which occupied about 17.54% of the total chromatin length. The DAPI-positive karyotype formula of this species was 12γ + 6φ + 3α + 4β + 53δ (Table 27).

Table 18. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. brevicornatus* Vollesen.

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	2.81±0.29	2.48±0.18	5.29±0.20	1.14	0.10	46.81	m
	2.67±0.24	1.91±0.10	4.58±0.23	1.39	0.09	41.77	m
II	2.40±0.30	2.05±0.06	4.45±0.35	1.17	0.08	46.05	m
	2.28±0.07	1.99±0.06	4.27±0.12	1.14	0.08	46.67	m
III	2.12±0.18	1.90±0.24	4.03±0.41	1.12	0.08	47.22	m
	2.06±0.18	1.85±0.18	3.91±0.23	1.12	0.08	47.22	m
IV	2.03±0.13	$1.46\pm0.12^{*0.92}$	$3.49\pm0.24^{*0.92}$	1.38	0.07	41.94	m
	2.00±0.13	$1.45\pm0.12^{*0.92}$	$3.45\pm0.30^{*0.92}$	1.38	0.07	41.94	m
V	1.98±0.24	1.43±0.07	3.41±0.18	1.38	0.07	41.94	m
	1.82±0.24	1.48±0.07	3.30±0.18	1.23	0.06	44.83	m
VI	1.60±0.12	$1.28\pm0.12^{*0.92}$	$2.88\pm0.20^{*0.92}$	1.25	0.06	44.44	m
	1.51±0.24	$1.20\pm0.18^{*0.92}$	$2.72\pm0.40^{*0.92}$	1.25	0.06	44.44	m
VII	1.35±0.24	1.26±0.27	2.61±0.46	1.08	0.06	48.15	m
	1.34±0.29	1.15±0.20	2.49±0.46	1.17	0.06	46.15	m
			TCL=50.87				

m = Metacentric chromosome
TCL = Total Chromatin Length
$\bar{x}$ = Mean
SD = Standard Deviation
* = Satellite

Table 19. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. septentrionalis* Planch

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	1.77±0.09	1.66±0.12	3.43±0.12	1.07	0.08	48.39	m
	1.77±0.09	1.66±0.12	3.43±0.12	1.07	0.08	48.39	m
II	1.69±0.20	1.59±0.15	3.28±0.32	1.07	0.08	48.39	m
	1.66±0.27	1.56±0.18	3.22±0.41	1.07	0.08	48.39	m
III	1.53±0.20	1.53±0.24	3.07±0.40	1.00	0.08	50.00	m
	1.53±0.20	1.53±0.24	3.07±0.40	1.00	0.08	50.00	m
IV	1.50±0.25	1.50±0.24$^{*0.46}$	3.01±0.46$^{*0.46}$	1.00	0.08	50.00	m
	1.54±0.25	1.43±0.18$^{*0.46}$	2.97±0.41$^{*0.46}$	1.07	0.07	48.28	m
V	1.39±0.21	1.39±0.24	2.78±0.43	1.00	0.07	50.00	m
	1.34±0.15	1.34±0.18	2.68±0.32	1.00	0.07	50.00	m
VI	1.39±0.23	1.19±0.13	2.59±0.36	1.17	0.07	46.15	m
	1.38±0.24	1.19±0.13	2.57±0.37	1.17	0.07	46.15	m
VII	1.12±0.18	1.02±0.12	2.15±0.29	1.10	0.05	47.62	m
	1.16±0.18	0.95±0.06	2.11±0.24	1.22	0.05	45.00	m
TCL=40.35							

m = Metacentric chromosome
TCL = Total Chromatin Length
$\bar{x}$ = Mean
SD = Standard Deviation
* = Satellite

Table 20. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. urticifolius* Wight & Arn.

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	2.60±0.53	1.98±0.17	4.58±0.69	1.30	0.09	43.48	m
	2.56±0.48	1.94±0.17	4.50±0.65	1.30	0.09	43.48	m
II	2.46±0.47	1.74±0.20	4.20±0.66	1.39	0.08	41.86	m
	2.29±0.37	1.77±0.18	4.06±0.54	1.28	0.08	43.90	m
III	2.27±0.35	1.75±0.27	4.03±0.61	1.28	0.08	43.90	m
	2.18±0.29	1.77±0.27	3.95±0.54	1.22	0.08	45.00	m
IV	2.03±0.24	1.52±0.17$^{*0.81}$	3.55±0.41$^{*0.81}$	1.31	0.07	43.28	m
	1.99±0.23	1.54±0.17$^{*0.81}$	3.53±0.40$^{*0.81}$	1.28	0.06	43.94	m
V	1.80±0.23	1.69±0.13	3.49±0.35	1.06	0.06	48.48	m
	1.80±0.23	1.69±0.13	3.49±0.35	1.06	0.06	48.48	m
VI	1.64±0.24	1.42±0.13$^{*0.81}$	3.07±0.37$^{*0.81}$	1.14	0.06	46.67	m
	1.64±0.24	1.42±0.13$^{*0.81}$	3.07±0.37$^{*0.81}$	1.14	0.06	46.67	m
VII	1.59±0.17	1.38±0.13	2.97±0.30	1.14	0.06	46.67	m
	1.45±0.18	1.35±0.12	2.80±0.29	1.07	0.06	48.28	m
TCL=51.27							

m = Metacentric chromosome
TCL = Total Chromatin Length
$\bar{x}$ = Mean
SD = Standard Deviation
* = Satellite

Table 21. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. hirtus* L.

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	1.87±0.30	1.56±0.15	3.43±0.45	1.18	0.04	45.95	m
	1.84±0.29	1.53±0.14	3.37±0.43	1.18	0.04	45.95	m
II	1.65±0.20	1.55±0.17	3.20±0.37	1.06	0.04	48.57	m
	1.65±0.20	1.55±0.17	3.20±0.37	1.06	0.04	48.57	m
III	1.57±0.27	1.52±0.23	3.09±0.50	1.03	0.04	49.28	m
	1.57±0.27	1.52±0.23	3.09±0.50	1.03	0.04	49.28	m
IV	1.50±0.22	1.50±0.21$^{*0.86}$	3.01±0.42$^{*0.86}$	1.00	0.04	50.00	m
	1.50±0.22	1.50±0.21$^{*0.86}$	3.01±0.42$^{*0.86}$	1.00	0.04	50.00	m
V	1.44±0.18	1.14±0.24	2.88±0.41	1.00	0.04	50.00	m
	1.44±0.18	1.14±0.24	2.88±0.41	1.00	0.04	50.00	m
VI	1.40±0.18	1.30±0.18	2.70±0.36	1.07	0.04	48.39	m
	1.40±0.18	1.30±0.18	2.70±0.36	1.07	0.04	48.39	m
VII	1.38±0.18	1.23±0.12	2.61±0.30	1.11	0.04	47.46	m
	1.38±0.18	1.23±0.12	2.61±0.30	1.11	0.04	47.46	m
VIII	1.34±0.18	1.23±0.12	2.57±0.29	1.07	0.03	48.28	m
	1.34±0.18	1.23±0.12	2.57±0.29	1.07	0.03	48.28	m
IX	1.32±0.18	1.21±0.13	2.53±0.30	1.07	0.03	48.28	m
	1.32±0.18	1.21±0.13	2.53±0.30	1.07	0.03	48.28	m
X	1.28±0.12	1.17±0.07	2.45±0.18	1.08	0.03	48.15	m
	1.28±0.12	1.17±0.07	2.45±0.18	1.08	0.03	48.15	
XI	1.25±0.14	1.15±0.12	2.40±0.26	1.08	0.03	48.15	m
	1.23±0.18	1.13±0.14	2.36±0.32	1.08	0.03	48.15	m

Table continued

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
XII	1.15±0.20	1.10±0.18	2.24±0.38	1.04	0.03	49.06	m
	1.15±0.20	1.10±0.18	2.24±0.38	1.04	0.03	49.06	m
XIII	1.08±0.23	1.03±0.23	2.21±0.46	1.04	0.03	49.06	m
	1.11±0.23	0.96±0.18	2.07±0.40	1.08	0.03	48.00	m
XIV	1.04±0.23	0.95±0.17	1.99±0.40	1.08	0.03	48.00	m
	0.96±0.14	0.96±0.14	1.92±0.29	1.00	0.03	50.00	m
TCL=74.19							

m = Metacentric chromosome
TCL = Total Chromatin Length
$\bar{x}$ = Mean
SD = Standard Deviation
* = Satellite

Table 22. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. siliquosus* L.

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	1.92±0.20	1.47±0.10	3.39±0.26	1.31	0.05	43.33	m
	1.81±0.23	1.39±0.03	3.20±0.22	1.31	0.05	43.33	m
II	1.58±0.13	1.37±0.11	2.95±0.24	1.15	0.04	46.43	m
	1.58±0.13	1.37±0.11	2.95±0.24	1.15	0.04	46.43	m
III	1.48±0.14	1.38±0.11	2.86±0.26	1.08	0.04	48.15	m
	1.48±0.14	1.38±0.11	2.86±0.26	1.08	0.04	48.15	m
IV	1.46±0.10	1.30±0.06	2.76±0.15	1.13	0.04	46.94	m
	1.44±0.10	1.28±0.03	2.72±0.12	1.13	0.04	46.94	m
V	1.41±0.09	1.25±0.09	2.66±0.13	1.13	0.04	46.94	m
	1.34±0.06	1.28±0.09	2.63±0.07	1.04	0.04	48.94	m
VI	1.26±0.10	$1.16\pm0.10^{*0.46}$	$2.42\pm1.20^{*0.46}$	1.09	0.04	47.83	m
	1.26±0.10	$1.16\pm0.10^{*0.46}$	$2.42\pm1.20^{*0.46}$	1.09	0.04	47.83	m
VII	1.23±0.03	1.23±0.10	2.45±0.13	1.00	0.04	50.00	m
	1.23±0.03	1.23±0.10	2.45±0.13	1.00	0.04	50.00	m
							m
VIII	1.25±0.07	1.14±0.09	2.40±0.14	1.10	0.03	47.62	m
	1.25±0.07	1.14±0.09	2.40±0.14	1.10	0.03	47.62	
							m
IX	1.22±0.13	1.11±0.12	2.34±0.24	1.10	0.03	47.62	m
	1.15±0.12	1.05±0.09	2.20±0.20	1.10	0.03	47.62	m
X	1.15±0.12	1.05±0.09	2.20±0.20	1.10	0.03	47.62	m
	1.06±0.09	1.06±0.12	2.13±0.21	1.00	0.03	50	m
XI	1.01±0.03	1.01±0.03	2.01±0.10	1.00	0.03	50	m
	1.01±0.71	0.92±0.07	1.94±0.78	1.10	0.03	47.62	m

Table continued

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
XII	1.01±0.07	0.90±0.12	1.92±0.18	1.13	0.03	47.06	m
	0.99±0.06	0.88±0.09	1.88±0.13	1.13	0.03	47.06	m
XIII	0.97±0.67	0.89±0.06	1.86±0.69	1.10	0.03	47.62	m
	0.97±0.67	0.89±0.06	1.86±0.69	1.10	0.03	47.62	m
XIV	0.92±0.12	0.81±0.12	1.73±0.23	1.14	0.02	46.67	m
	0.82±0.09	0.82±0.09	1.65±0.18	1.00	0.02	50.00	m
			TCL=67.22				

m = Metacentric chromosome
TCL = Total Chromatin Length
$\bar{x}$ = Mean
SD = Standard Deviation
* = Satellite

Table 23. Length (in µm), arm ratio, centromeric index, relative length and centromeric type of metaphase chromosomes of *C. pinnatipartitus* Wild.

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
I	2.76±0.46	1.61±0.20	4.37±0.48	1.71	0.02	36.84	sm
	2.76±0.55	1.61±0.10	4.37±0.55	1.71	0.02	36.84	sm
II	2.42±0.42	1.96±0.20	4.37±0.61	1.24	0.02	44.74	m
	2.19±0.28	1.96±0.20	4.14±0.48	1.12	0.02	47.22	m
III	1.96±0.23	1.84±0.23	3.80±0.46	1.06	0.02	48.48	m
	2.07±0.29	1.73±0.18	3.80±0.46	1.20	0.02	45.45	m
IV	2.36±0.47	1.44±0.12	3.80±0.46	1.64	0.02	37.88	sm
	2.30±0.44	1.44±0.12	3.74±0.43	1.60	0.02	38.46	sm
V	1.84±0.18	1.61±0.12	3.45±0.30	1.14	0.02	46.67	m
	1.73±0.06	1.61±0.12	3.44±0.17	1.00	0.01	50.00	m
VI	1.73±0.12	1.61±0.12	3.34±0.23	1.07	0.02	48.28	m
	1.73±0.14	1.61±0.18	3.34±0.32	1.07	0.02	48.28	m
VII	1.73±0.15	1.61±0.18	3.34±0.33	1.07	0.02	48.28	m
	1.73±0.15	1.61±0.18	3.34±0.33	1.07	0.02	48.28	m
VIII	1.84±0.24	1.50±0.12	3.34±0.35	1.23	0.02	44.83	m
	1.84±0.24	1.50±0.12	3.34±0.35	1.23	0.02	44.83	m
IX	1.84±0.24	1.50±0.12	3.34±0.35	1.23	0.02	44.83	m
	1.84±0.24	1.50±0.12	3.34±0.35	1.23	0.02	44.83	m
X	1.84±0.24	1.50±0.12	3.34±0.35	1.23	0.02	44.83	m
	1.84±0.24	1.50±0.12	3.34±0.35	1.23	0.02	44.83	m
XI	1.61±0.15	1.50±0.13	3.11±0.28	1.08	0.01	48.15	m
	1.61±0.15	1.50±0.13	3.11±0.28	1.08	0.01	48.15	m
XII	1.61±0.15	1.50±0.13	3.11±0.28	1.08	0.01	48.15	m
	1.61±0.15	1.50±0.13	3.11±0.28	1.08	0.01	48.15	m
XIII	1.61±0.18	1.50±0.15	3.11±0.34	1.08	0.01	48.15	m
	1.61±0.18	1.50±0.15	3.11±0.34	1.08	0.01	48.15	m

Table continued

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
XIV	1.50±0.13	1.50±0.15	2.99±0.28	1.00	0.01	50.00	m
	1.50±0.13	1.50±0.15	2.99±0.28	1.00	0.01	50.00	m
XV	1.50±0.13	1.50±0.15	2.99±0.28	1.00	0.01	50.00	m
	1.50±0.13	1.50±0.15	2.99±0.28	1.00	0.01	50.00	m
XVI	1.50±0.13	1.38±0.09	2.88±0.22	1.08	0.01	48.00	m
	1.50±0.13	1.38±0.09	2.88±0.22	1.08	0.01	48.00	m
XVII	1.50±0.13	1.38±0.10	2.88±0.23	1.08	0.01	48.00	m
	1.50±0.13	1.38±0.10	2.88±0.23	1.08	0.01	48.00	m
XVIII	1.38±0.09	1.38±0.12	2.76±0.21	1.00	0.01	50.00	m
	1.38±0.09	1.38±0.12	2.76±0.21	1.00	0.01	50.00	m
XIX	1.38±0.09	1.38±0.12	2.76±0.21	1.00	0.01	50.00	m
	1.38±0.09	1.38±0.12	2.76±0.21	1.00	0.01	50.00	m
XX	1.38±0.12	1.38±0.15	2.76±0.27	1.00	0.01	50.00	m
	1.38±0.12	1.38±0.15	2.76±0.27	1.00	0.01	50.00	m
XXI	1.38±0.13	1.38±0.15	2.76±0.28	1.00	0.01	50.00	m
	1.38±0.13	1.38±0.15	2.76±0.28	1.00	0.01	50.00	m
XXII	1.38±0.13	1.38±0.15	2.76±0.28	1.00	0.01	50.00	m
	1.38±0.13	1.38±0.15	2.76±0.28	1.00	0.01	50.00	m
XXIII	1.38±0.13	1.38±0.15	2.76±0.28	1.00	0.01	50.00	m
	1.38±0.13	1.27±0.09	2.65±0.22	1.09	0.01	47.83	m
XXIV	1.38±0.13	1.27±0.10	2.65±0.23	1.09	0.01	47.83	m
	1.38±0.13	1.27±0.10	2.65±0.23	1.09	0.01	47.83	m
XXV	1.38±0.15	1.27±0.10	2.65±0.25	1.09	0.01	47.83	m
	1.38±0.15	1.27±0.10	2.65±0.25	1.09	0.01	47.83	m
XXVI	1.38±0.15	1.27±0.10	2.65±0.25	1.09	0.01	47.83	m
	1.38±0.15	1.27±0.10	2.65±0.25	1.09	0.01	47.83	m
XXVII	1.38±0.18	1.27±0.12	2.65±0.30	1.09	0.01	47.83	m
	1.38±0.18	1.27±0.12	2.65±0.30	1.09	0.01	47.83	m
XXVIII	1.27±0.12	1.27±0.13	2.53±0.24	1.00	0.01	50.00	m
	1.27±0.12	1.27±0.13	2.53±0.24	1.00	0.01	50.00	m

Table continued

Pair	Long arm (l) µm ($\bar{x}$ ± SD)	Short arm (s) µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$ ± SD)	Arm ratio (l/s)	Relative length (RL)	Centro-meric index (CI)	Centro-meric type (CT)
XXIX	1.27±0.12	1.27±0.13	2.53±0.24	1.00	0.01	50.00	m
	1.27±0.12	1.27±0.13	2.53±0.24	1.00	0.01	50.00	m
XXX	1.27±0.12	1.27±0.15	2.53±0.26	1.00	0.01	50.00	m
	1.27±0.12	1.27±0.15	2.53±0.26	1.00	0.01	50.00	m
XXXI	1.27±0.12	1.15±0.09	2.42±0.20	1.10	0.01	47.62	m
	1.27±0.12	1.15±0.09	2.42±0.20	1.10	0.01	47.62	m
XXXII	1.27±0.13	1.15±0.09	2.42±0.22	1.10	0.01	47.62	m
	1.27±0.13	1.15±0.09	2.42±0.22	1.10	0.01	47.62	m
XXXIII	1.15±0.09	1.15±0.09	2.30±0.18	1.00	0.01	50.00	m
	1.15±0.09	1.15±0.09	2.30±0.18	1.00	0.01	50.00	m
XXXIV	1.15±0.09	1.15±0.09	2.30±0.18	1.00	0.01	50.00	m
	1.15±0.09	1.15±0.09	2.30±0.18	1.00	0.01	50.00	m
XXXV	1.15±0.10	1.09±0.07	2.24±0.17	1.05	0.01	48.72	m
	1.15±0.10	1.09±0.07	2.24±0.17	1.05	0.01	48.72	m
XXXVI	1.15±0.12	1.09±0.09	2.24±0.21	1.05	0.01	48.72	m
	1.15±0.12	1.09±0.09	2.24±0.21	1.05	0.01	48.72	m
XXXVII	1.15±0.14	1.09±0.12	2.24±0.26	1.05	0.01	48.72	m
	1.15±0.14	1.09±0.12	2.24±0.26	1.05	0.01	48.72	m
XXXVIII	1.04±0.09	1.04±0.09	2.07±0.17	1.00	0.01	50.00	m
	1.04±0.09	1.04±0.09	2.07±0.17	1.00	0.01	50.00	m
XXXIX	0.98±0.07	0.98±0.06	1.96±0.12	1.00	0.01	50.00	m
	0.98±0.07	0.98±0.06	1.96±0.12	1.00	0.01	50.00	m
TCL=220.28							

m = Metacentric chromosome
sm = Sub-metacentric chromosome
* = Satellite
TCL = Total Chromatin Length
$\bar{x}$ = Mean
SD = Standard Deviation

Table 24. Comparative orcein-staind karyotype analysis of six wild *Corchorus* L. species.

Species	2n	Range of chromosomal length µm ($\bar{x}$ ± SD)	Total length (T) µm ($\bar{x}$)	Arm ratio (l/s)	Relative length (RL)
C. brevicornatus	14	2.49±0.46–5.29±0.20	50.87	14m	Symmetric
C. septentrionalis	14	2.11±0.24–3.43±0.12	40.35	14m	Symmetric
C. urticifolius	14	2.80±0.29–4.58±0.69	51.27	14m	Symmetric
C. hirtus	28	1.92±0.29–3.43±0.45	74.19	28m	Symmetric
C. siliquosus	28	1.65±0.18–3.39±0.26	67.22	28m	Symmetric
C. pinnatipartitus	78	1.96±0.12–4.37±0.48	220.28	72m+4sm	Relatively Asymmetric

$\bar{x}$ = Mean
SD = Standard Deviation

Table 25. Comparative karyosystematic analysis of six wild *Corchorus* species.

Features	*C. brevicornatus*	*C. septentrionalis*	*C. urticifolius*	*C. hirtus*	*C. siliquosus*	*C. pinnatipartitus*
2n	14	14	14	28	28	78
Satellite	4	2	4	2	2	--
CF	14m	14m	14m	28m	28m	76m + 2sm
THL(µm)	25.43	20.17	25.63	36.80	33.59	112.26
CVCI	4.52	3.22	4.51	3.21	3.36	5.87
CVCL	23.34	15.39	15.49	15.88	19.26	19.23
MCA	10	3.35	9.98	4.40	4.65	4.22
AsK%	54.99	51.56	55.21	52.22	52.49	52.49
TF%	45.01	48.44	44.79	47.78	47.51	47.51
Syi%	81.84	93.94	81.13	91.49	90.53	90.51
Rec%	68.67	84.01	79.94	76.64	70.78	65.72
A1	0.18	0.06	0.18	0.08	0.09	0.08
A2	0.23	0.15	0.15	0.16	0.19	0.19
A	0.10	0.03	0.10	0.04	0.05	0.04
DI	9.79	7.55	7.13	7.52	9.21	9.61
AI	1.06	0.50	0.70	0.51	0.65	1.13
Category	1B	1A	1A	1A	1B	1B

2n = Somatic chromosome number, CF = Centromeric formula, THL = Total haploid chromosome length (µm), CV_{CI} = Coeffient of variation of centromeric index, CV_{CL} = Coefficient of chromosome length, M_{CA} = Mean centromeric asymmetry, AsK% = Karyotype asymmetry index, TF% = Total form value, Syi% = karyotype symmetry index, Rec% = The index of chromosomal size resemblance, A_1 = Intrachromosomal asymmetry index, A_2 = Interchromosomal asymmetry index, A = Degree of asymmetry of karyotypes, AI = asymmetry index, DI = The dispersion index, m = metacentric chromosome, sm = Sub-metacentric chromosome.

Table 26. Comparative CMA-banding analysis of six wild *Corchorus* L. species.

Species	No. of CMA-bands	Total length of CMA-positive banded region (µm) ($\bar{x}$)	% of CMA-positive banded region (µm)	CMA- banded karyotypic formulae
C. brevicornatus	8	5.64	11.09	$4\alpha + 4\gamma + 6\delta$
C. septentrionalis	4	3.22	7.98	$2\alpha + 2\gamma + 10\delta$
C. urticifolius	8	6.79	13.24	$4\gamma + 4\lambda + 6\delta$
C. hirtus	9	7.13	9.61	$3\gamma + 6\phi + 19\delta$
C. siliquosus	6	2.99	4.45	$2\gamma + 2\beta + 24\delta$
C. pinnatipartitus	23	27.72	12.58	$4\alpha + 12\gamma + 2\varphi + 2\,\Omega + 3\phi + 55\delta$

Classification of CMA-positive bands

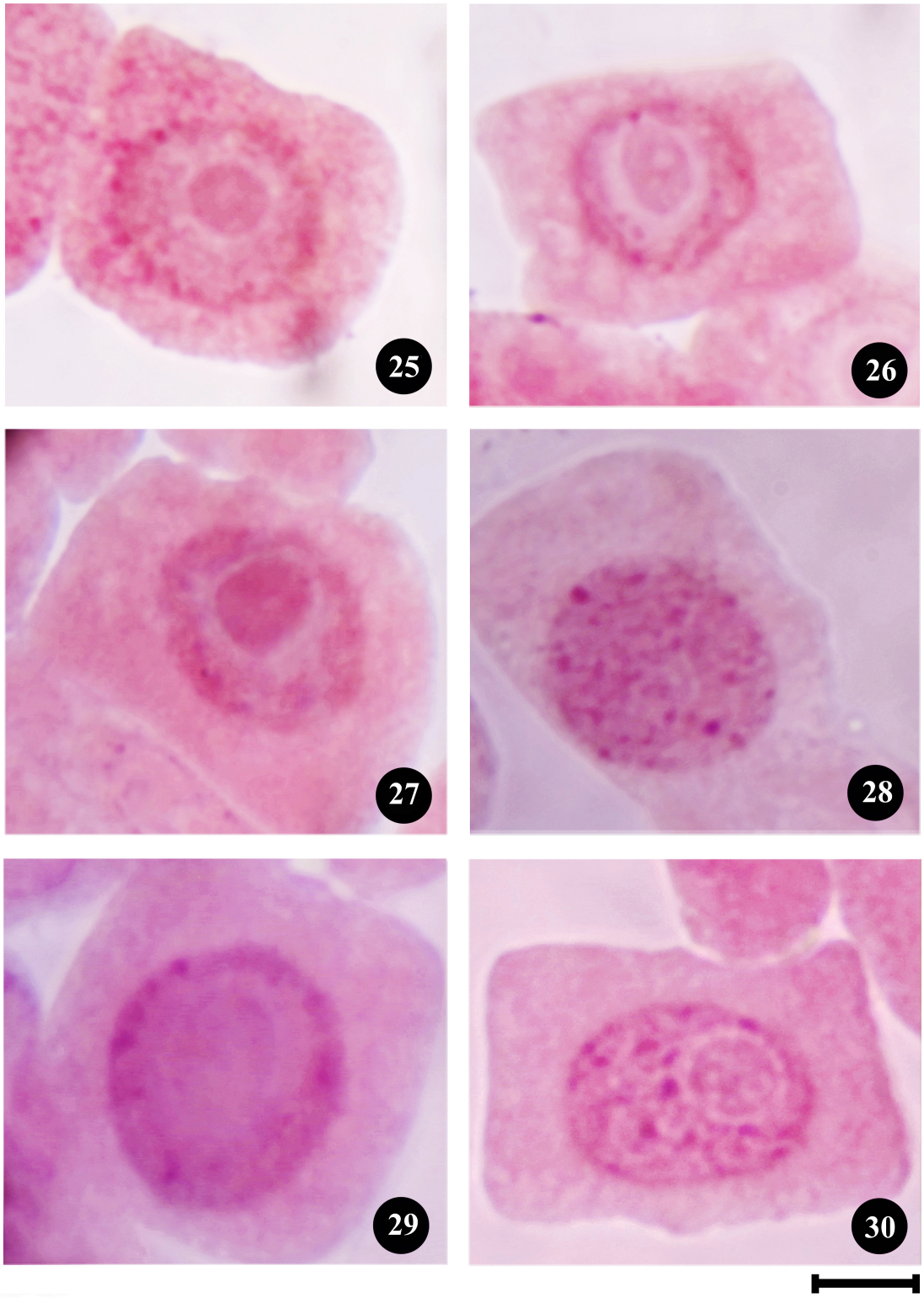
α = Band in whole chromosome
γ = Band in whole short arm
λ = Band in short arm with centromere
ϕ = band in centromere
β = Band in two terminal
φ = Band in long arm
Ω = Band in terminal of long arm
δ = No band
$\bar{x}$ = Mean

Table 27. Comparative DAPI-banding analysis of six wild *Corchorus* L. species.

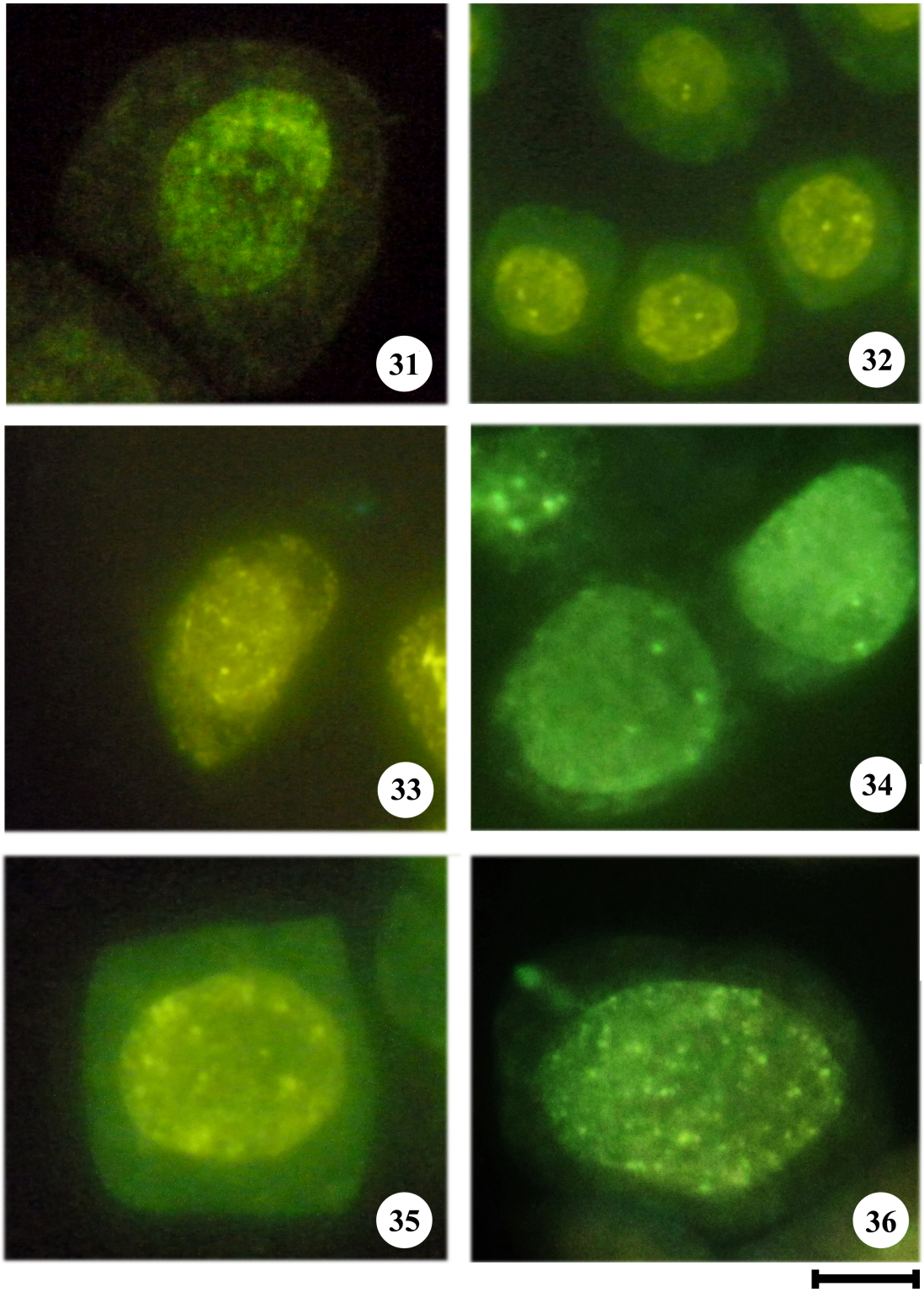
Species	No. of DAPI-bands	Total length of DAPI-positive banded region (µm) ($\bar{x}$)	% of DAPI-positive banded region (µm)	DAPI- banded karyotypic formulae
C. brevicornatus	3	3.22	6.33	3α + 11δ
C. septentrionalis	6	3.68	9.12	4α + 2γ + 8δ
C. urticifolius	6	4.60	8.97	2ϕ + 4γ + 8δ
C. hirtus	6	11.5	15.50	2α + 4γ + 22δ
C. siliquosus	3	2.19	3.26	1α + 2ϕ + 25δ
C. pinnatipartitus	25	38.64	17.54	12γ +6φ + 3α+ 4β + 53δ

Classification of DAPI-positive bands

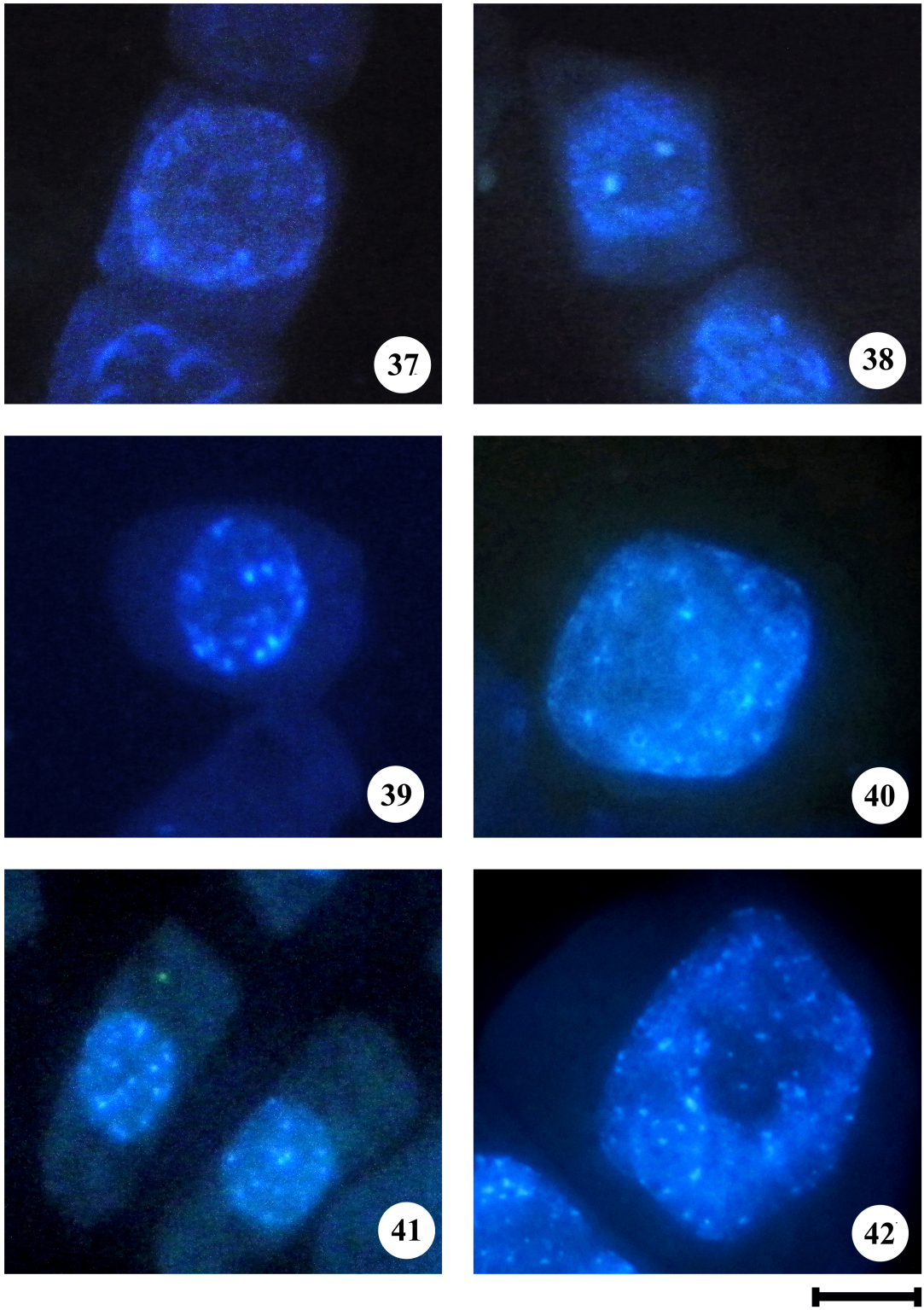
α = Band in whole chromosome
γ = Band in whole short arm
λ = Band in short arm with centromere
ϕ = band in centromere
β = Band in two terminal
φ = Band in long arm
δ = No band
$\bar{x}$ = Mean



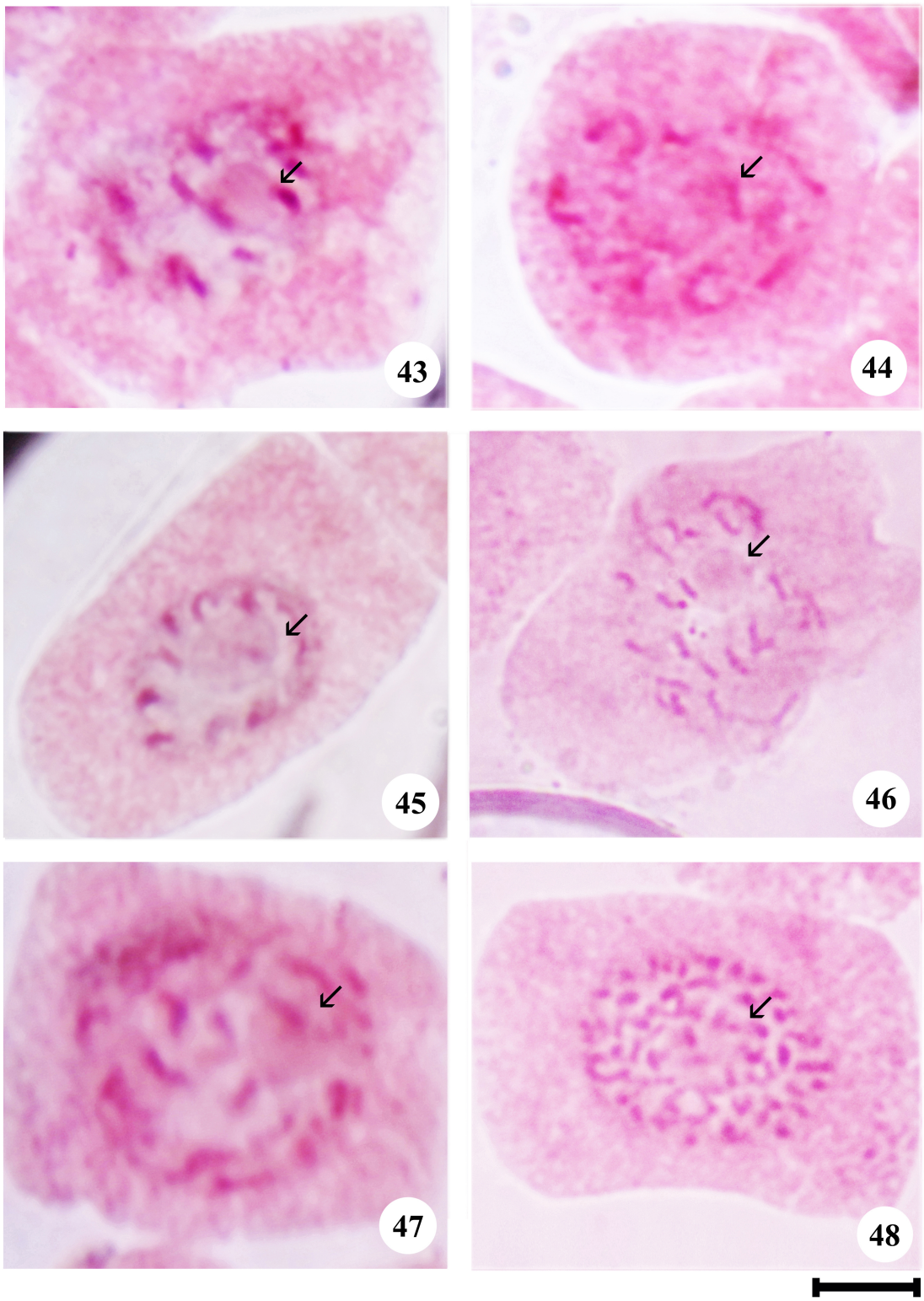
Figs 25-30. Orcein-stained mitotic interphase nuclei of six wild *Corchorus* L. species. 25. *C. brevicornatus*, 26. *C. septentrionalis*, 27. *C. urticifolius*, 28. *C. hirtus*, 29. *C. siliquosus* and 30. *C. pinnatipartitus*. Bar = 10 µm.



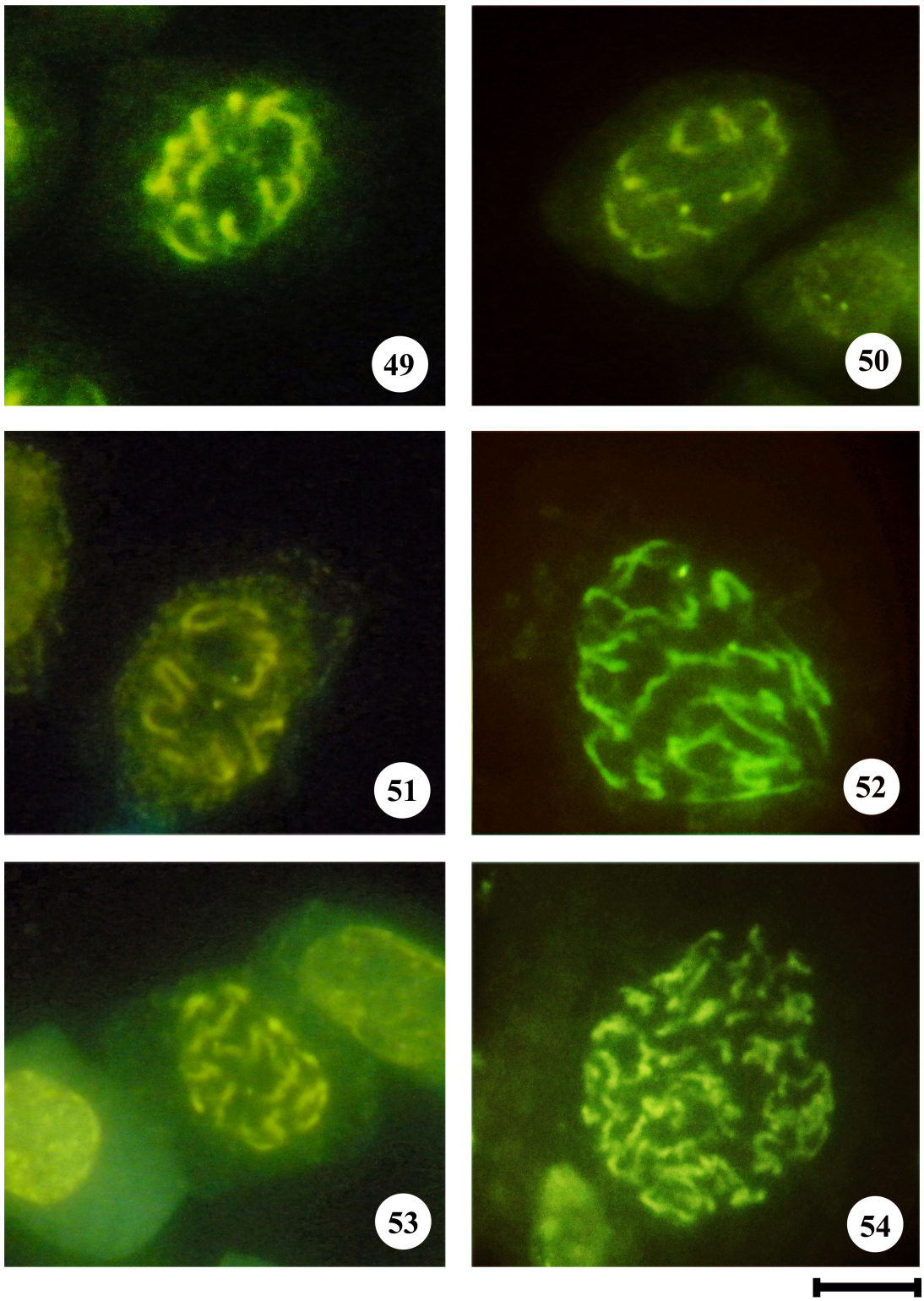
Figs 31-36. CMA-stained mitotic interphase nuclei of six wild *Corchorus* L. species. 31. *C. brevicornatus*, 32. *C. septentrionalis*, 33. *C. urticifolius*, 34. *C. hirtus*, 35. *C. siliquosus* and 36. *C. pinnatipartitus*. Bar = 10 µm.



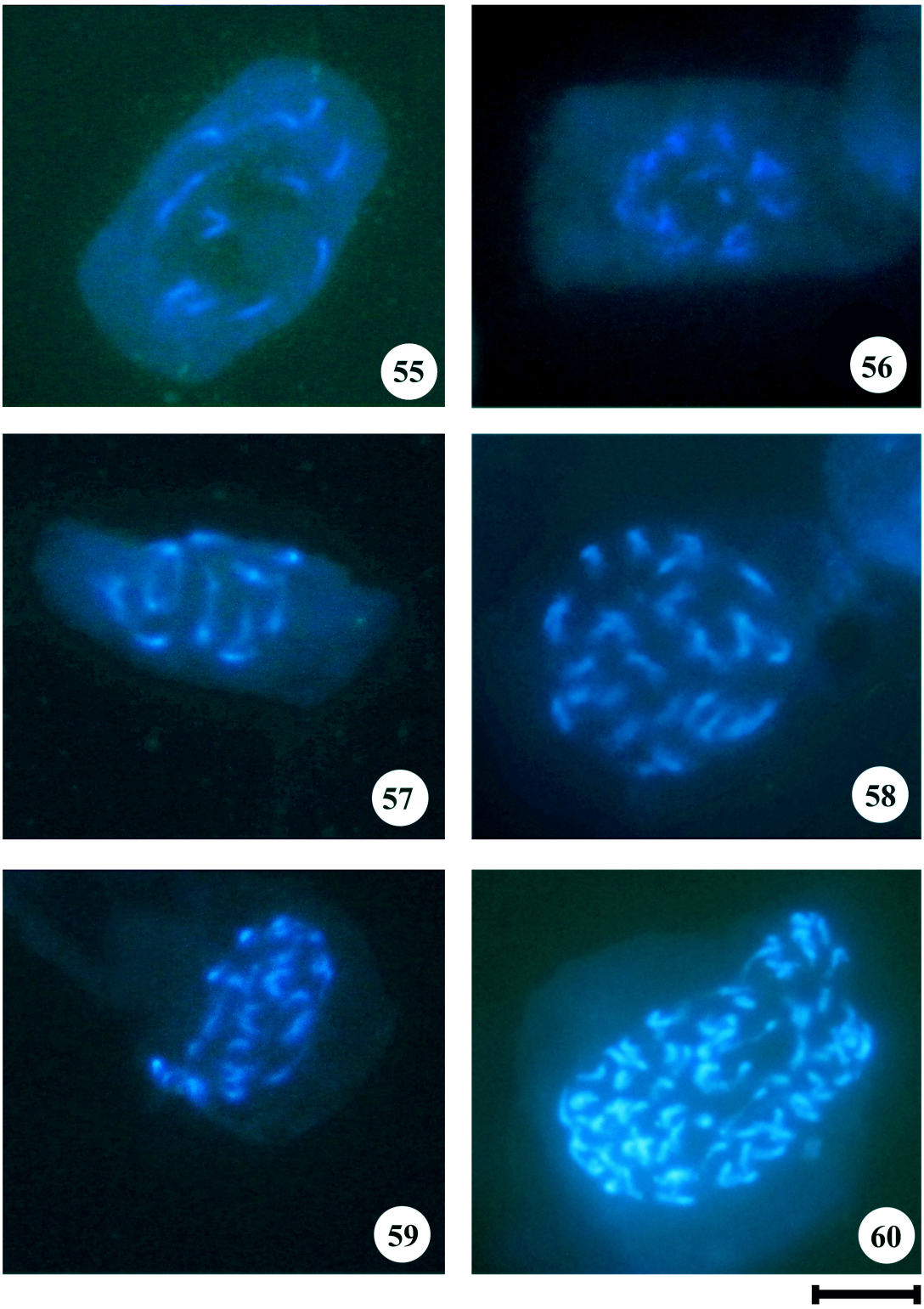
Figs 37-42. DAPI-stained mitotic interphase nuclei of six wild *Corchorus* L. species. 37. *C. brevicornatus*, 38. *C. septentrionalis*, 39. *C. urticifolius*, 40. *C. hirtus*, 41. *C. siliquosus* and 42. *C. pinnatipartitus*. Bar = 10 µm.



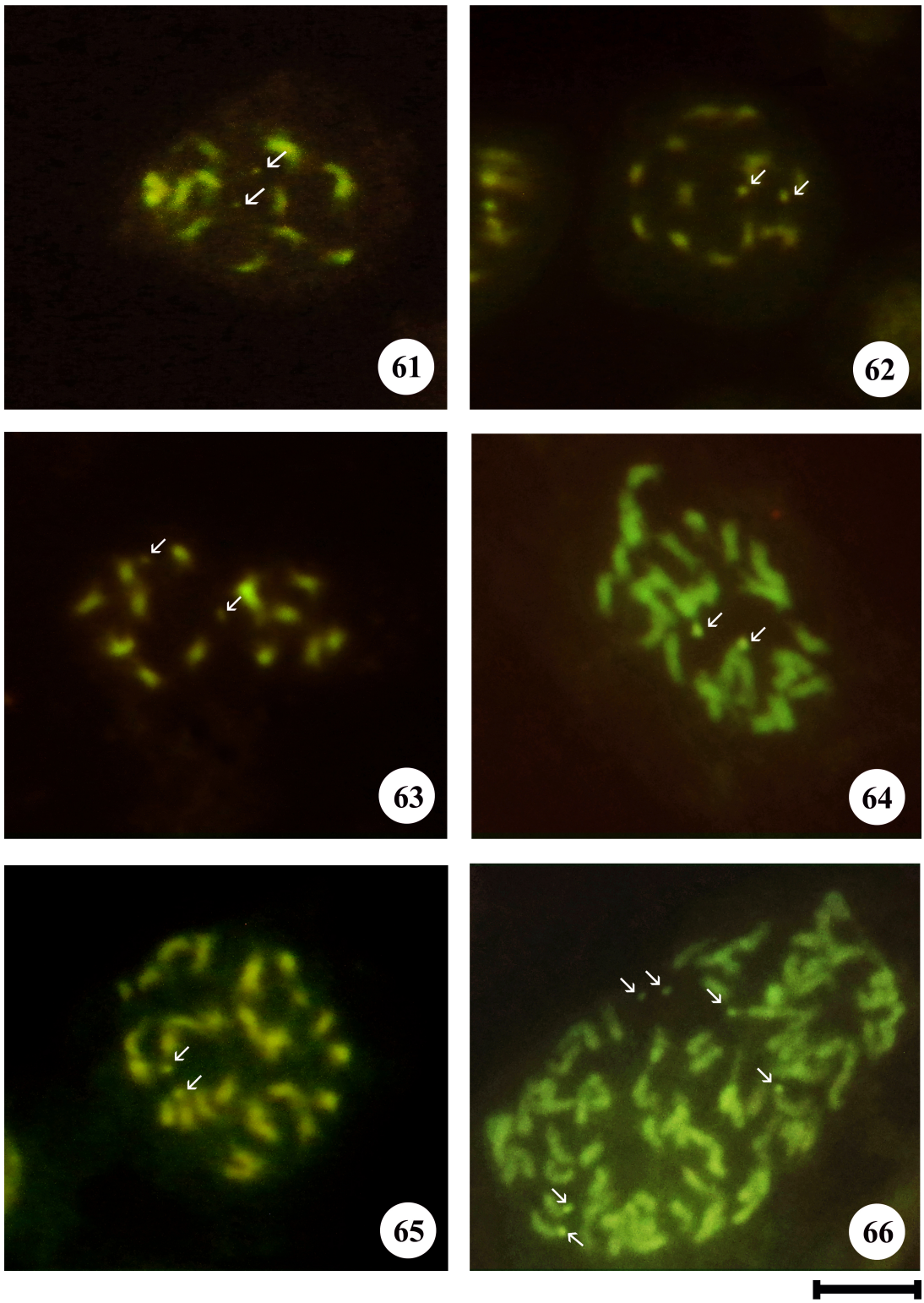
Figs 43-48. Orcein-stained mitotic prophase chromosomes of six wild *Corchorus* L. species. 43. *C. brevicornatus*, 44. *C. septentrionalis*, 45. *C. urticifolius*, 46. *C. hirtus*, 47. *C. siliquosus* and 48. *C. pinnatipartitus*. Arrow indicates nucleolus. Bar = 10 µm.



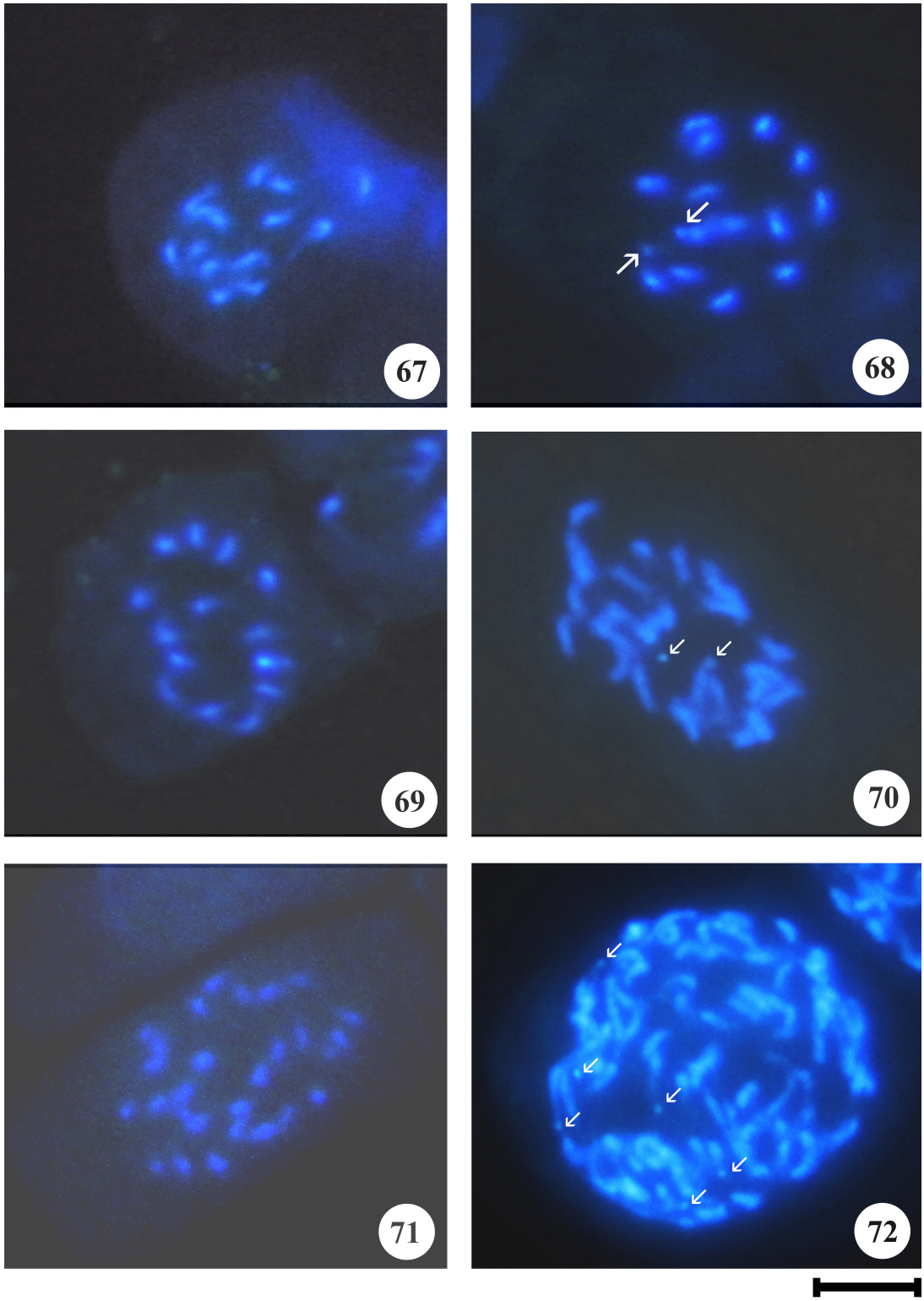
Figs 49-54. CMA-stained mitotic prophase chromosomes of six wild *Corchorus* L. species. 49. *C. brevicornatus*, 50. *C. septentrionalis*, 51. *C. urticifolius*, 52. *C. hirtus*, 53. *C. siliquosus* and 54. *C. pinnatipartitus*. Bar = 10 µm.



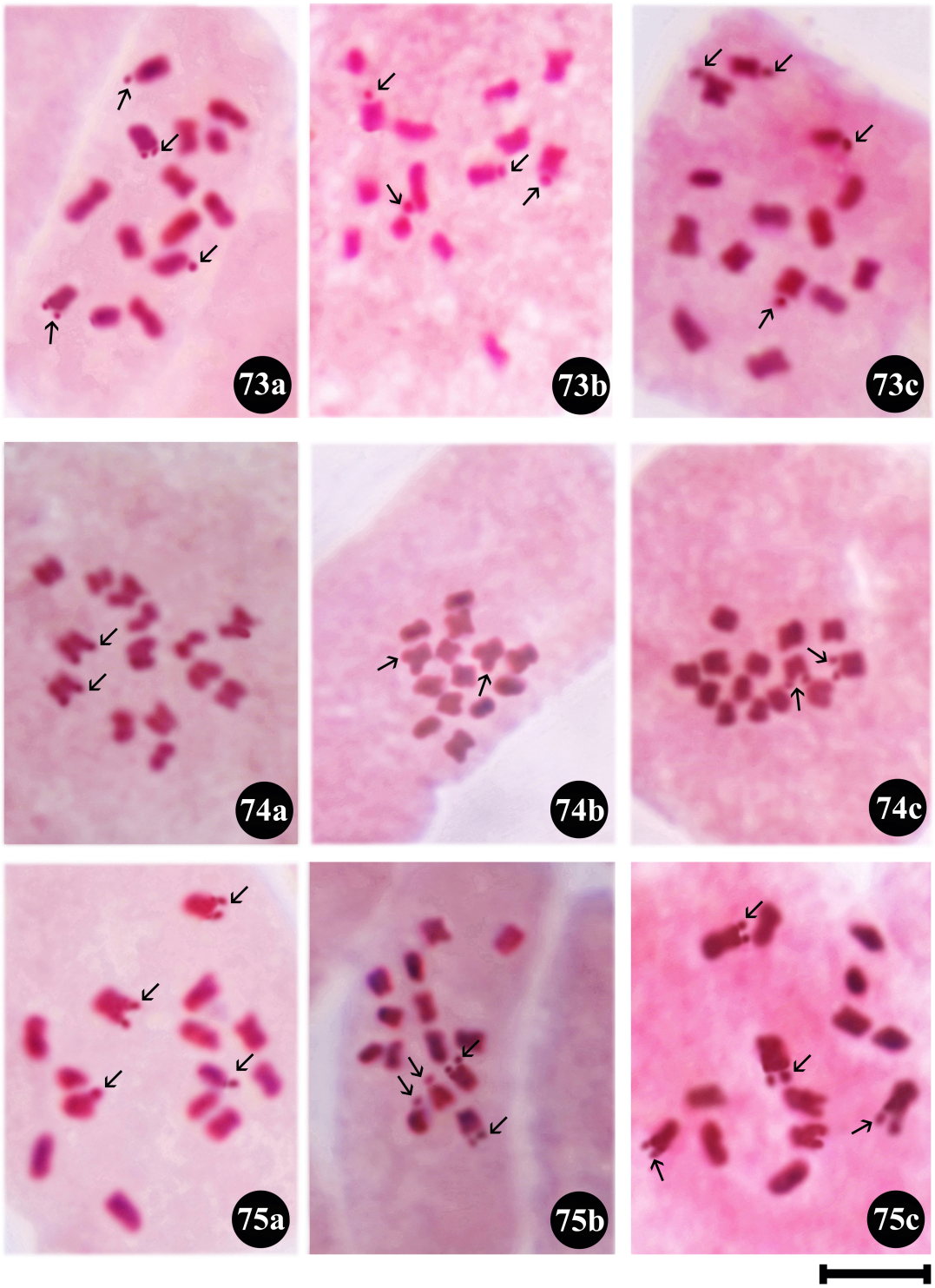
Figs 55-60. DAPI-stained mitotic prophase chromosomes of six wild *Corchorus* L. species. 55. *C. brevicornatus*, 56. *C. septentrionalis*, 57. *C. urticifolius*, 58. *C. hirtus*, 59. *C. siliquosus* and 60. *C. pinnatipartitus*. Bar = 10 µm.



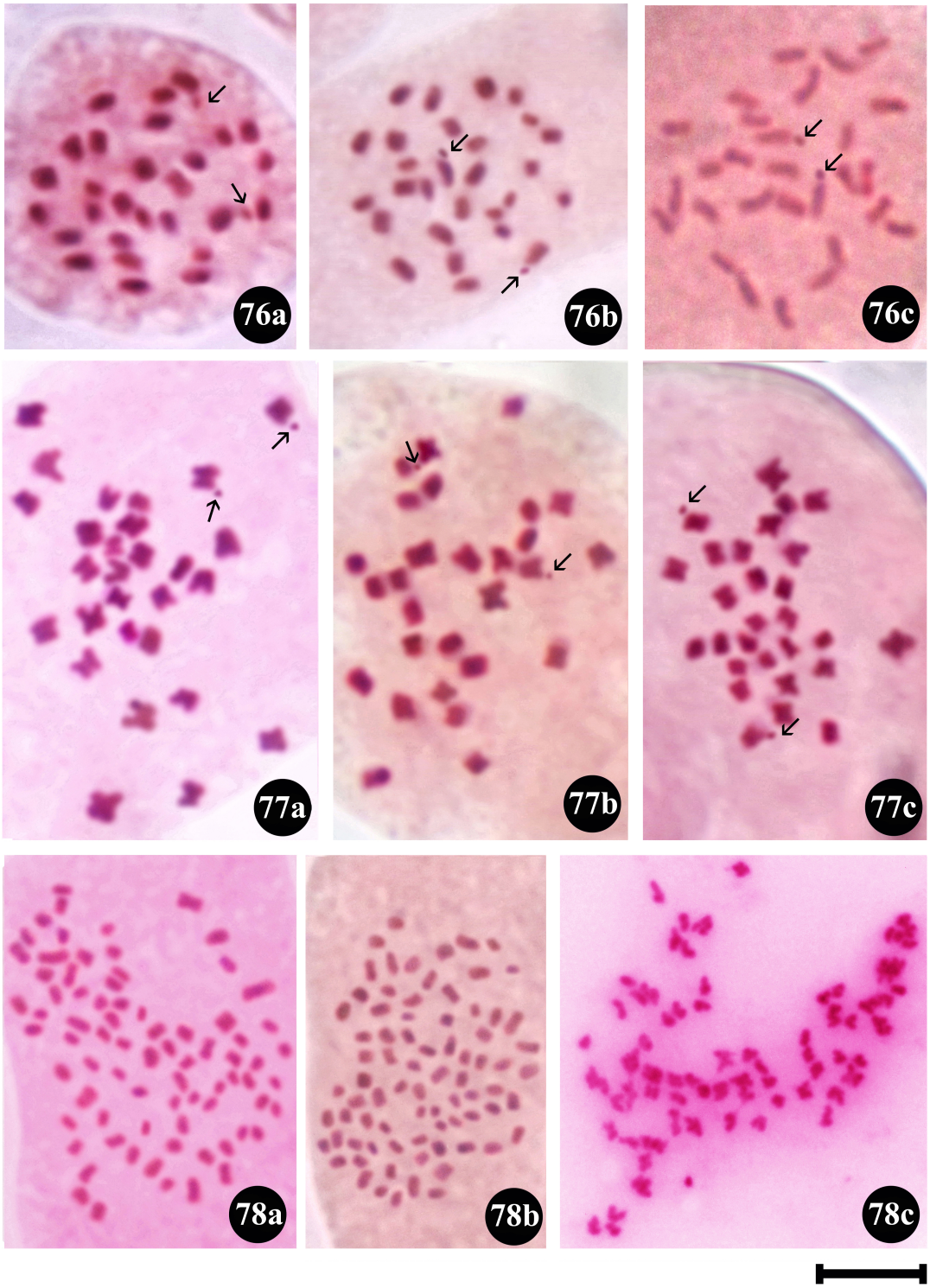
Figs 61-66. CMA-stained mitotic pro-metaphase chromosomes of six wild *Corchorus* L. species. 61. *C. brevicornatus*, 62. *C. septentrionalis*, 63. *C. urticifolius*, 64. *C. hirtus*, 65. *C. siliquosus* and 66. *C. pinnatipartitus.* Arrow indicates satellites. Bar = 10 µm.



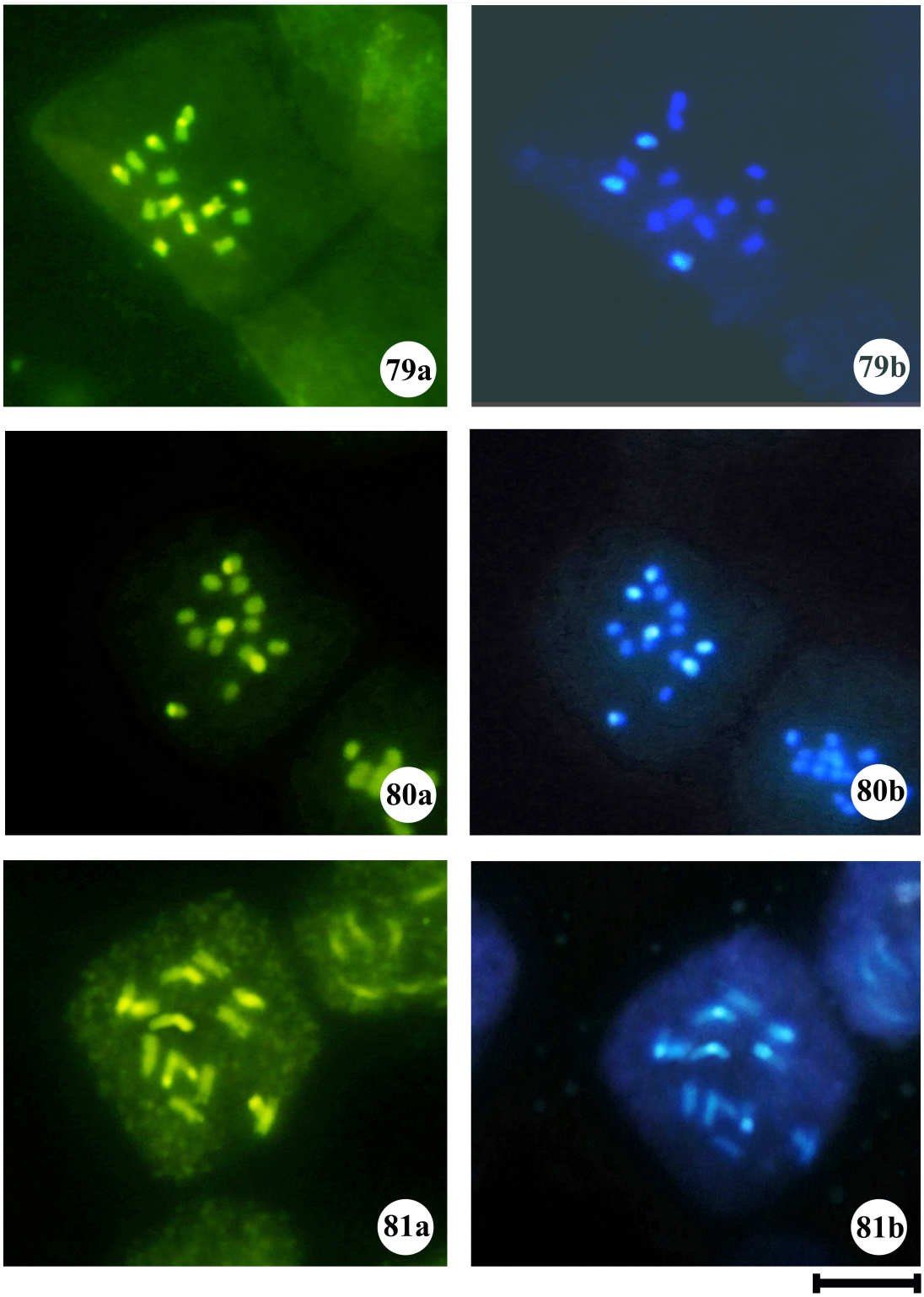
Figs 67-72. DAPI-stained mitotic pro-metaphase chromosomes of six wild *Corchorus* L. species. 67. *C. brevicornatus*, 68. *C. septentrionalis*, 69. *C. urticifolius*, 70. *C. hirtus*, 71. *C. siliquosus* and 72. *C. pinnatipartitus.* Arrow indicates satellites. Bar = 10 µm.



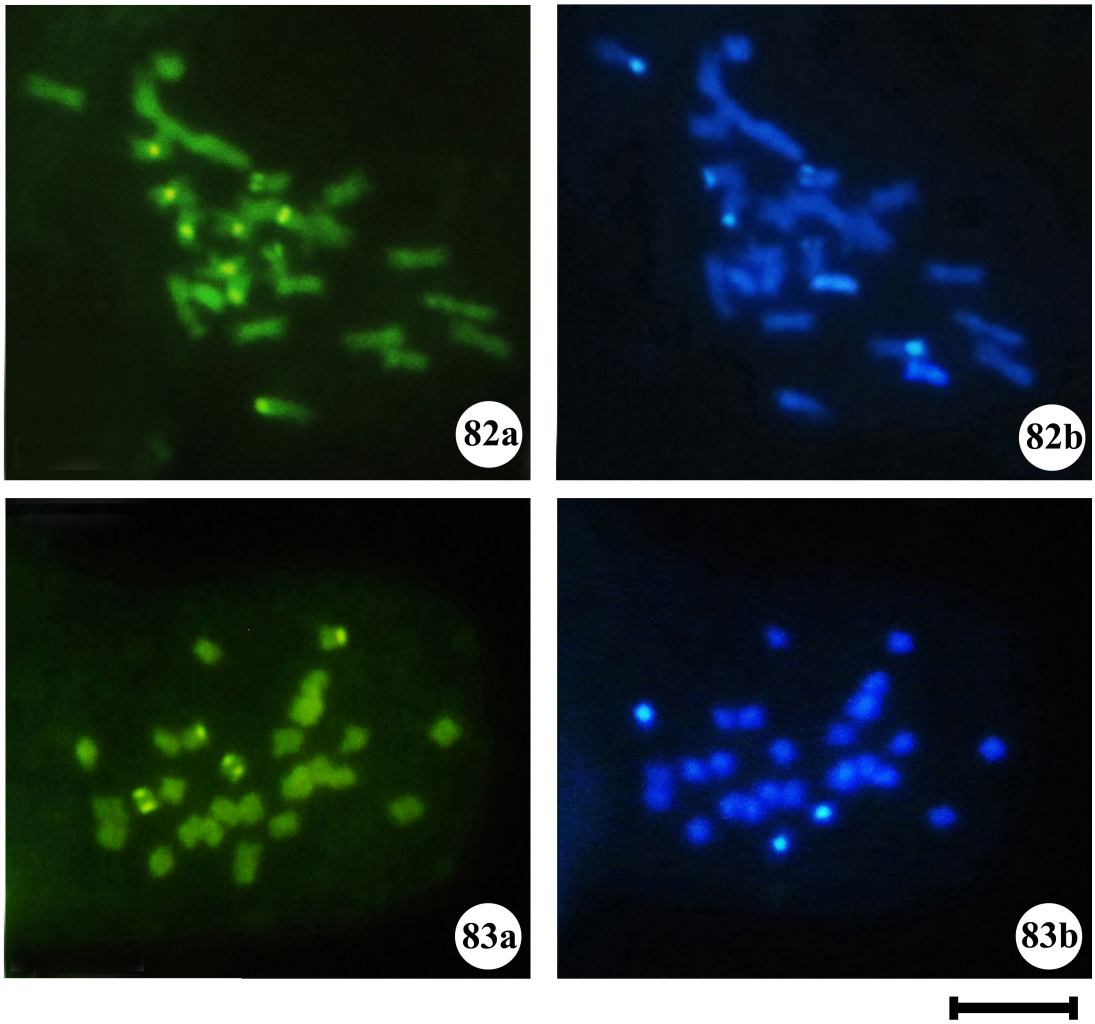
Figs 73-75. Orcein-stained mitotic metaphase chromosomes (three replicates of each showing in a, b and c) of three wild *Corchorus* L. species. 73. *C. brevicornatus*, 74. *C. septentrionalis* and 75. *C. urticifolius*. Arrow indicates satellites. Bar = 10 µm.



Figs 76-78. Orcein-stained mitotic metaphase chromosomes (three replicates of each showing in a, b and c) of three wild *Corchorus* L. species. 76. *C. hirtus*, 77. *C. siliquosus* and 78. *C. pinnatipartitus*. Arrow indicates satellites. Bar = 10 µm.



Figs 79-81. Counter-stained mitotic metaphase chromosomes of three wild *Corchorus* L. species with CMA (a) and DAPI (b) banding. 79. *C. brevicornatus*, 80. *C. septentrionalis* and 81. *C. urticifolius*. Bar = 10 μm.



Figs 82-83. Counter-stained mitotic metaphase chromosomes of two wild *Corchorus* L. species with CMA (a) and DAPI (b) banding. 82. *C. hirtus* and 83. *C. siliquosus*. Bar = 10 μm.

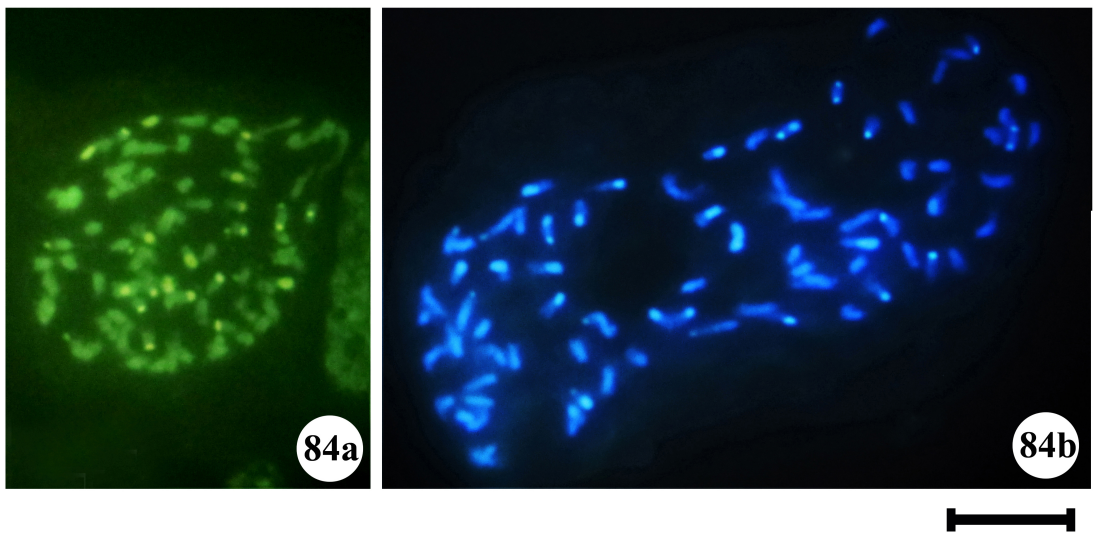
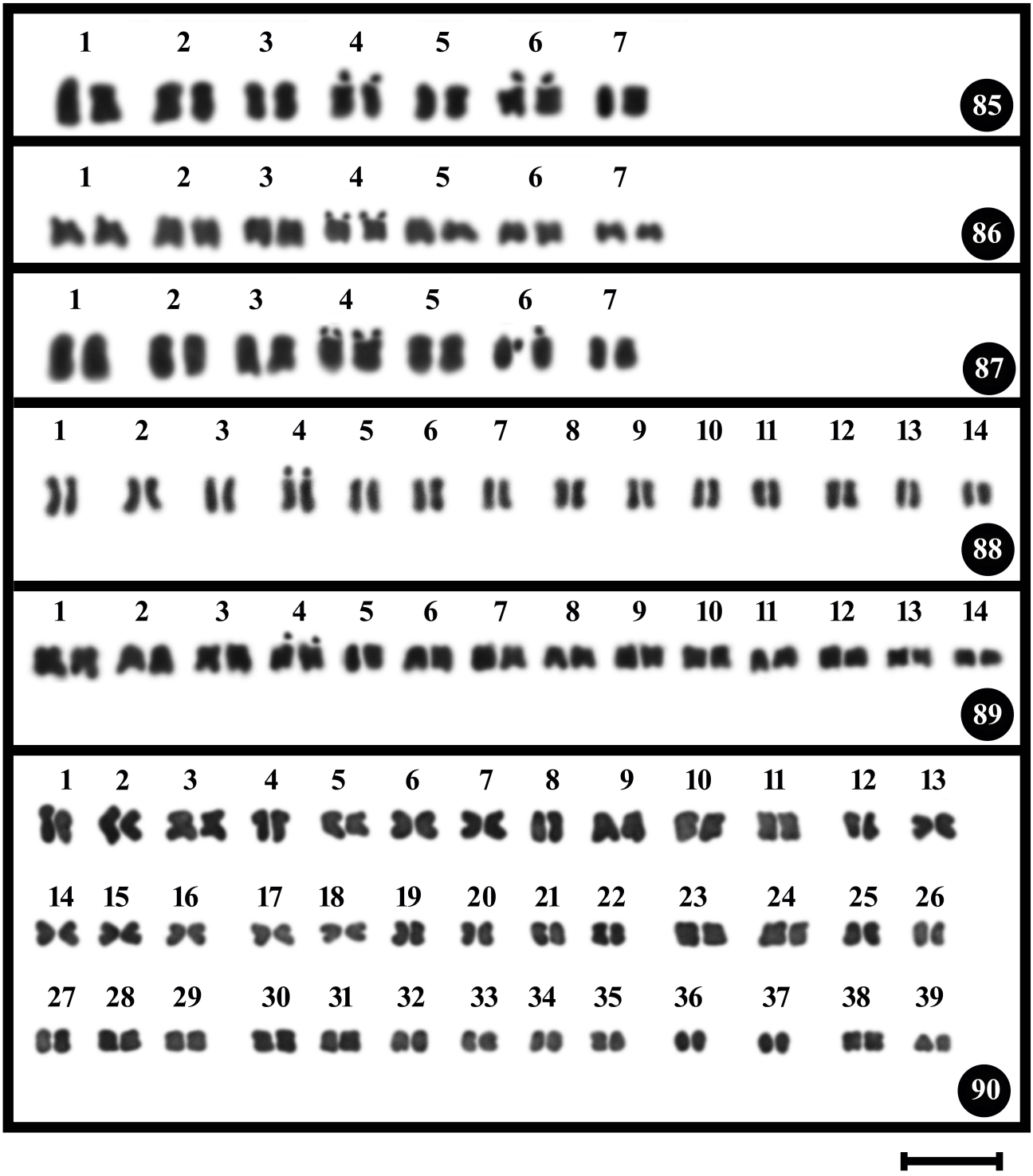


Fig. 84. Mitotic metaphase chromosomes of *C. pinnatipartitus* with CMA (a) and DAPI (b) banding. Bar = 10 μm.



Figs 85-90. Karyotype prepared from orcein-stained mitotic metaphase chromosomes of six wild *Corchorus* L. species. 85. *C. brevicornatus*, 86. *C. septentrionalis*, 87. *C. urticifolius*, 88. *C. hirtus*, 89. *C. siliquosus* and 90. *C. pinnatipartitus.* Bar = 10 µm.

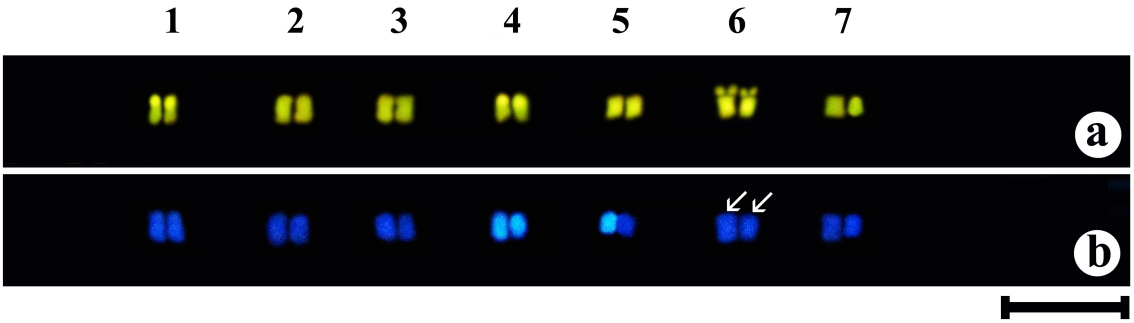


Fig. 91. Karyotype prepared from counter-stained mitotic metaphase chromosomes of *C. brevicornatus* with CMA (a) and DAPI (b) banding, arrow indicates negative DAPI- bands. Bar = 10 μm.

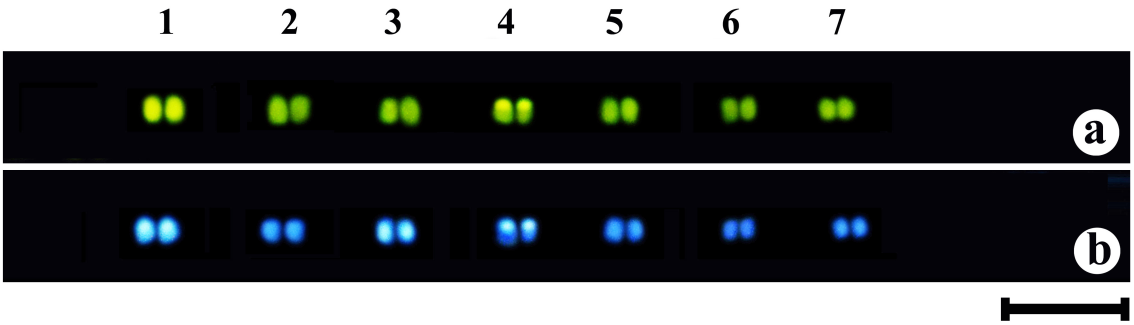


Fig. 92. Karyotype prepared from counter-stained mitotic metaphase chromosomes of *C. septentrionalis* with CMA (a) and DAPI (b) banding. Bar = 10 μm.

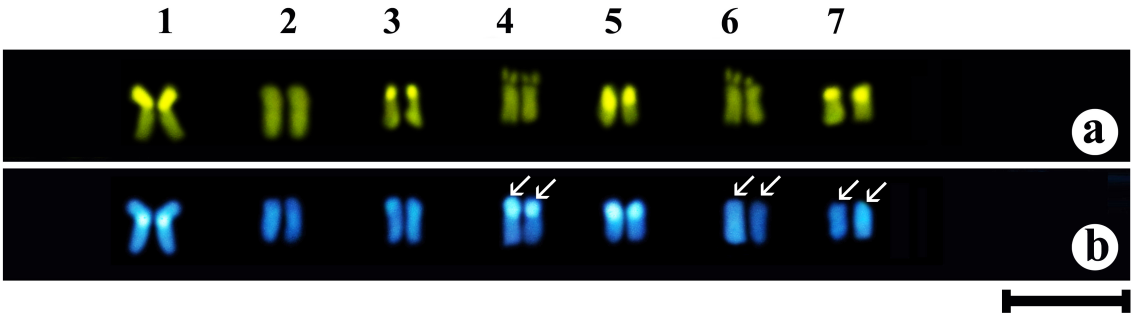


Fig. 93. Karyotype prepared from counter-stained mitotic metaphase chromosomes of *C. urticifolius* with CMA (a) and DAPI (b) banding, arrow indicates negative DAPI- bands. Bar = 10 μm.

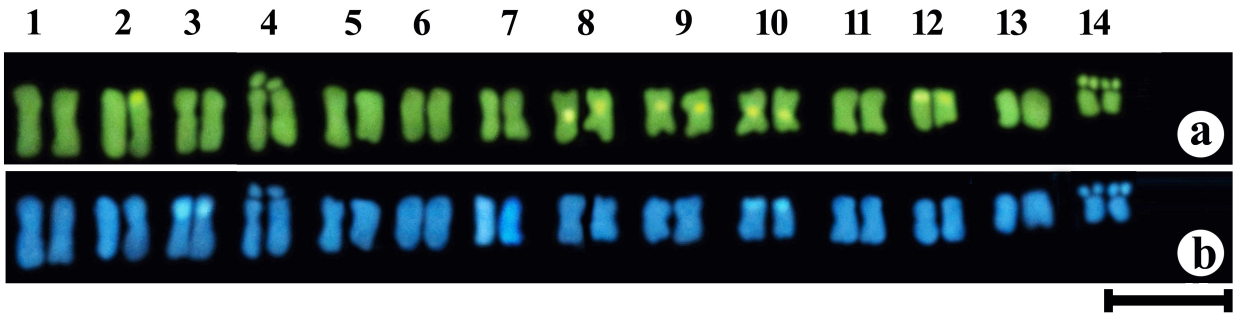


Fig. 94. Karyotype prepared from counter-stained mitotic metaphase chromosomes of *C. hirtus* with CMA (a) and DAPI (b) banding. Bar = 10 µm.

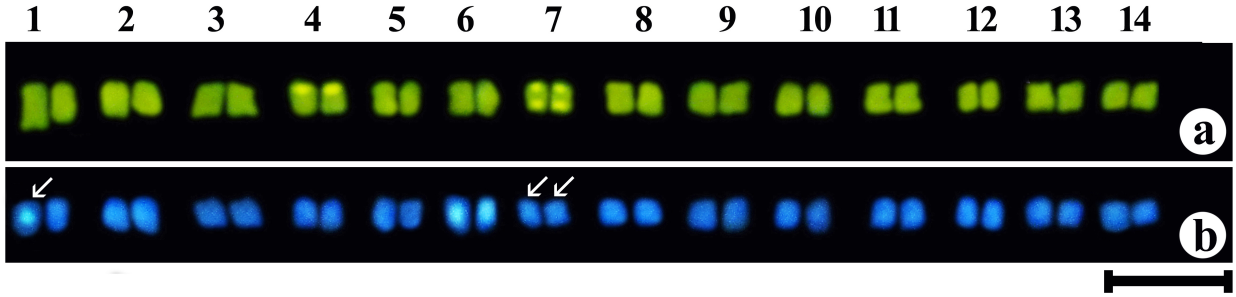


Fig. 95. Karyotype prepared from counter-stained mitotic metaphase chromosomes of *C. siliquosus* with CMA (a) and DAPI (b) banding, arrow indicates negative DAPI- bands. Bar = 10 µm.

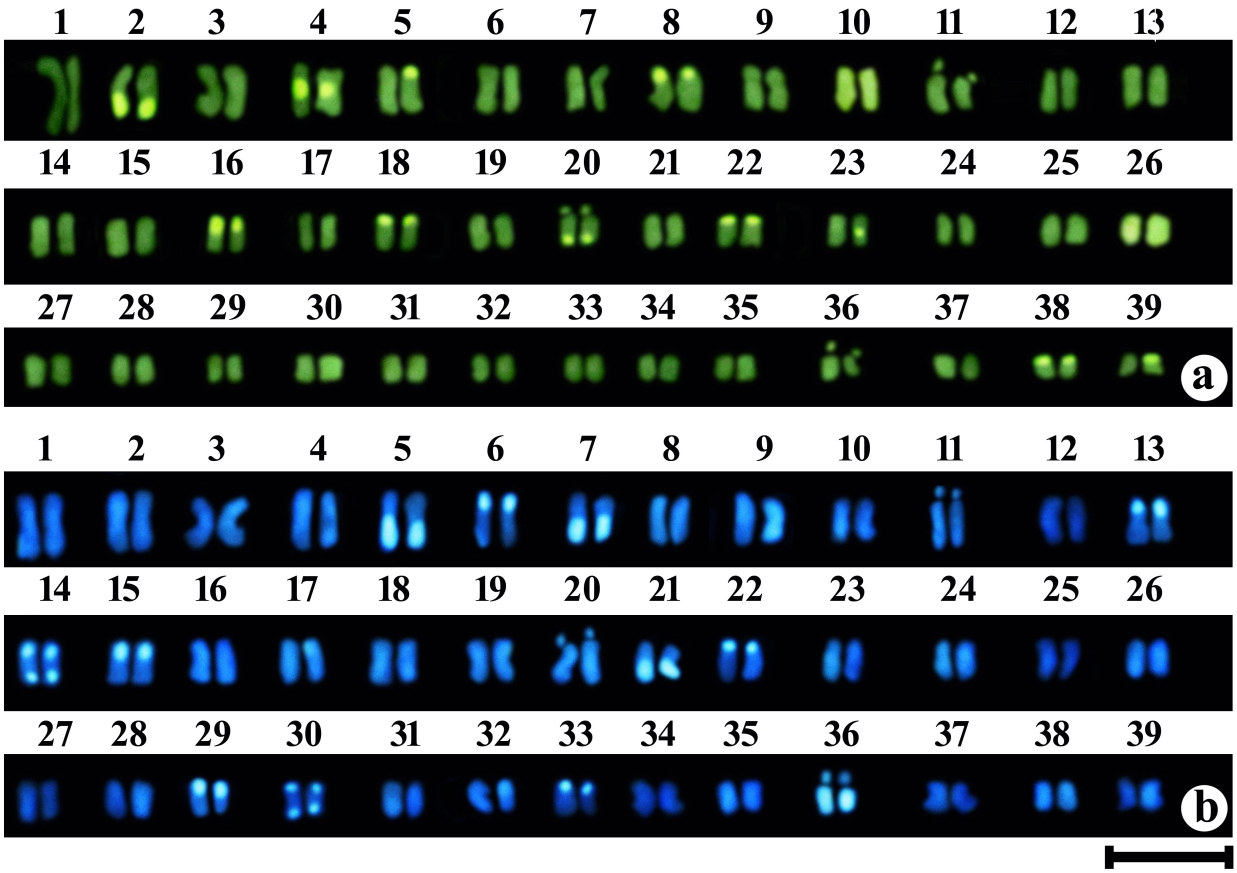
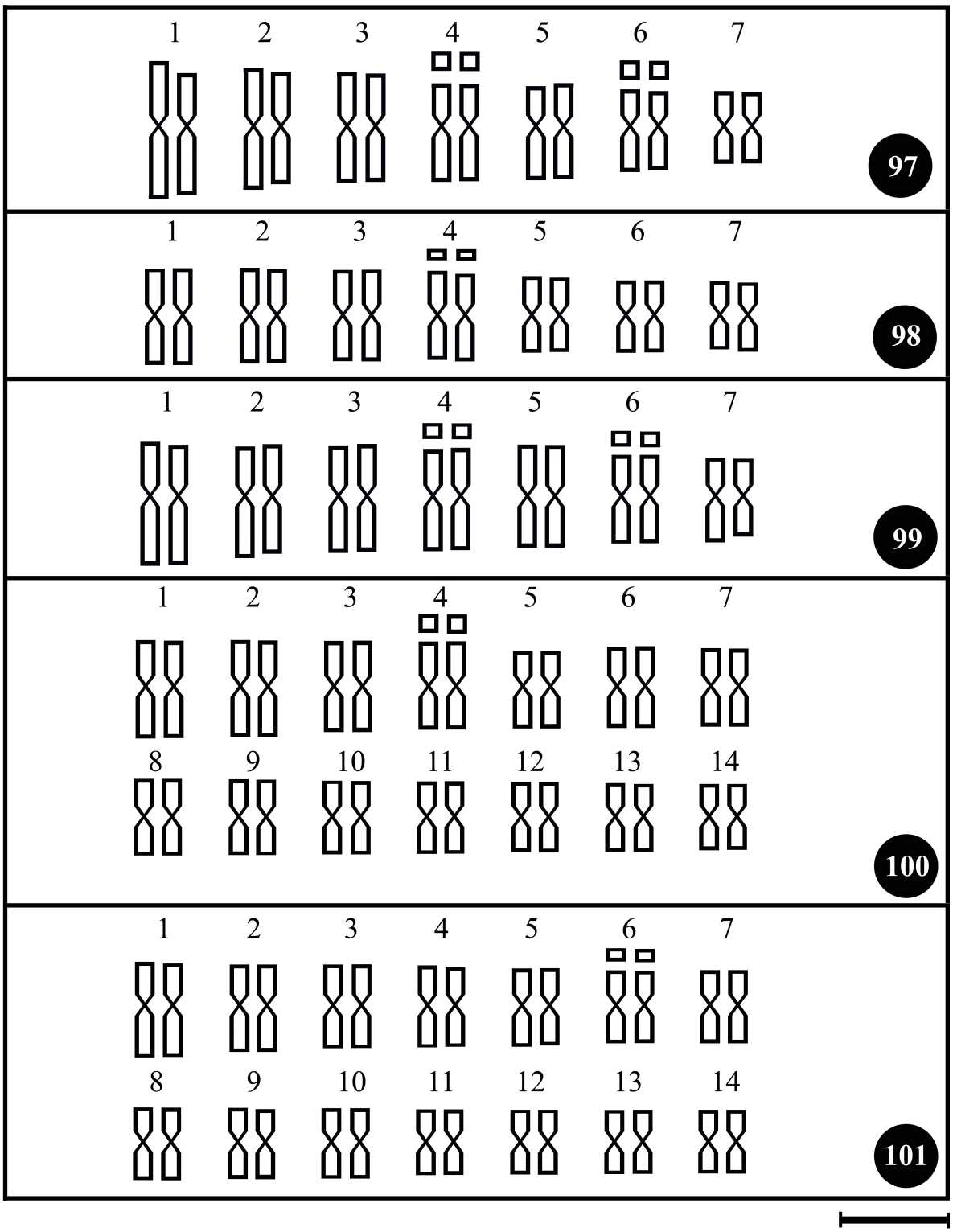


Fig. 96. Karyotype prepared from mitotic metaphase chromosomes of *C. pinnatipartitus* with CMA (a) and DAPI (b) banding. Bar = 10 µm.



Figs 97-101. Idiogram prepared from orcein-stained mitotic metaphase chromosomes of five wild *Corchorus* L. species. 97. *C. brevicornatus*, 98. *C. septentrionalis*, 99. *C. urticifolius*, 100. *C. hirtus* and 101. *C. siliquosus*. Bar = 5 µm.
□ = satellites

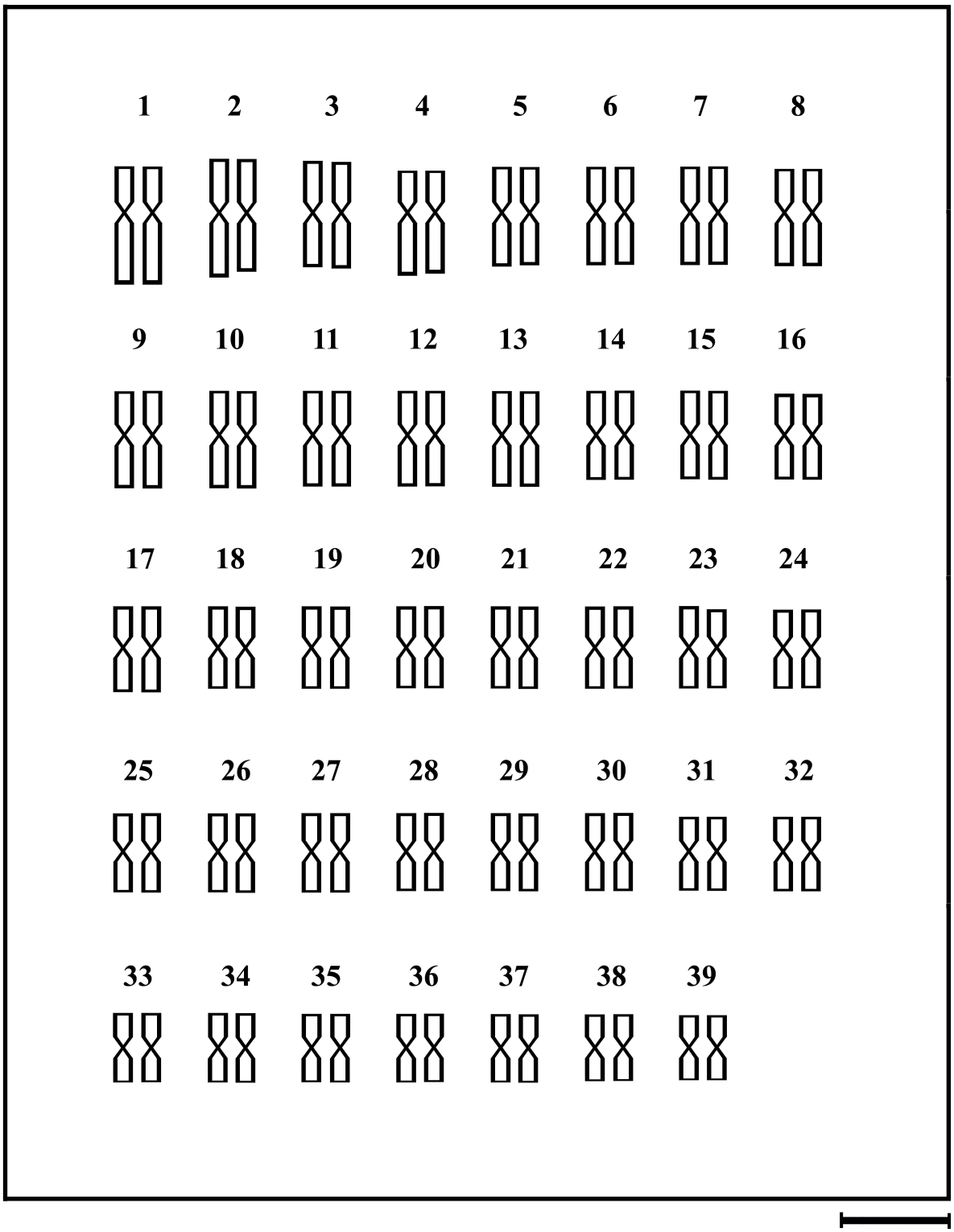
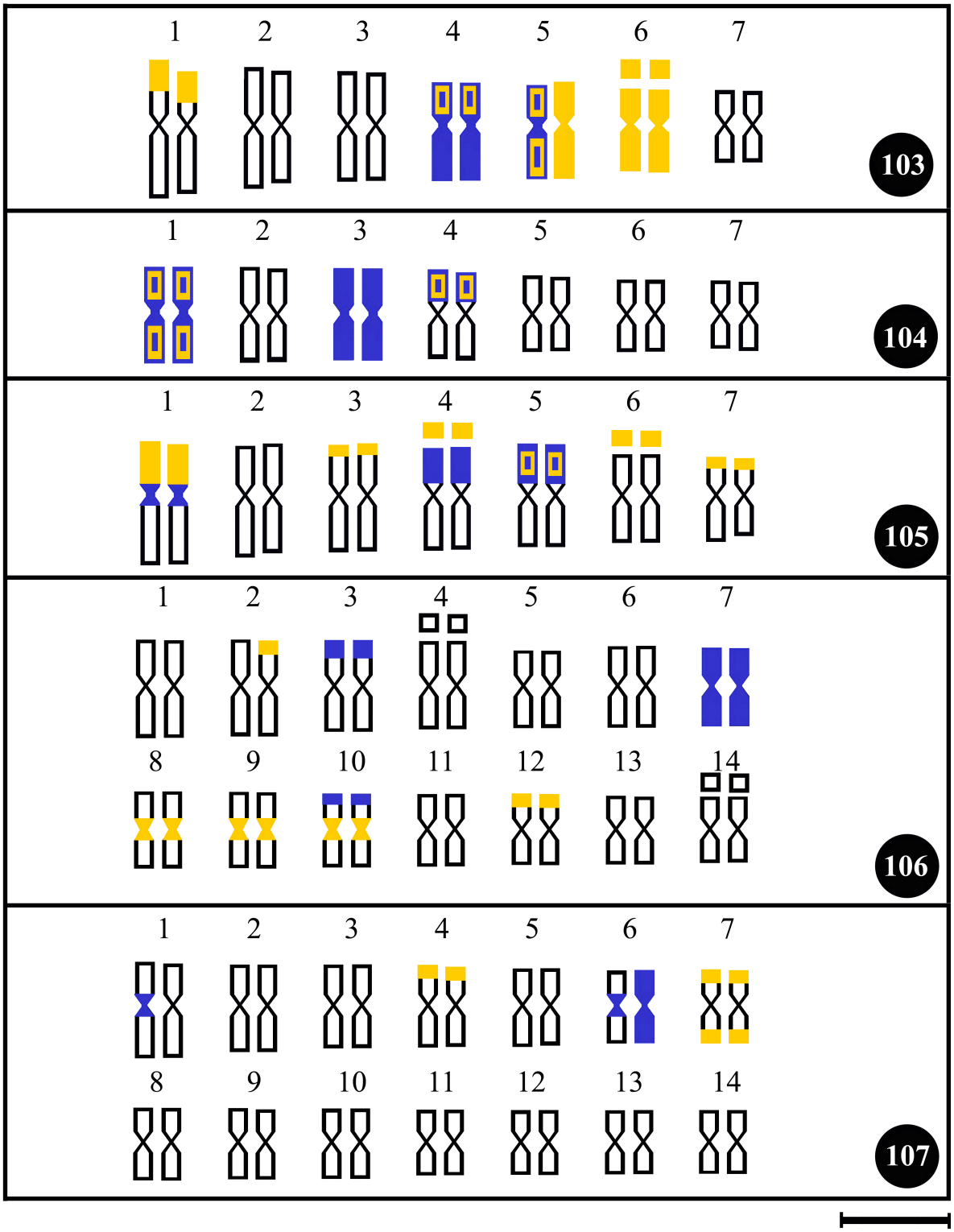


Fig. 102. Idiogram prepared from orcein-stained mitotic metaphase chromosomes of *C. pinnatipartitus*. Bar = 5 μm.



Figs 103-107. Idiogram prepared from counter-stained mitotic metaphase chromosomes of five wild *Corchorus* L. species with CMA- and DAPI-banding. 103. *C. brevicornatus*, 104. *C. septentrionalis*, 105. *C. urticifolius,* 106. *C. hirtus* and 107. *C. siliquosus.* Bar = 5 µm.

= CMA-positive bands
= DAPI-positive bands
= Coexistance of CMA- and DAPI-positive bands

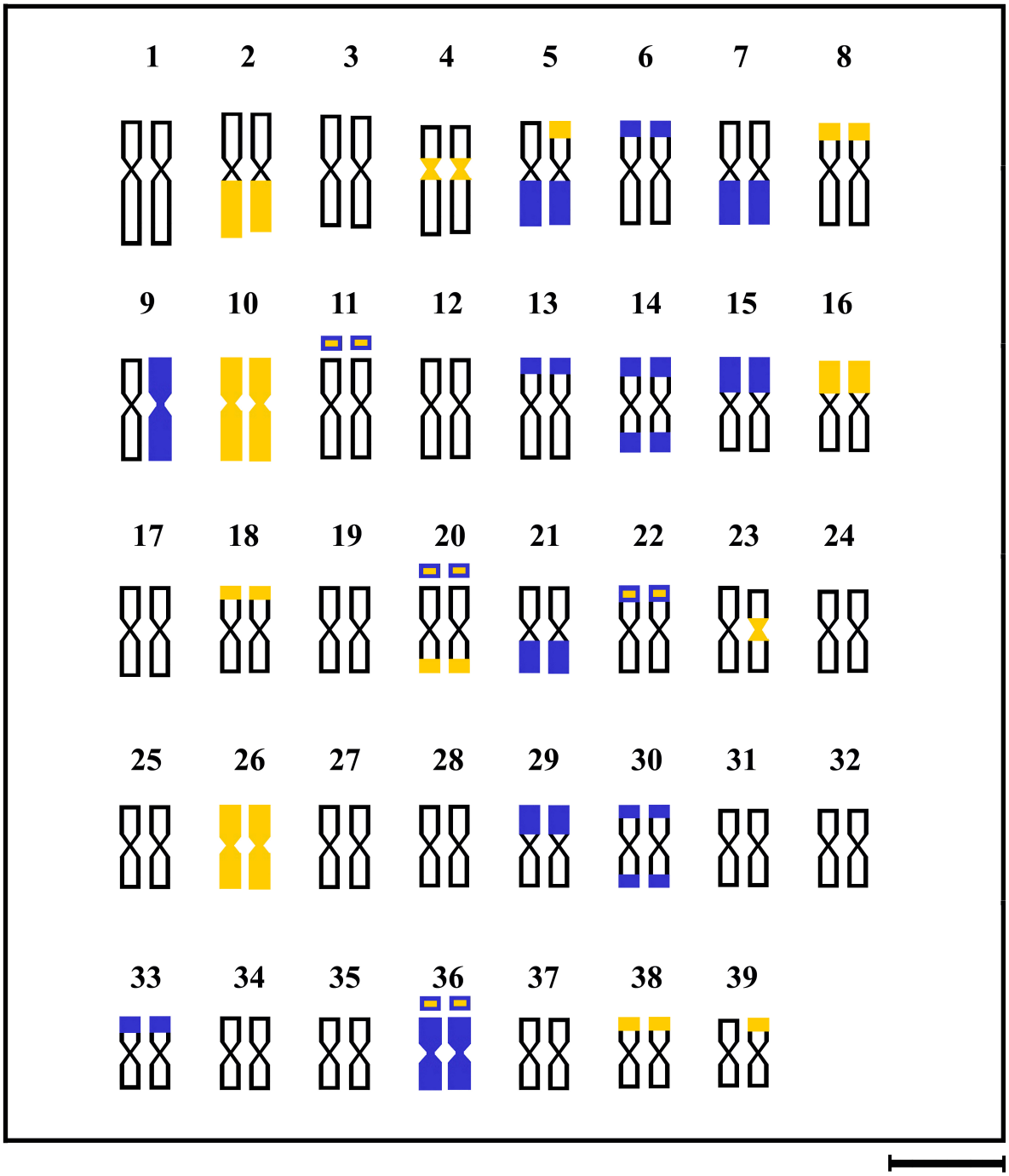


Fig. 108. Idiogram prepared from mitotic metaphase chromosomes of *C. pinnatipartitus* with CMA- and DAPI-banding. Bar = 5 µm.

3.9. RAPD analysis

Initially, twenty five arbitrary primers were used for RAPD amplification of which thirteen primers exhibited good quality banding patterns with sufficient variability. Therefore, thirteen primers were used for further analysis. Description of primers used in this study is presented in Materials and Methods section (Table 7). The primer wise RAPD profiles and banding patterns of six wild *Corchorus* species are given below:

3.9.1. Primer OPA-18

The primer OPA-18 generated a total of 4 RAPD bands (1400, 1100, 500 and 400 bp) only in *C. septentrionalis* among the six species of *Corchorus*. All these bands could be considered as unique as absent in other species (Fig. 110; Table 26). Among these bands, the bands of 1400 bp and 1100 bp were appeared to be light whereas 500 bp and 400 bp bands were bright (Fig. 110, Tables 28, 41).

3.9.2. Primer OPC-10

Total six bands of different size were observed using this primer in two *Corchorus* species. Five light bands were found in *C. hirtus* (1500, 1300, 750, 450 and 250 bp). *Corchorus siliquosus* showed only one bright band (350 bp). All of these bands were unique since they were absent in other species. No RAPD band was observed in *C. septentrionalis*, *C. brevicornatus*, *C. urticifolius* and *C. pinnatipartitus* (Fig. 111, Tables 29, 41).

3.9.3. Primer OPC-13

The primer OPC-13 generated a total of 7 RAPD bands (1500, 1000, 700, 600, 500, 350 and 180 bp) only in *C. septentrionalis* among the six *Corchorus* species. All the bands were unique due to absence of those bands in rest five species (Fig. 112, Table 28). In this species the bands of 600 bp and 500 bp were light and the rest five bands were bright (Fig. 112, Tables 30, 41).

3.9.4. Primer OPC-14

Total of 12 different bands were observed with this primer among the studied species except *C. hirtus* and *C. pinnatipartitus* (Fig. 113, Table 31). Six bands were found in *C. siliquosus* (1800, 1400, 800, 600, 300 and 250 bp) of which 300 bp and 250 bp were light and the rest four bands were bright. *Corchorus septentrionalis* showed seven bands (1800, 1200, 1100, 800, 700, 550 and 500 bp) where 1800 bp and 800 bp were bright. Three bands (1800, 800 and 500 bp) were observed in *C. brevicornatus* of which 800 bp were bright. *Corchorus urticifolius* possesed seven bands (2500, 1100, 800, 700, 600, 500 and 250 bp) where four bands were bright (2500, 1100, 800 and 700 bp). *Corchorus siliquosus*, *C. septentrionalis* and *C. urticifolius* had two (1400, 300 bp), one (1200 bp) and one (2500 bp) unique bands, respectively as these bands were absent in other species (Fig. 113, Table 31, 41).

3.9.5. Primer OPC-16

The primer OPC-16 generated a total of 2 RAPD bands (800 and 400 bp) only in *C. brevicornatus* among the six *Corchorus* species. As a result these two bands could be considered as unique bands (Fig. 114, Table 32). Among these bands, 400 bp was light and 800 bp were bright (Fig. 114, Tables 32, 41).

3.9.6. Primer OPC-26

The primer OPC-26 generated a total of 7 RAPD bands (1500, 1400, 950, 700, 600, 500 and 350 bp) in three within the six species of *Corchorus*. Four bands were found in *C. siliquosus* (1500, 1400, 950 and 350 bp) of which 1500 bp, 950 bp and 350 bp were bright whereas 1400 bp were light. *Corchorus septentrionalis* showed three bright bands (600, 500 and 350 bp). Two bright bands (700 and 350 bp) were observed in *C. urticifolius*. *Corchorus siliquosus*, *C. septentrionalis* and *C. urticifolius* had three (1500, 1400 and 950 bp), two (600 and 500 bp) and one (700 bp) unique bands, respectively as these bands were absent in other species (Fig. 115, Tables 33, 41).

3.9.7. Primer OPC-96

The primer OPC-96 generated a total of 3 RAPD bands (900, 500 and 150 bp) only in *C. septentrionalis* within the six *Corchorus* species. All these bands could be considered as unique bands as absent in other species (Fig. 192, Table 34). Among these bands, 900 bp was bright whereas 500 and 150 bp was light (Fig. 116, Tables 34, 41).

3.9.8. Primer OPD-20

Total four different molecular weight bands were found with this primer in two *Corchorus* species. Only one 5000 bp light band was found in *C. brevicornatus*. *Corchorus pinnatipartitus* showed three unique RAPD bands (1300, 950 and 700 bp) of which 950 bp band was bright and 1300 bp and 700 bp bands were light. No RAPD band was observed in *C. hirtus*, *C. siliquosus*, *C. septentrionalis*, *C. brevicornatus* and *C. urticifolius* (Fig. 117, Tables 35, 41)

3.9.9. Primer OPD-96

The primer OPD-96 generated a total of four RAPD bands (900, 750, 500 and 250 bp) only in *C. septentrionalis* within the six *Corchorus* species. By detecting those bands only in that species made them unique in nature (Fig. 118, Tables 36, 41). Among these, 900 bp and 250 bp bands were lighter then bands of 750 bp and 500 bp (Fig. 118, Tables 36, 41).

3.9.10. Primer OPF-22

The primer OPF-22 generated a total of 2 RAPD bands (500 and 400 bp) only in *C. septentrionalis* within the six *Corchorus* species. These two bands could be considered as unique bands as absent in other species (Fig. 119, Table 37). Among these bands, 400 bp was light and 500 bp were bright (Fig. 119, Tables 37, 41).

3.9.11. Primer OPG-03

RAPD bands of six different size (1500, 1200, 1000, 900, 700 and 300 bp) were visualized in *C. hirtus*, *C. septentrionalis* and *C. brevicornatus*. *Corchorus hirtus* showed four light bands (1200, 1000, 900 and 700 bp). Five bands (1500, 1200, 900, 700 and 300 bp) were observed in *C. septentrionalis* where 1500 bp band was light and rest four bands were bright. Four light (1500, 1200, 1000 and 300 bp) and two bright (900 and 700 bp) bands were found in *C. brevicornatus*. No unique band was observed with this primer. No RAPD band was generated in *C. siliquosus*, *C. urticifolius* and *C. pinnatiparttitus* (Fig. 120, Tables 38, 41).

3.9.12. Primer OPG-06

With this primer, a total of four RAPD bands were generated among the studied materials except *C. pinnatipartitus*. *Corchorus hirtus* showed two bands (1500 and 600 bp) where 1500 bp was bright and 600 bp was light. Four bands were found in *C. siliquosus* (2500, 1500, 850 and 600 bp) of which 2500 bp was light and the rest three bands were bright. *Corchorus septentrionalis* showed three bands (2500, 1500 and 600 bp) where 1500 bp was bright. Three bands (2500, 1500 and 600 bp) were observed in *C. brevicornatus* of which 2500 bp was light. *Corchorus urticifolius* posses four bands (2500, 1500, 850 and 600 bp) where two bands were bright (1500 and 600 bp). No unique band was observed with this primer (Fig. 121, Tables 39, 41).

3.9.13. Primer OPG-09

With the primer OPG-09, total eight different sized RAPD bands (2500, 1500, 1400, 1000, 900, 750, 550 and 400 bp) were found in five species of *Corchorus* except *C. pinnatipartitus*. Among the five species, *C. hirtus* showed only a 1400 bp sized bright RAPD band. Five RAPD bands (1400, 1000, 900, 750, 550 and 400 bp) were observed in *C. siliquosus* where three bands (1000, 750 and 550 bp) were bright and two were bright bands (1400 and 400 bp). *C.*

*septentrionalis*posses five bands (2500, 1400, 900, 750 and 550 bp) of which 2500 bp, 900 bp and 550 bp bands were light. Three bright RAPD bands were observed in *C. brevicornatus* (1400, 1000 and 550 bp) and *C. urticifolius* (1500, 1400 and 1000 bp). *Corchorus siliquosus*, *C. septentrionalis* and *C. urticifolius* had one (400 bp), two (2500 and 900 bp) and one (1500 bp) unique bands, respectively as these bands were absent in other species (Fig. 122, Tables 40, 41).

3.9.14. Genetic distances and cluster analysis

Cluster analysis and the values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among six species of *Corchorus* were computed using combined data for the thirteen RAPD primers on the basis of DNA fingerprinting by RAPD. According to the developed dendogram, *C. septentrionalis* is distantly related from other 5 wild species and formed a separate cluster at highest genetic distance 1.1431. Cluster-2 further differentiated into 2 separate clusters. In Cluster-3, *C. hirtus* and *C. pinnatipartitus* are close to each other at 0.2638 and *C. brevicornatus* seperated at 0.3629 genetic distance. Another cluster had *C. siliquosus* and *C. urticifolius* with 0.3629 genetic distances from each other (Fig. 123, Table 42).

3.10. SSR analysis

Initially, twelve selected primer pairs were used for SSR analysis of six wild *Corchorus* species. Eight primer pairs exhibited good quality banding patterns with sufficient variability. The details of primer pairs used in this study have been given previously in Materials and Methods section (Table 8). The pair-wise SSR profiles and banding patterns of six species of *Corchorus* were as follows:

3.10.1. Primer AW-584539

The primer AW-584539 generated a total of 2 SSR bands (400 and 300 bp) observed in three species of *Corchorus*. In *C. hirtus* only a light (400 bp) band

was observed. In *C. brevicornatus* and *C. urticifolius*, two bands (400 and 300 bp) were found of which 400 bp was bright and 300 bp was light (Fig. 124, Tables 43, 51). No unique band band was observed with this primer.

3.10.2. Primer CEDG-30

The primer CEDG-30 generated a total of three light SSR bands (1000, 750 and 550 bp) only in *C. urticifolius* within the six species of *Corchorus*. These three bands could be considered as unique bands as absent in other species (Fig. 125, Tables 44, 51).

3.10.3. Primer CEDG-56

The primer pair CEDG-56 generated two bands in three *Corchorus* species. No unique band was observed with this primer. Both *C. hirtus* and *C. septentrionalis* posses two bands (400 and 200 bp) of which 400 bp were bright and 200 bp was light. On the other hand, *C. urticifolius* had only one bright band (400 bp). No RAPD band was observed in *C. siliquosus*, *C. brevicornatus* and *C. pinnatiparttitus* (Fig. 126, Tables 45, 51).

3.10.4. Primer HK-7

Two different sized bright SSR bands (275 and 100 bp) were observed only in two *Corchorus* species. *Corchorus urticifolius* posses teo bright bands (275 and 100 bp) where 275 bp was unique. On the other hand, only 100 bp band was found in *C. brevicornatus*. There was no band found in *C. hirtus*, C. siliquosus, *C. septentrionalis* and *C. pinnatipartitus* with this primer (Fig. 127, Tables 46, 51).

3.10.5. Primer HK-12

Only one band (400 bp) was observed in *C. siliquosus* and *C. septentrionalis* with primer HK-12. There was no band found in *C. hirtus*, *C. brevicornatus*, *C. urticifolius* and *C. pinnatipartitus* with this primer (Fig. 128, Tables 47, 51).

3.10.6. Primer HK-16

The primer pair HK-16 generated two bands (200 and 100 bp) in five *Corchorus* species. No band was found in *C. pinnatipartitus*. No unique band was observed with this primer. 200 bp was found in *C. brevicornatus, C. septentrionalis, C. urticifolius, C. hirtus* and *C. siliquosus.* In addition 100 bp was present in *C. brevicornatus* and *C. urticifolius* (Fig. 129, Tables 48, 51).

3.10.7. Primer HK-18

With the primer HK-18, total seven different sized SSR bands (500, 400, 350, 300, 250, 200 and 100 bp) were found in four species of *Corchorus*.

Among the four species, *C. hirtus* showed four SSR bands (400, 250, 200 and 100 bp) of which 400 bp was light. Three bright SSR bands (500, 350 and 200 bp) was observed in *C. septentrionalis*. *Corchorus brevicornatus* posses five bright SSR bands (500, 400, 350, 300, 250, 200 and 100 bp). Three bands (300, 200 and 100 bp) was observed in *C. urticifolius* where two bands (300 and 200 bp) were light and one was bright band (100 bp). *Corchorus septentrionalis* and *C. urticifolius* had one (350 bp and 300 bp) unique band, respectively as this bands were absent in other species (Fig. 130, Tables 49, 51).

3.10.8. Primer HK-30

Eight different sized SSR bands (1500, 900, 800, 600, 400, 250, 200 and 100 bp) were observed in six wild species of *Corchorus*. Only one 200 bp bright SSR band was observed *C. hirtus, C. siliquosus* and *C. pinnatipartitus*. *Corchorus septentrionalis* showed four bright bands (800, 400, 200 and 100 bp) of which two bands (800 and 100 bp) were unique in nature. Three bands (600, 400 and 200 bp) were observed in *C. brevicornatus* where 200 bp band was bright and rest two bands were light. Five bright bands (900, 600, 400, 250 and 200 bp) and one light SSR bands (1500 bp) were found in *C. urticifolius* of which three were unique bands, respectively as these bands were absent in other species (Fig. 131, Tables 50, 51).

3.10.9. Genetic distances and cluster analysis

Cluster analysis and the values of pair-wise Nei's (1972) genetic distances analyzed by using computer software "popgene32" among six species of *Corchorus* were computed using combined data for the eight SSR primers on the basis of DNA fingerprinting. According to dendogram, *Corchorus urticifolius* is distantly related from other 5 wild species and formed a separate cluster at highest genetic distance 1.3499. Cluster-2 further differentiated into 2 separate clusters. *Corchorus brevicornatus* separated at genetic distance 0.5878 from the rest. In cluster-3, *C. septentrionalis* separated at genetic distance 0.7309 whereas *C. hirtus*, *C. siliquosus* and *C. pinnatipartitus* made Cluster-4. In Cluster-4, *C. siliquosus* and *C. pinnatipartitus* are close to each other at 0.0770 lowest genetic distances whereas *C. hirtus* is distant at 0.3514 (Fig. 132, Table 52).

Table 28. RAPD bands of six wild *Corchorus* L. species with primer OPA-18.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	4 (1400, 1100, 500, 400)	2 (1400, 1100)	2 (500, 400)	--	4 (1400, 1100, 500, 400)
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 29. RAPD bands of six wild *Corchorus* L. species with primer OPC-10.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	5 (1500, 1300, 750, 450, 250)	5 (1500, 1300, 750, 450, 250)	--		5 (1500, 1300, 750, 450, 250)
C. siliquosus	1 (350)	--	1 (350)	--	1 (350)
C. septentrionalis	--	--	--		--
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 30. RAPD bands of six wild *Corchorus* L. species with primer OPC-13.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	7 (1500, 1000, 700, 600, 500, 350, 180)	6 (1500, 1000, 600, 500, 350, 180)	1 (700)	--	7 (1500, 1000, 700, 600, 500, 350, 180)
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 31. RAPD bands of six wild *Corchorus* L. species with primer OPC-14.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	6 (1800, 1400, 800, 600, 300, 250)	2 (300, 250)	4 (1800, 1400, 800, 600)		2 (1400, 300)
C. septentrionalis	7 (1800, 1200 1100, 800, 700, 550, 500)	5 (1200 1100, 700, 550, 500)	2 (1800, 800)	--	1 (1200)
C. brevicornatus	3 (1800, 800, 500)	2 (1800, 500)	1 (800)		--
C. urticifolius	7 (2500, 1100, 800, 700, 600, 500, 250)	3 (600, 500, 250)	4 (2500, 1100, 800, 700)		1 (2500)
C. pinnatipartitus	--	--	--		--

Table 32. RAPD bands of six wild *Corchorus* L. species with primer OPC-16.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	--	--	--	--	--
C. brevicornatus	2 (800, 400)	1 (400)	1 (800)		2 (800, 400)
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 33. RAPD bands of six wild *Corchorus* L. species with primer OPC-26.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	4 (1500, 1400, 950, 350)	1 (1400)	3 (1500, 950, 350)		3 (1500, 1400, 950)
C. septentrionalis	3 (600, 500, 350)	--	3 (600, 500, 350)	--	2 (600, 500)
C. brevicornatus	--	--	--		--
C. urticifolius	2 (700, 350)	--	2 (700, 350)		1 (700)
C. pinnatipartitus	--	--	--		

Table 34. RAPD bands of six wild *Corchorus* L. species with primer OPC-96.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	3 (900, 500, 150)	2 (500, 150)	2 (900)	--	3 (900, 500, 150)
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 35. RAPD bands of six wild *Corchorus* L. species with primer OPD-20.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	--	--	--		--
C. brevicornatus	1 (5000)	1 (5000)	--	--	1 (5000)
C. urticifolius	--	--	--		--
C. pinnatipartitus	3 (1300, 950, 700)	2 (1300, 700)	1 (950)		3 (1300, 950, 700)

Table 36. RAPD bands of six wild *Corchorus* L. species with primer OPD-69.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Unique Band (bp)	Common Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	4 (900,750, 500, 250)	2 (900,250)	2 (750,500)	--	4 (900, 750, 500, 250)
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 37. RAPD bands of six wild *Corchorus* L. species with primer OPF-22.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	2 (500,400)	1 (400)	1 (500)	--	2 (500,400)
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 38. RAPD bands of six wild *Corchorus* L. species with primer OPG-03.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	4 (1200, 1000, 900, 700)	4 (1200, 1000, 900, 700)	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	5 (1500, 1200, 900, 700, 300)	1 (1500)	4 (1200, 900, 700, 300)	--	--
C. brevicornatus	6 (1500, 1200, 1000 900, 700, 300)	4 (1500, 1200, 1000, 300)	2 (900, 700)		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 39. RAPD bands of six wild *Corchorus* L. species with primer OPG-06.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	2 (1500, 600)	1 (600)	1 (1500)		--
C. siliquosus	4 (2500, 1500, 850, 600)	1 (2500)	3 (1500, 850, 600)		--
C. septentrionalis	3 (2500, 1500, 600)	2 (2500, 600)	1 (1500)	--	--
C. brevicornatus	3 (2500, 1500, 600)	1 (2500)	2 (1500, 600)		--
C. urticifolius	4 (2500, 1500, 850, 600)	2 (2500, 850)	2 (1500, 600)		--
C. pinnatipartitus	--	--	--		--

Table 40. RAPD bands of six wild *Corchorus* L. species with primer OPG-09.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	1 (1400)	--	1 (1400)		--
C. siliquosus	5 (1400, 1000, 750, 550, 400)	2 (1400, 400)	3 (1000, 750, 550)		1 (400)
C. septentrionalis	5 (2500, 1400, 900, 750, 550)	2 (1400, 750)	3 (2500, 900, 550)	--	2 (2500, 900)
C. brevicornatus	3 (1400, 1000, 550)	--	3 (1400, 1000, 550)		--
C. urticifolius	3 (1500, 1400, 1000)	--	3 (1500, 1400, 1000)		1 (1500)
C. pinnatipartitus	--	--	--		--

Table 41. RAPD bands generated through the use of different primer in six wild species of *Corchorus* L.

Primer codes	Total loci	Size ranges (bp)	Number of Polymorphic loci	Number of unique bands	Polymorphism (%)
OPA-18	4	400-1400	4	4	100
OPC-10	6	250-1500	6	6	100
OPC-13	7	180-1500	7	7	100
OPC-14	12	250-2500	12	4	100
OPC-16	2	400-800	2	2	100
OPC-26	7	350-1500	7	6	100
OPC-96	3	150-900	3	3	100
OPD-20	4	700-5000	4	4	100
OPD-69	4	250-900	4	4	100
OPF-22	2	400-500	2	2	100
OPG-3	6	300-1500	6	-	100
OPG-6	4	600-2500	4	-	100
OPG-9	8	400-2500	8	4	100
Grand Total	**69**	**150-5000**	**69**	**46**	**100**

Table 42. Genetic distances of six wild species of *Corchorus* L. by RAPD analysis according to Nei's (1972).

Species ID	I	II	III	IV	V	VI
I	****					
II	0.4729	****				
III	0.9760	1.0561	****			
IV	0.3423	0.4274	0.7077	****		
V	0.4499	0.3629	0.7376	0.3629	****	
VI	0.2638	0.3840	1.1431	0.3840	0.3629	****

Here,

I : *C. hirtus*
II : *C. siliquous*
III : *C. septentrionalis*
IV : *C. brevicornatus*
V : *C. urticifolius*
VI : *C. pinnatipartitus*

Table 43. SSR bands of six wild *Corchorus* L. species with primer AW-584539.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	1 (400)	1 (400)	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	--	--	--	--	--
C. brevicornatus	2 (400, 300)	1 (300)	1 (400)		--
C. urticifolius	2 (400, 300)	1 (300)	1 (400)		--
C. pinnatipartitus	--	--	--		--

Table 44. SSR bands of six wild *Corchorus* L. species with primer CEDG-30.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	--	--	--		--
C. brevicornatus	--	--	--	--	--
C. urticifolius	3 (1000, 750, 550)	3 (1000, 750, 550)	--		3 (1000, 750, 550)
C. pinnatipartitus	--	--	--		--

Table 45. SSR bands of six wild *Corchorus* L. species with primer CEDG-56.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	2 (400, 200)	1 (200)	1 (400)		--
C. siliquosus	--	--	--		--
C. septentrionalis	2 (400, 200)	1 (200)	1 (400)	--	--
C. brevicornatus	--	--	--		--
C. urticifolius	1 (400)	--	1 (400)		--
C. pinnatipartitus	--	--	--		--

Table 46. SSR bands of six wild *Corchorus* L. species with primer HK-07.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	--	--	--		--
C. septentrionalis	--	--	--	--	--
C. brevicornatus	1 (100)	--	1 (100)		--
C. urticifolius	2 (275, 100)	--	2 (275, 100)		1 (275)
C. pinnatipartitus	--	--	--		--

Table 47. SSR bands of six wild *Corchorus* L. species with primer HK-12.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	--	--	--		--
C. siliquosus	1 (400)	1 (400)	--		--
C. septentrionalis	1 (400)	--	1 (400)	--	--
C. brevicornatus	--	--	--		--
C. urticifolius	--	--	--		--
C. pinnatipartitus	--	--	--		--

Table 48. SSR bands of six wild *Corchorus* L. species with primer HK-16.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	1 (200)	--	1 (200)		--
C. siliquosus	1 (200)	1 (200)	--		--
C. septentrionalis	1 (200)	--	1 (200)	--	--
C. brevicornatus	2 (200, 100)	2 (200, 100)	--		--
C. urticifolius	2 (200, 100)	--	2 (200, 100)		--
C. pinnatipartitus	--	--	--		--

Table 49. SSR bands of six wild *Corchorus* L. species with primer HK-18.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	4 (400, 250, 200, 100)	1 (400)	3 (250, 200, 100)		--
C. siliquosus	--	--	--		--
C. septentrionalis	3 (500, 350, 200)	--	3 (500, 350, 200)	--	1 (350)
C. brevicornatus	5 (500, 400, 250, 200, 100)	--	5 (500, 400, 250, 200, 100)		--
C. urticifolius	3 (300, 200, 100)	2 (300, 200)	1 (100)		1 (300)
C. pinnatipartitus	--	--	--		--

Table 50. SSR bands of six wild *Corchorus* L. species with primer HK-30.

Species	Total Band (bp)	Light Band (bp)	Bright Band (bp)	Common Band (bp)	Unique Band (bp)
C. hirtus	1 (200)	--	1 (200)		--
C. siliquosus	1 (200)	--	1 (200)		--
C. septentrionalis	4 (800, 400, 200, 100)	--	4 (800, 400, 200, 100)	--	2 (800, 100)
C. brevicornatus	3 (600, 400, 200)	2 (600, 400)	1 (200)		--
C. urticifolius	6 (1500,900, 600, 400, 250, 200)	1 (1500)	5 (900, 600, 400, 250, 200)		3 (1500, 900, 250)
C. pinnatipartitus	1 (200)	--	1 (200)		--

Table 51. SSR bands generated through the use of different primer in six wild species of *Corchorus* L.

Primer codes	Total loci	Size ranges (bp)	Number of Polymorphic loci	Number of unique bands	Polymorphism (%)
AW-584539	2	300-400	2	--	100
CEDG-30	3	550-1000	3	3	100
CEDG-56	2	200-400	2	--	100
HK-7	2	100-300	2	1	100
HK-12	1	400	1	--	100
HK-16	2	100-200	2	--	100
HK-18	7	150-500	7	2	100
HK-30	8	100-1500	7	5	87.5
Grand Total	**27**	**100-1500**	**26**	**11**	**96.30**

Table 52. Genetic distances of six wild species of *Corchorus* L. by SSR analysis according to Nei's (1972).

Species ID.	I	II	III	IV	V	VI
I	****					
II	0.3514	****				
III	0.4626	0.3514	****			
IV	0.3514	0.5878	0.7309	****		
V	0.8979	1.0986	1.3499	0.5878	****	
VI	0.3514	0.0770	0.4626	0.5878	1.0986	****

Here,

I : *C. hirtus*
II : *C. siliquous*
III : *C. septentrionalis*
IV : *C. brevicornatus*
V : *C. urticifolius*
VI : *C. pinnatipartitus*

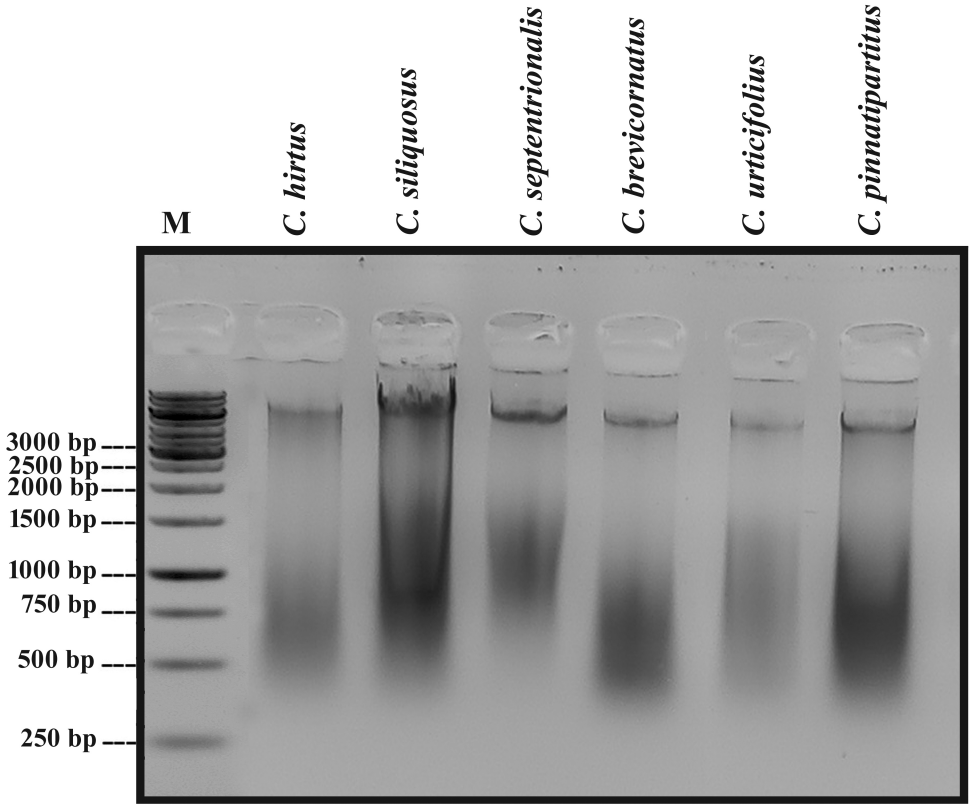


Fig. 109. Electrophoregram of ethidium bromide stained genomic DNA samples of six wild *Corchorus* L. species, M=1 Kb DNA ladder.

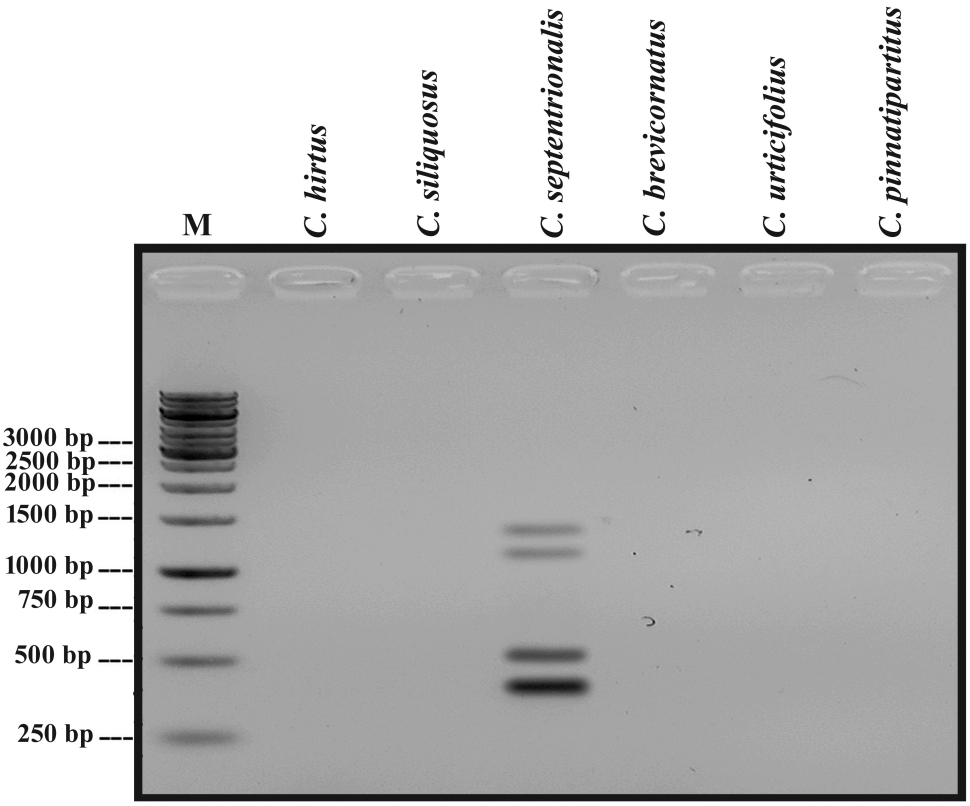


Fig. 110. RAPD profile of six wild *Corchorus* L. species with primer OPA-18, M=1 Kb DNA ladder.

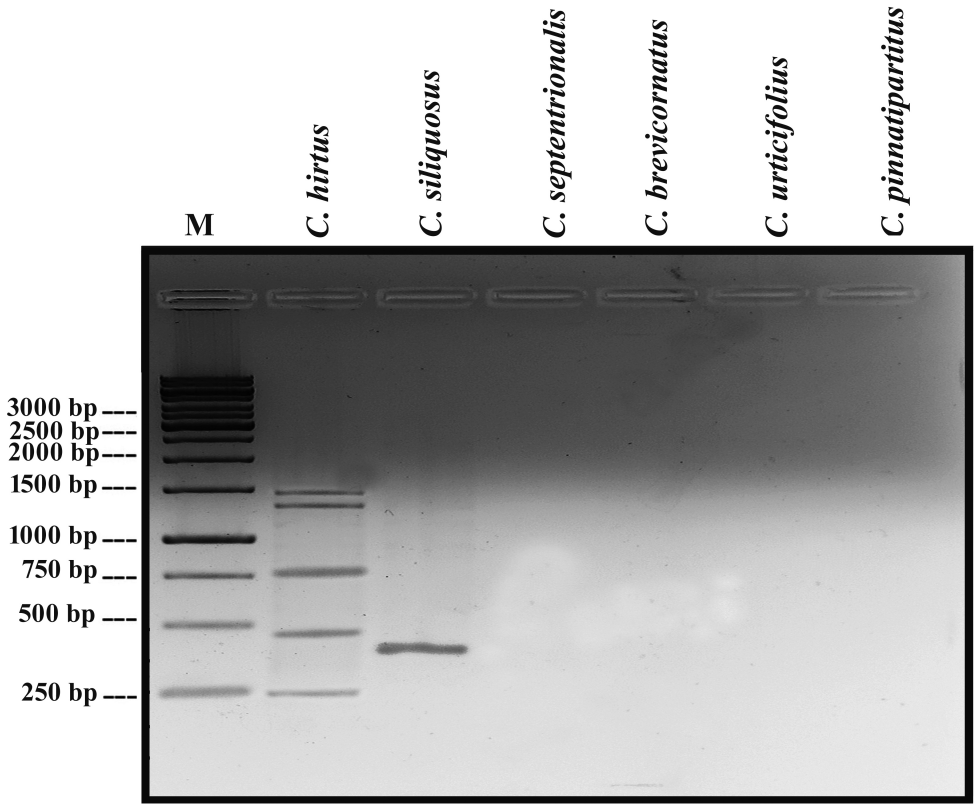


Fig. 111. RAPD profile of six wild *Corchorus* L. species with primer OPC-10, M=1 Kb DNA ladder.

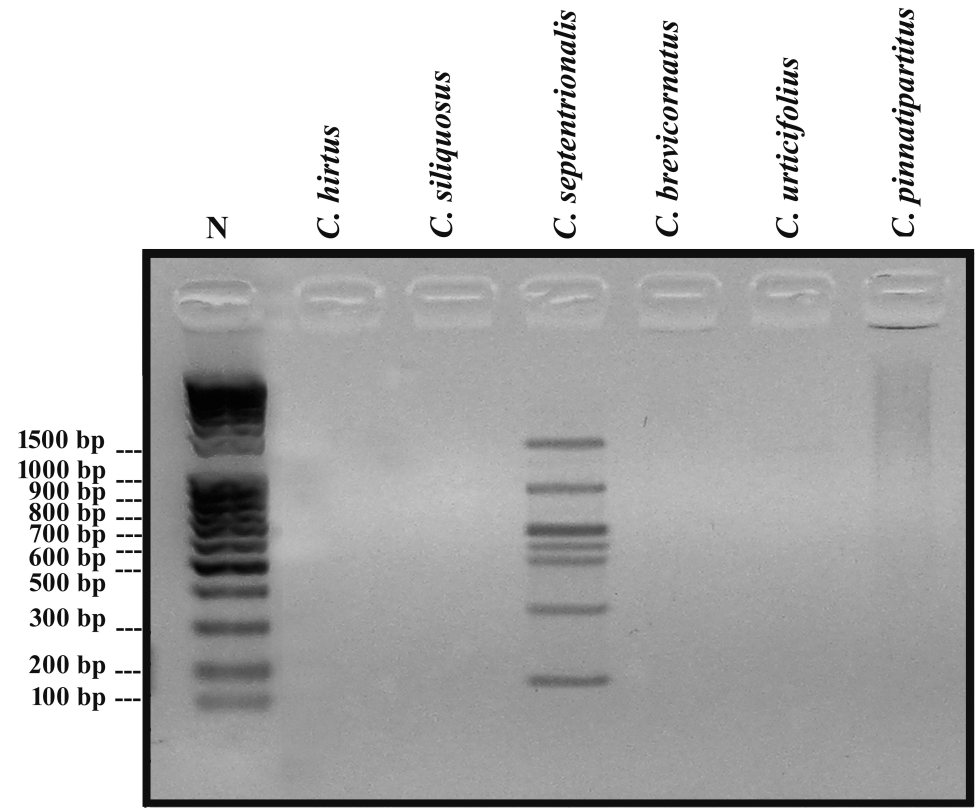


Fig. 112. RAPD profile of six wild *Corchorus* L. species with primer OPC-13, N=100 bp DNA ladder.

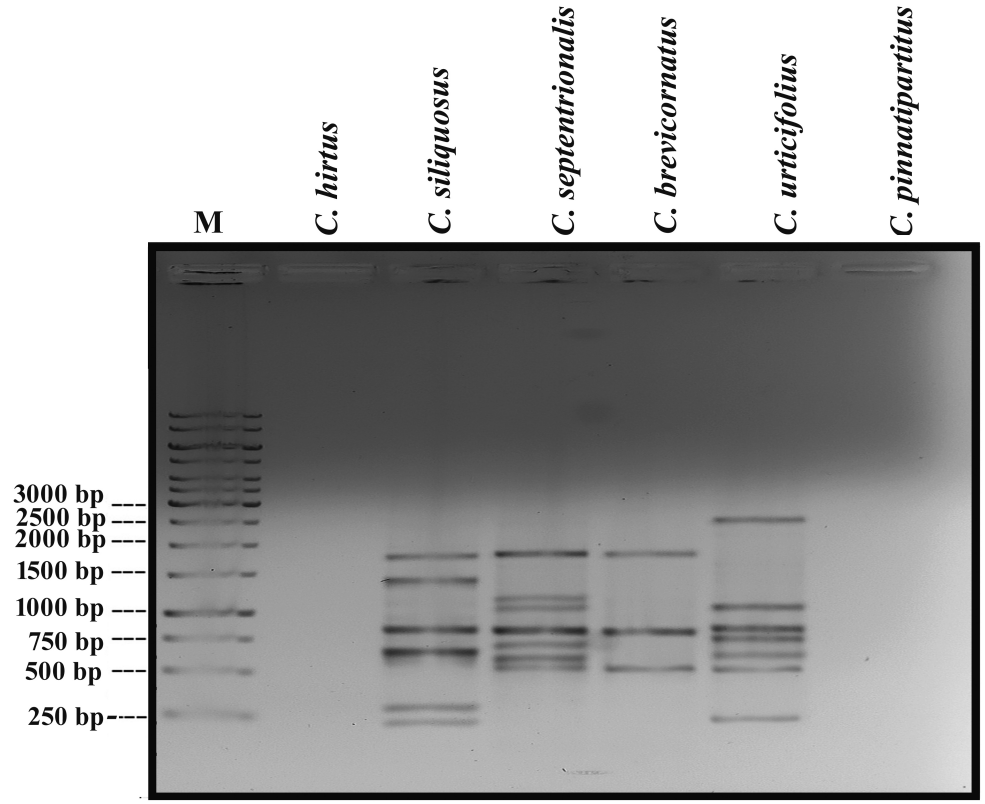


Fig. 113. RAPD profile of six wild *Corchorus* L. species with primer OPC-14, M=1 Kb DNA ladder.

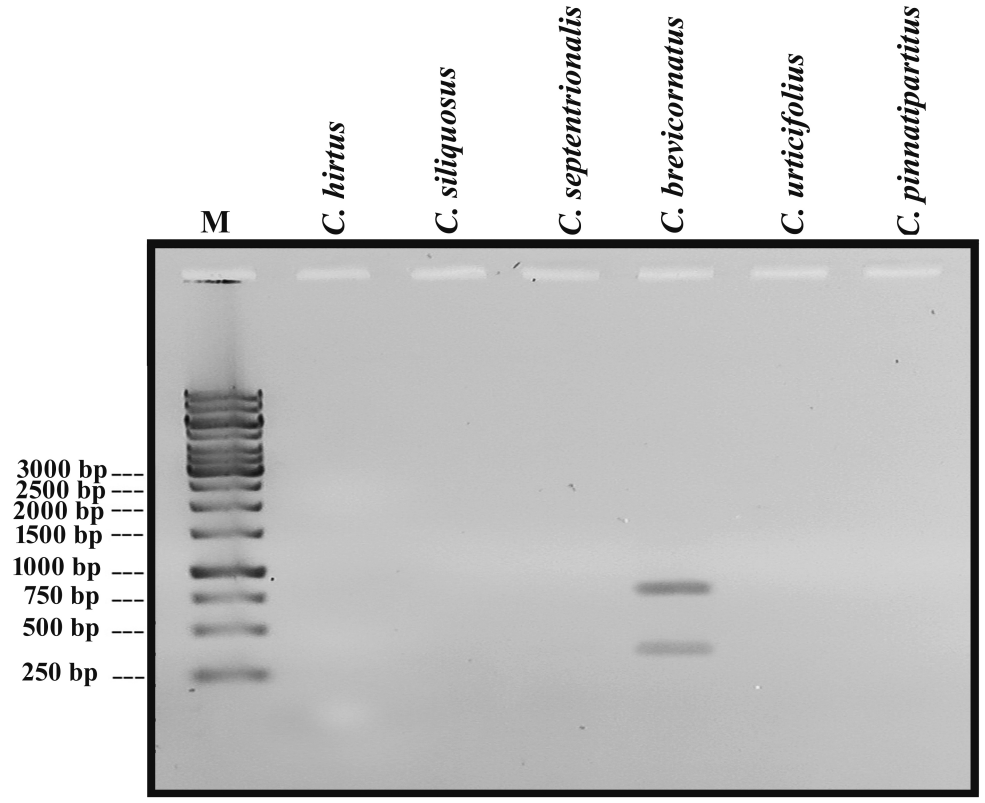


Fig. 114. RAPD profile of six wild *Corchorus* L. species with primer OPC-16, M=1 Kb DNA ladder.

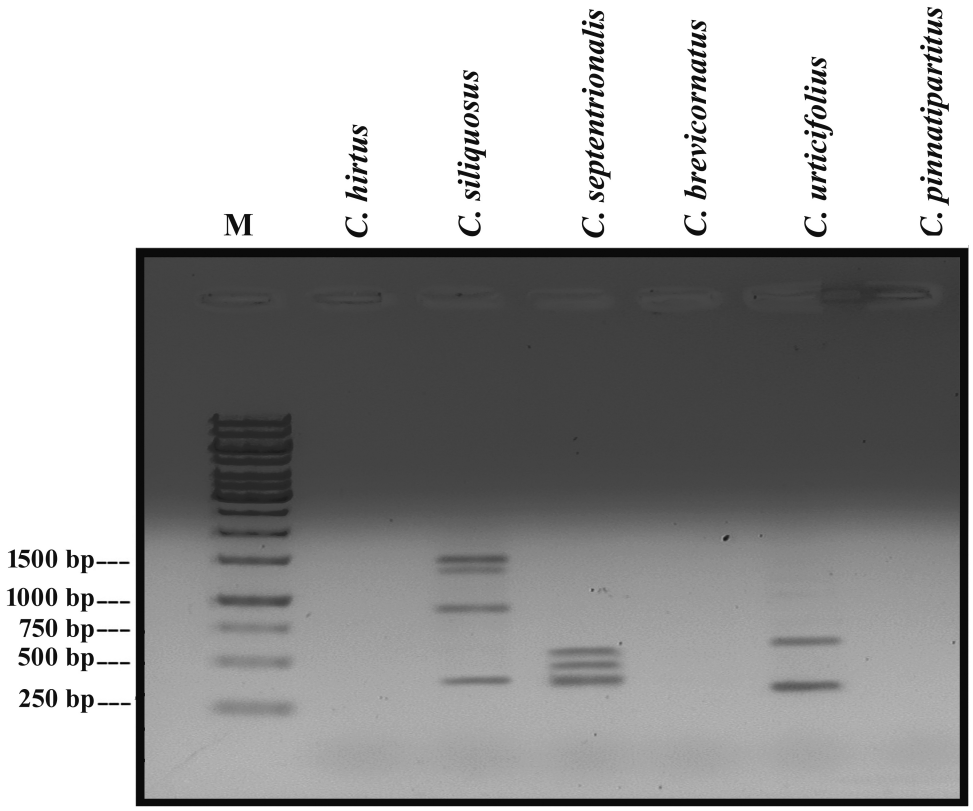


Fig. 115. RAPD profile of six wild *Corchorus* L. species with primer OPC-26, M=1 Kb DNA ladder.

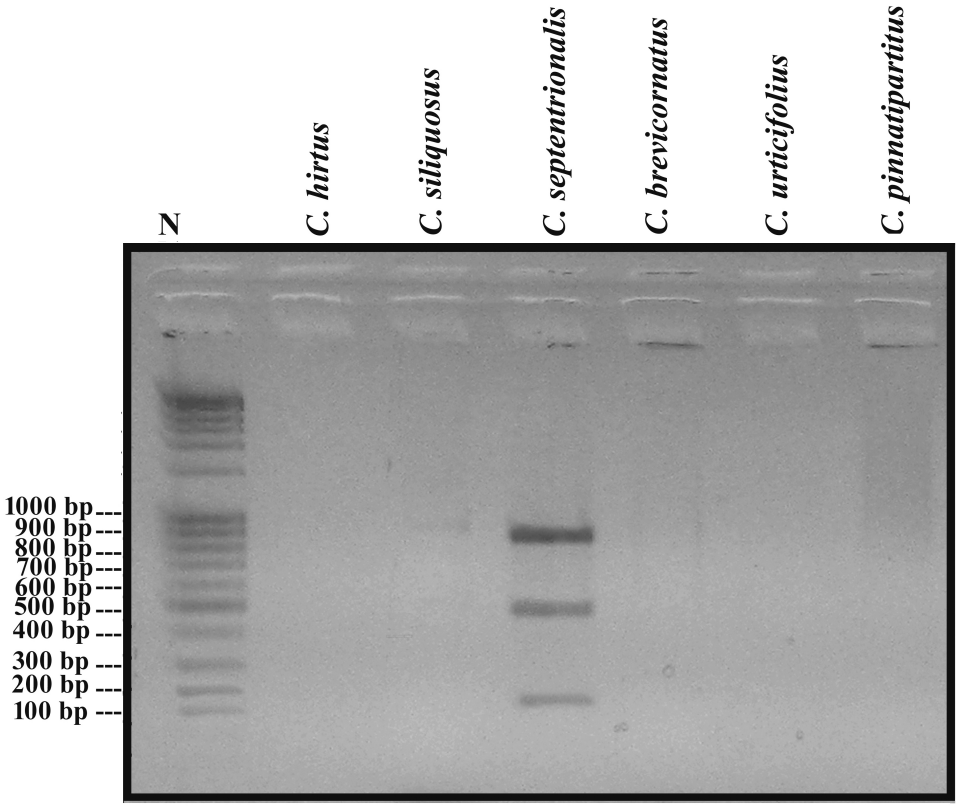


Fig. 116. RAPD profile of six wild *Corchorus* L. species with primer OPC-96, N=100 bp DNA ladder.

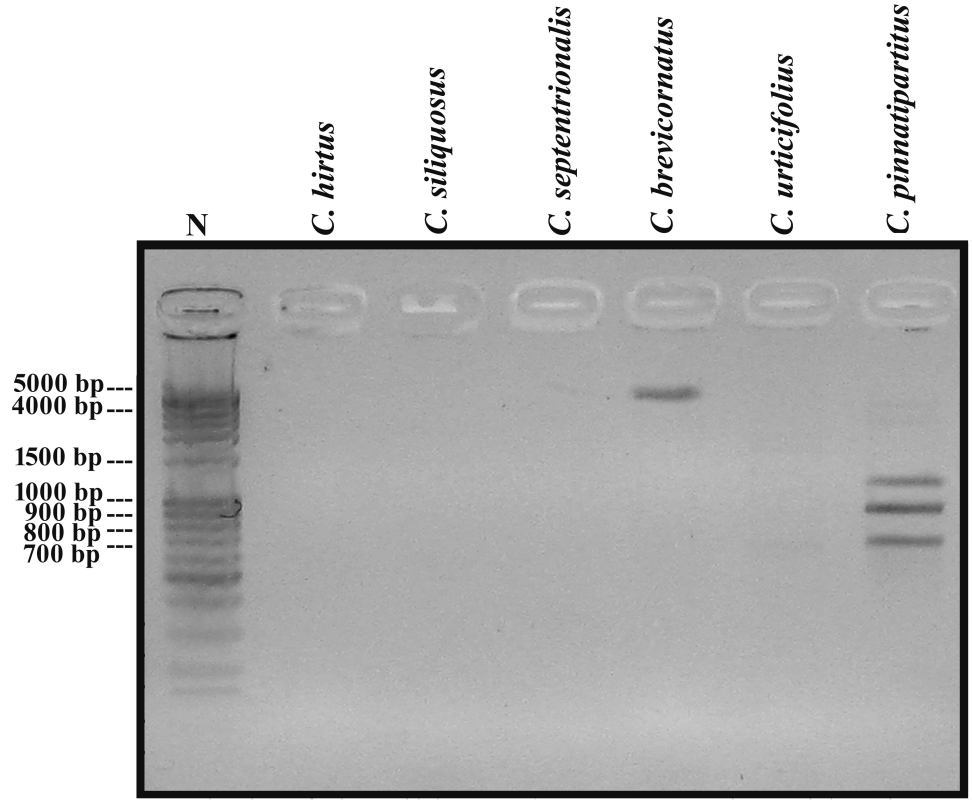


Fig. 117. RAPD profile of six wild *Corchorus* L. species with primer OPD-20, N=100 bp DNA ladder.

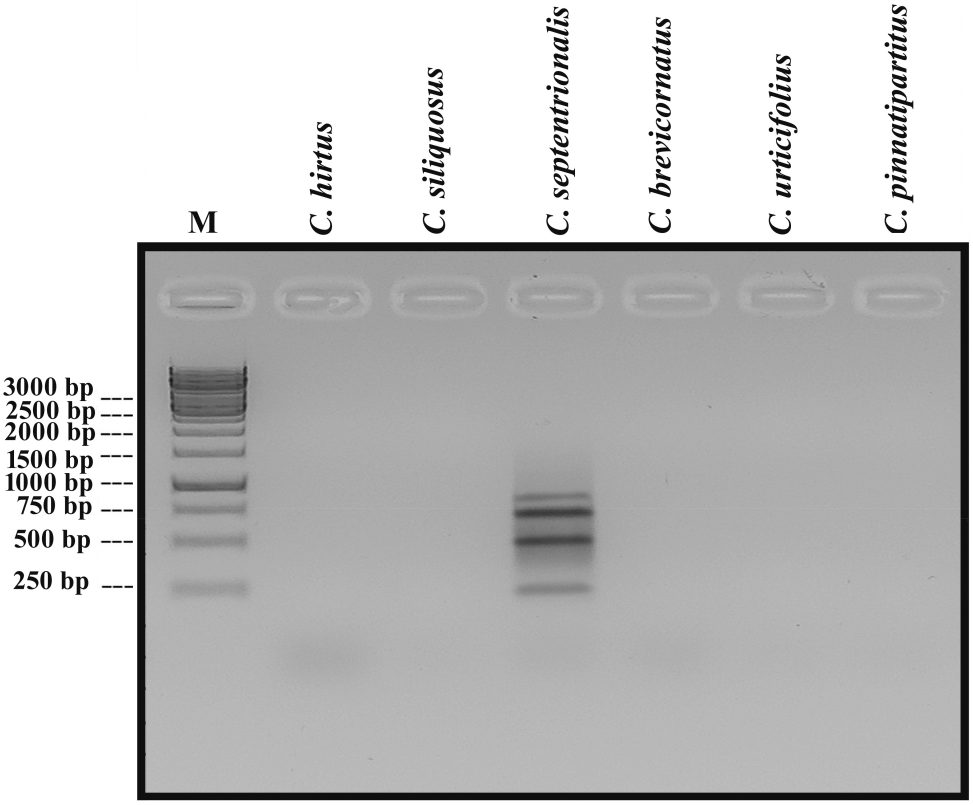


Fig. 118. RAPD profile of six wild *Corchorus* L. species with primer OPD-69, M=1 Kb DNA ladder.

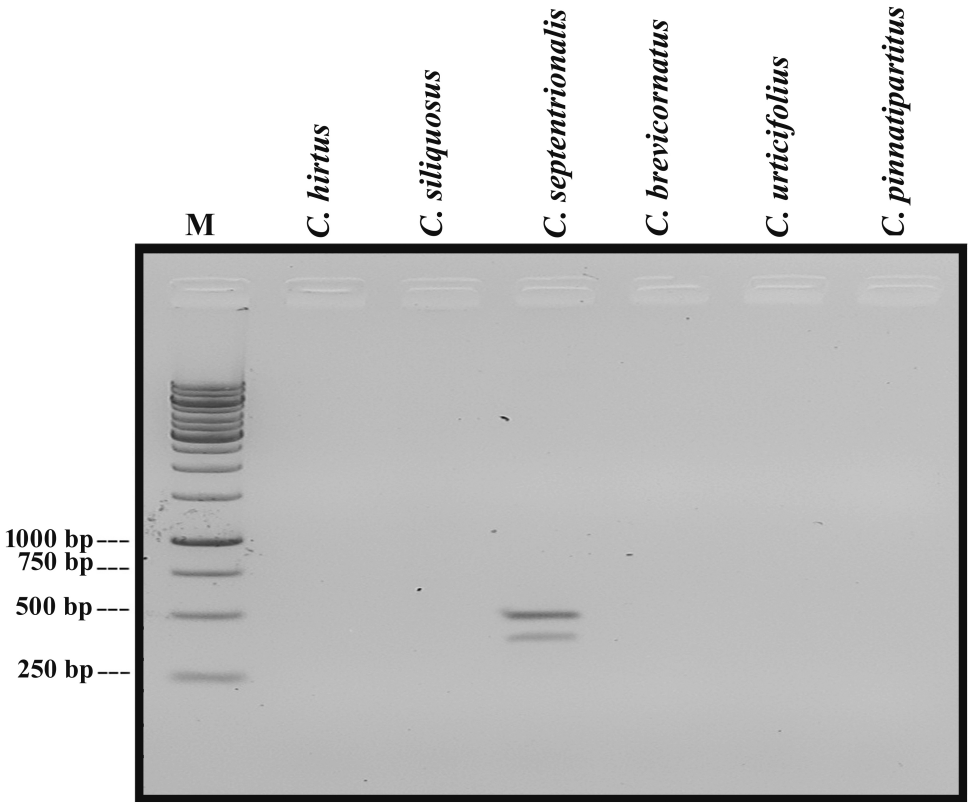


Fig. 119. RAPD profile of six wild *Corchorus* L. species with primer OPF-22, M=1 Kb DNA ladder.

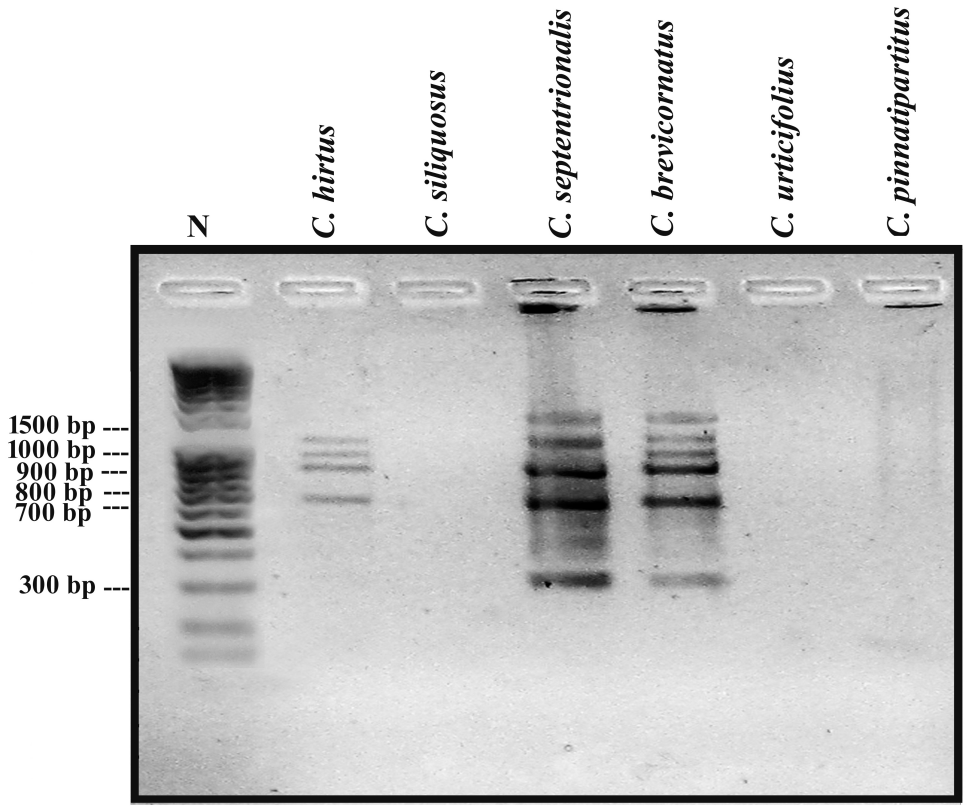


Fig. 120. RAPD profile of six wild *Corchorus* L. species with primer OPG-03, N=100 bp DNA ladder.

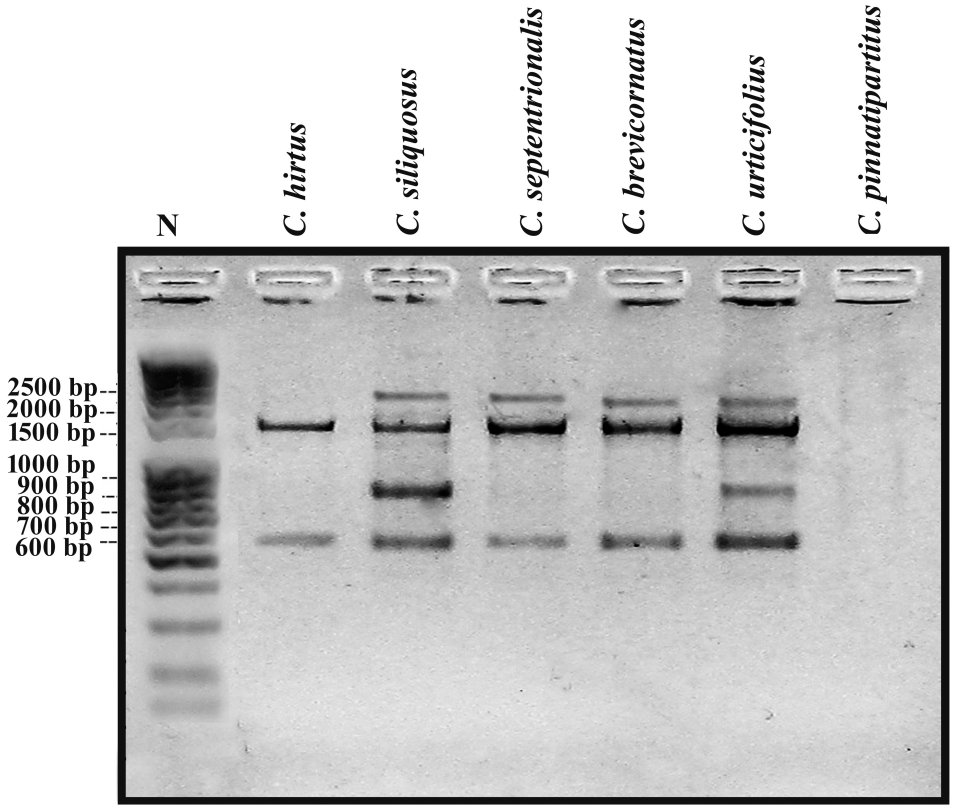


Fig. 121. RAPD profile of six wild *Corchorus* L. species with primer OPG-06, N=100 bp DNA ladder.

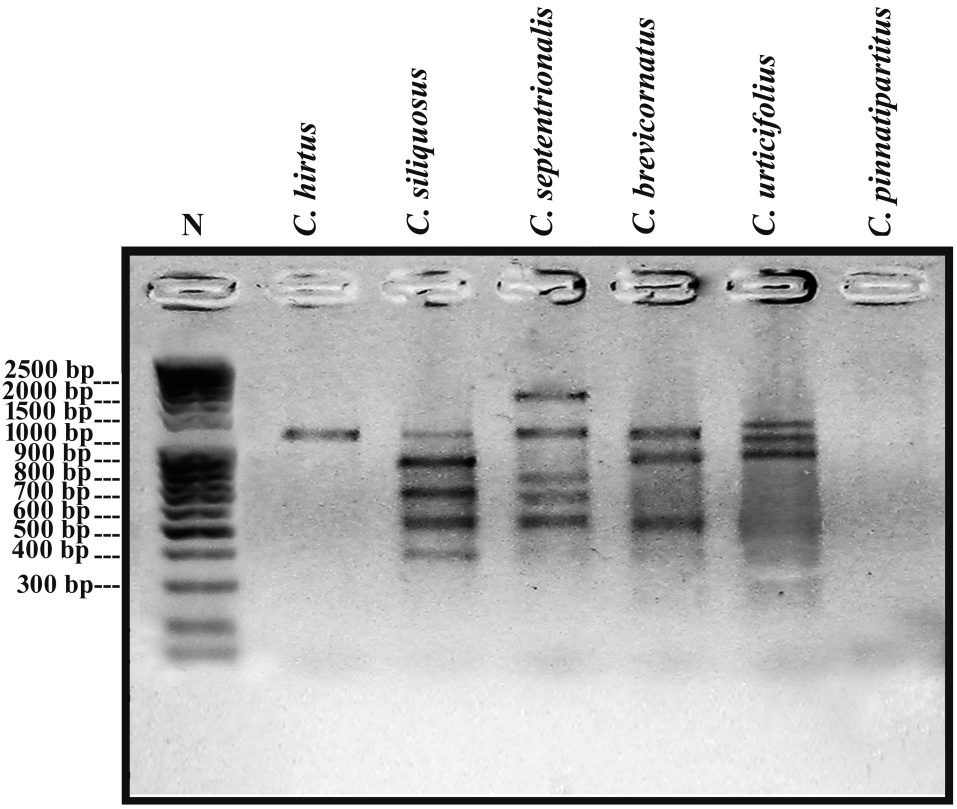


Fig. 122. RAPD profile of six wild *Corchorus* L. species with primer OPG-09, N=100 bp DNA ladder.

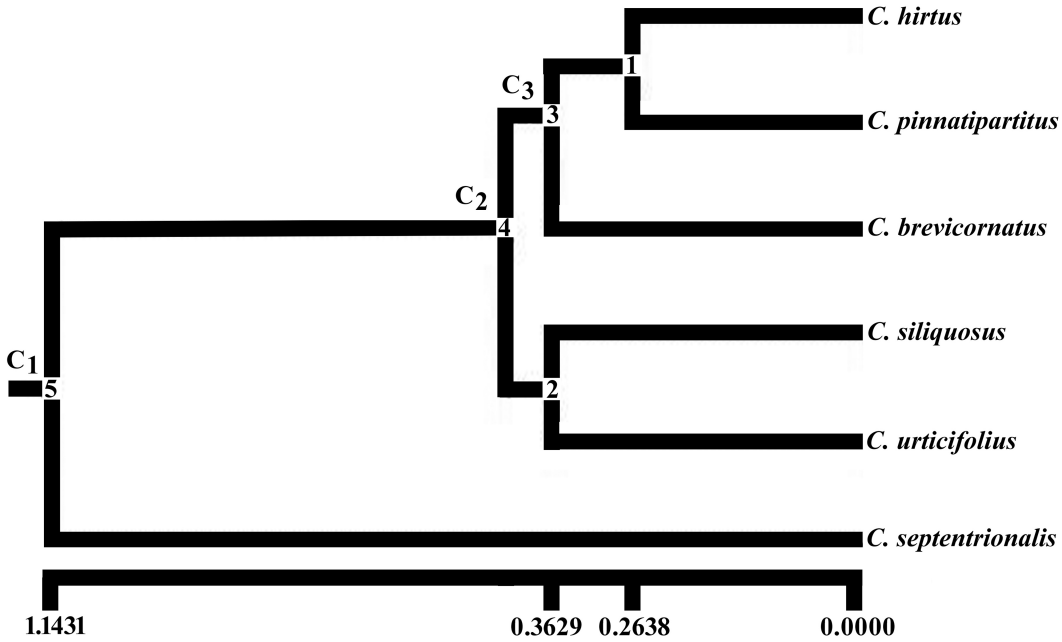


Fig. 123. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among six wild *Corchorus* L. species by RAPD analysis.

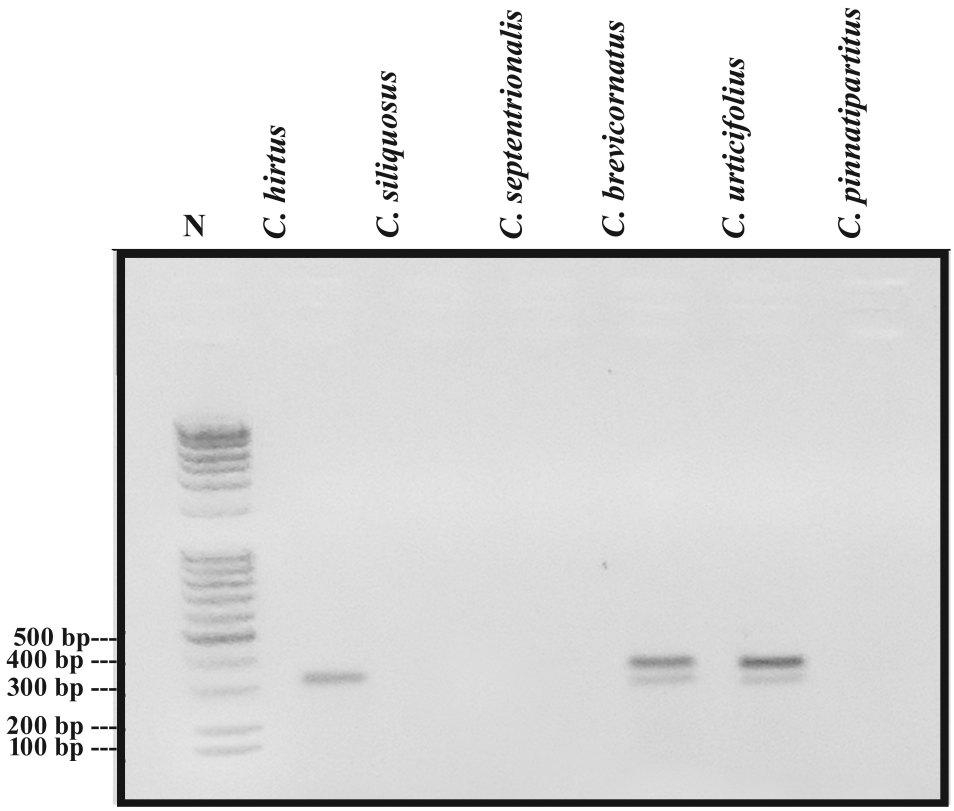


Fig. 124. SSR profile of six wild *Corchorus* L. species with primer AW-584539, N=100 bp DNA ladder.

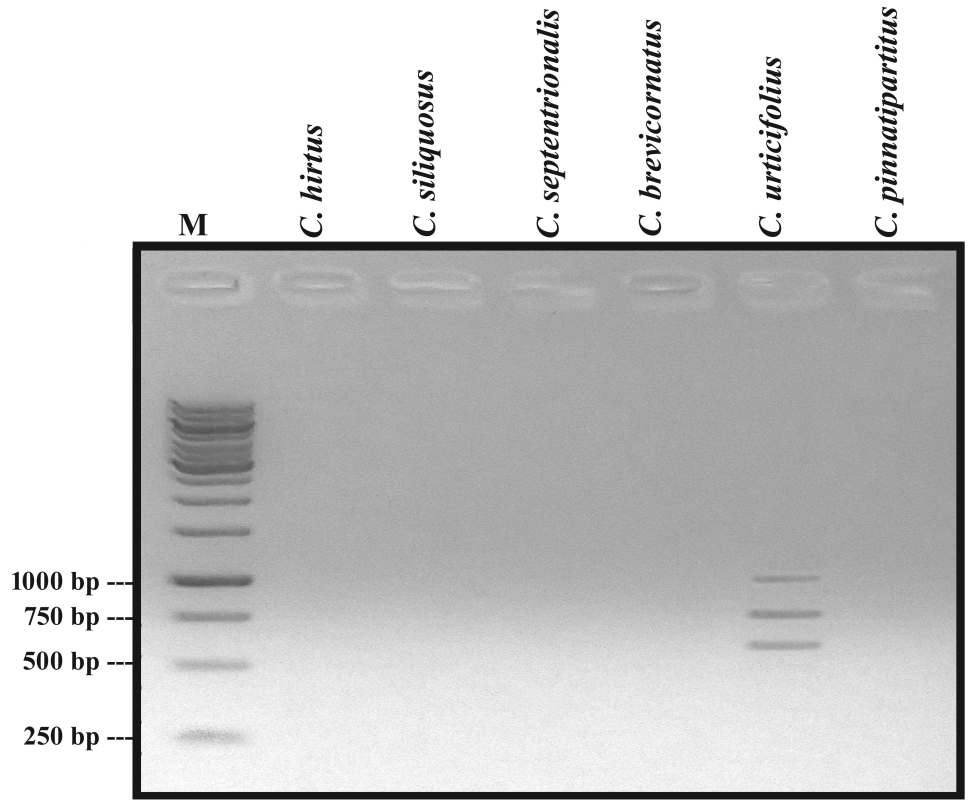


Fig. 125. SSR profile of six wild *Corchorus* L. species with primer CEDG-30, M=1 Kb DNA ladder.

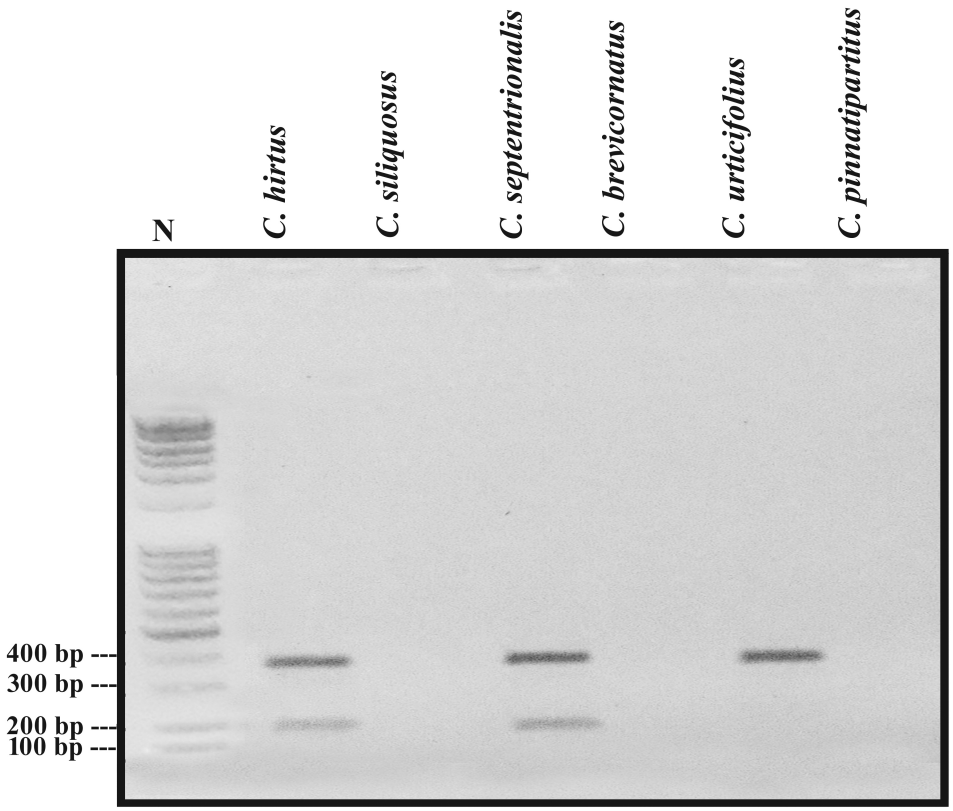


Fig. 126. SSR profile of six wild *Corchorus* L. species with primer CEDG-56, N=100 bp DNA ladder.

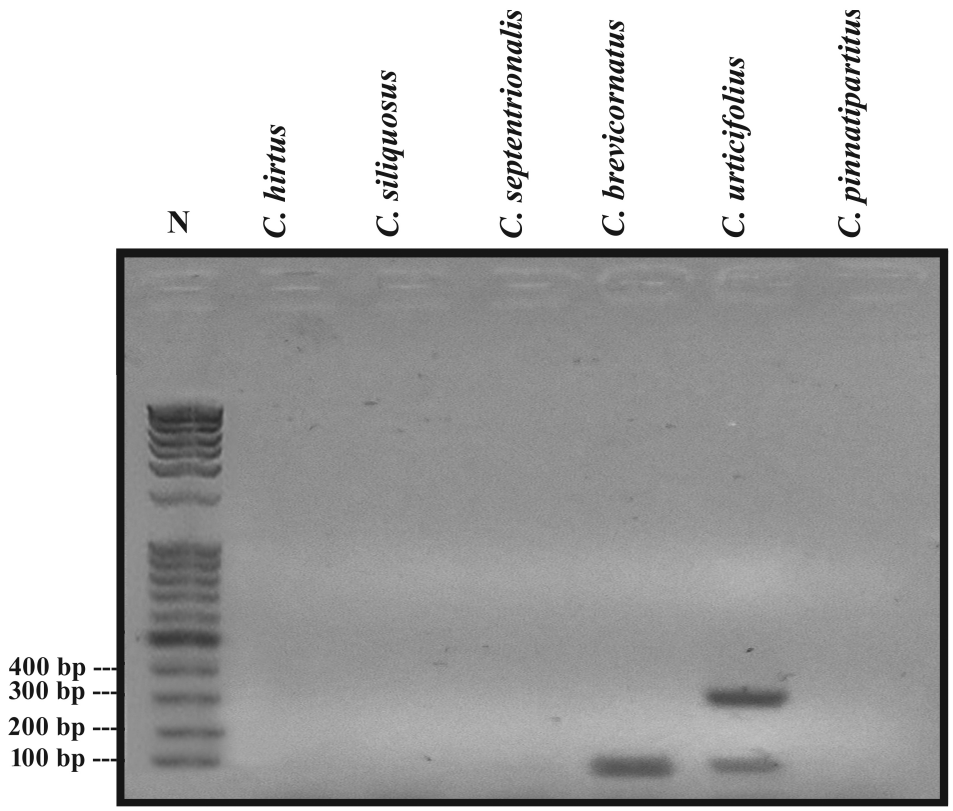


Fig. 127. SSR profile of six wild *Corchorus* L. species with primer HK-07, N=100 bp DNA ladder.

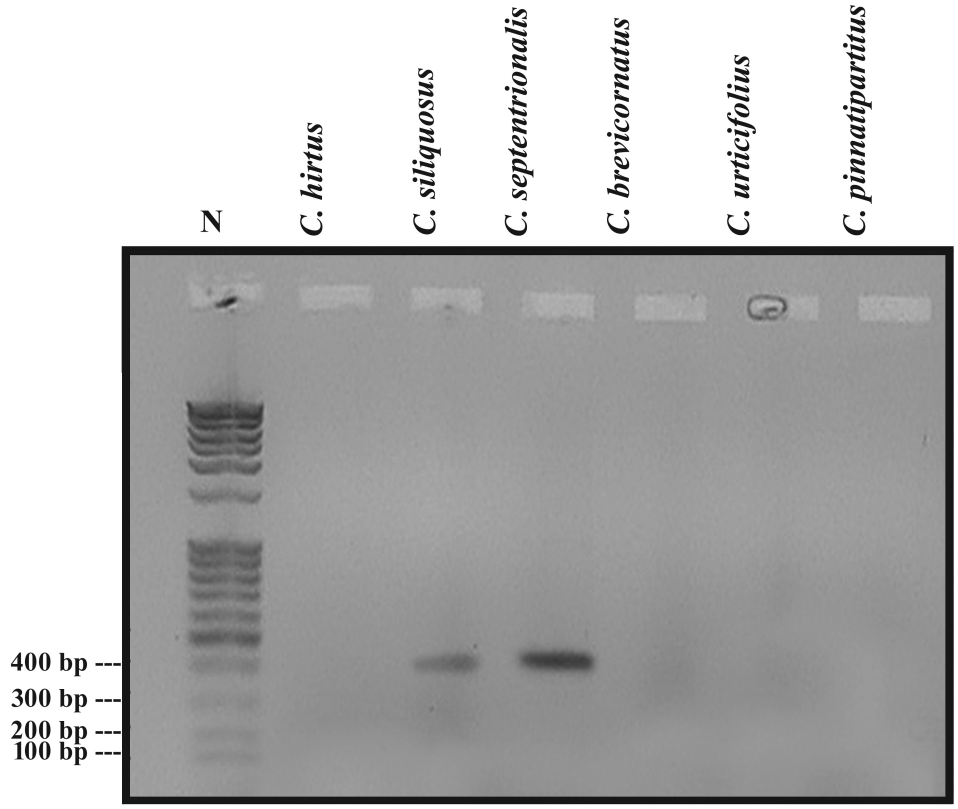


Fig. 128. SSR profile of six wild *Corchorus* L. species with primer HK-12, N=100 bp DNA ladder.

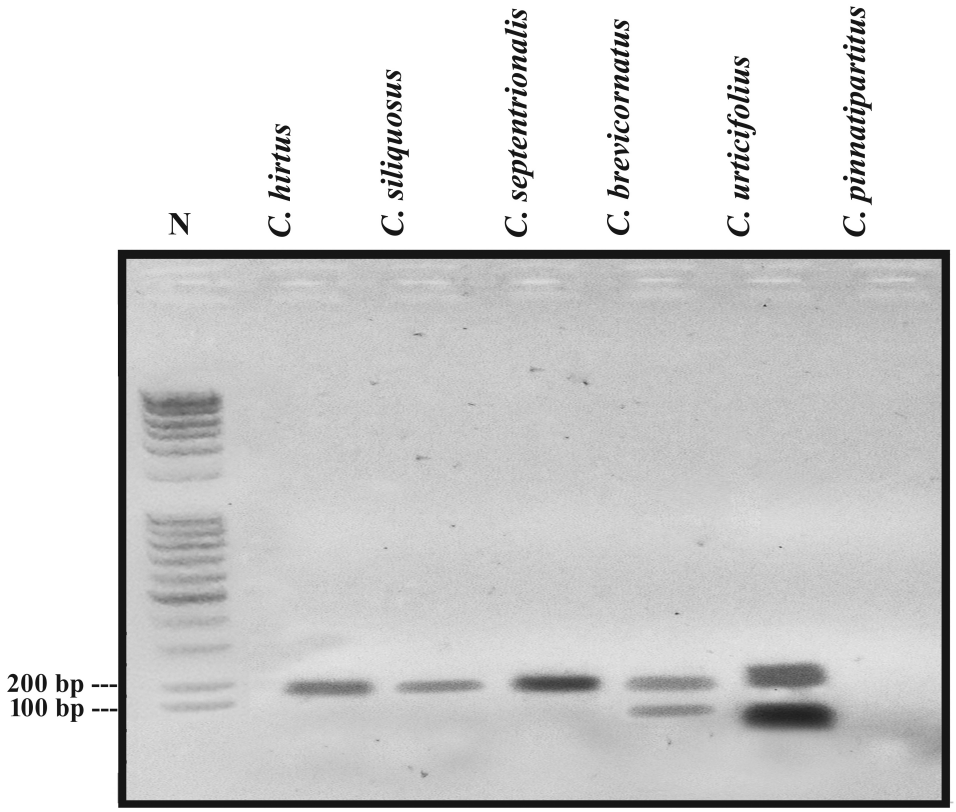


Fig. 129. SSR profile of six wild *Corchorus* L. species with primer HK-16, N=100 bp DNA ladder.

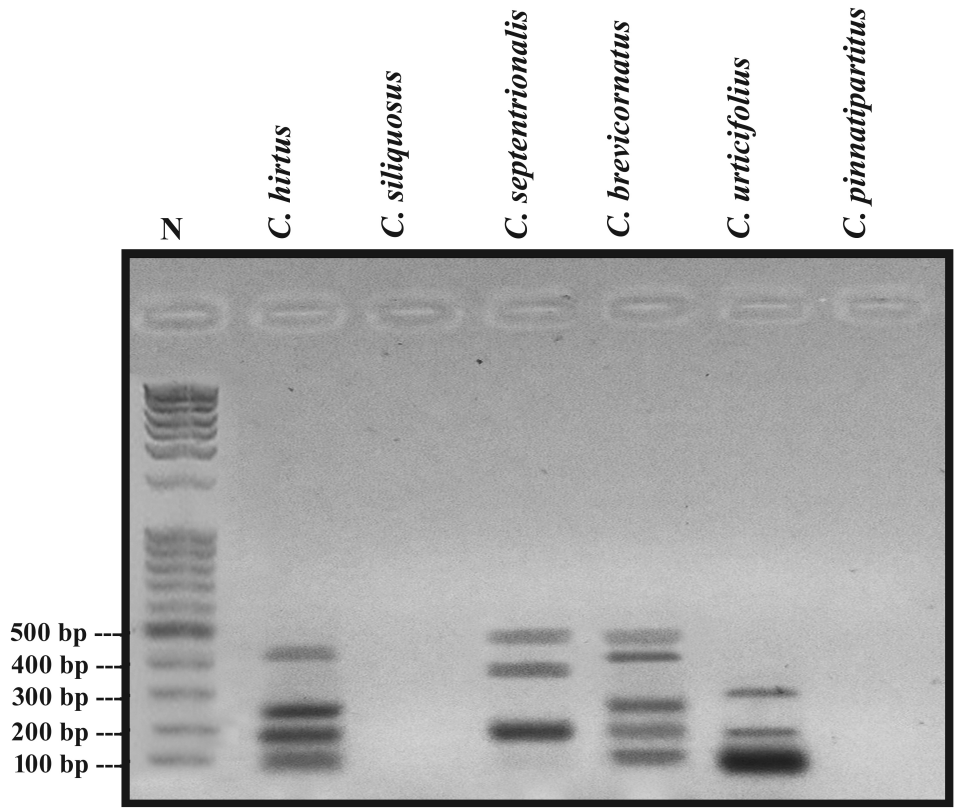


Fig. 130. SSR profile of six wild *Corchorus* L. species with primer HK-18, N=100 bp DNA ladder.

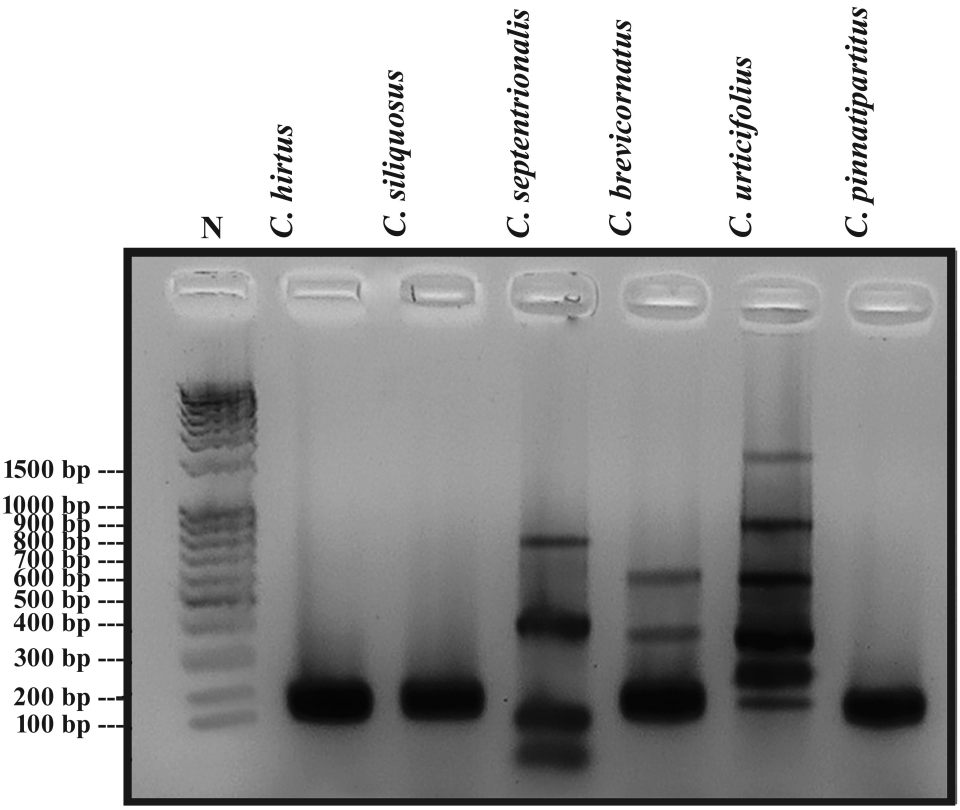


Fig. 131. SSR profile of six wild *Corchorus* L. species with primer HK-30, N=100 bp DNA ladder.

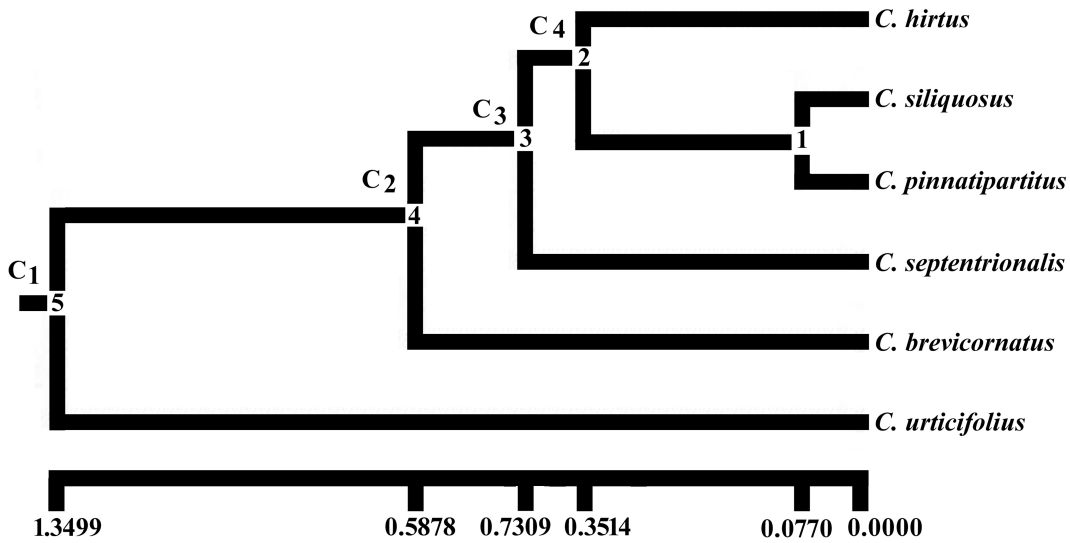


Fig. 132. UPGMA dendrogram constructed based on Nei's (1972) genetic distance summarizing the data on differentiation among six wild *Corchorus* L. species by SSR analysis.

3.11. Molecular phylogeny analysis with DNA barcode primers

Three barcode primers were used to make molecular phylogeny by sequence analysis. Description of primers used in this study was given in Materials and Methods section (Table 13). PCR amplification was 100% successful with three Bar-code primers of six wild *Corchorus* species (Figs 133-135). The DNA sequences obtained with different primers of six wild jute species in this study are listed in Table 53.The properties obtained from sequence analysis to phylogenetic tree are tabulated in Table 59. The majority-rule consensus trees using Maximum Likelihood (ML) methods (1000 bootstrap replicates) are illustrated in Figures 136 to 139. Several other genera of both Malvaceae and Sparrmanniaceae family were downloaded from NCBI to test the monophyly of genera *Corchorus* (Table 54). The primer wise Maximum Likelihood tree analysis for *Corchorus* species are given below:

3.11.1. ML tree from ITS data matrices

The ML analysis of ITS sequence data matrices resulted in a bootstrap consensus tree with the log likelihood of -5007.84 [SBL (Sum of Branch Length) = 2.758] (Fig. 136). A well defined clades were constructed after this analysis for six wild *Corchorus* species with strong bootstrap support (97%) except for *C. pinnatipartitus* and no outgroup member (Fig. 136). *Corchorus pinnatipartitus* make a different sub-clade from rest five *Corchorus* species. A terminal sub-clade includes *C. hirtus* and *C. septentrionalis* with less bootstrap significance (Fig. 136). Percentage (%) of guanine (G) and cytosine (C) is higher than adenine (A) and thymine (T) in case of ITS sequence analysis for the studied six *Corchorus* species (Table 55)

3.11.2. ML tree from *MatK* data matrices

The ML analysis of *MatK* sequence data matrices resulted in a bootstrap consensus tree with the log likelihood of -2160.87 [SBL (Sum of Branch Length) = 0.268] (Fig. 137). A well defined clades were constructed after this

analysis for five wild *Corchorus* species with strong bootstrap support (99%) except for *C. pinnatipartitus* and no outgroup member (Fig. 137). On the other hand, *C. pinnatipartitus* make a well defined clade (bootstrap 100%) with other genus of Malvaceae which used as out group member. With this primer, terminal sub-clade includes *C. hirtus* and *C. siliquosus* with strong bootstrap value (Fig. 137). Percentage (%) of adenine (A) and thymine (T) is higher than guanine (G) and cytosine (C) in case of *MatK* data analysis for the studied six *Corchorus* species (Table 56).

3.11.3. ML tree from *rbcL* data matrices

The ML analysis of *rbcL* sequence data matrices resulted in a bootstrap consensus tree with the log likelihood of -1264.18 [SBL (Sum of Branch Length) = 0.104] (Fig. 138). A well defined clades were constructed after this analysis for five wild *Corchorus* species with strong bootstrap support (100%) except for *C. pinnatipartitus* and no outgroup member (Fig. 138). On the other hand, *C. pinnatipartitus* make a well defined clade (bootstrap 96%) with other genus of Malvaceae which used as out group member. With this primer, terminal sub-clade includes *C. brevicornatus* and *C. urticifolius* with less bootstrap significance (Fig. 138). Percentage (%) of adenine (A) and thymine (T) is higher than guanine (G) and cytosine (C) in case of *rbcL* data analysis for the studied six *Corchorus* species (Table 57).

3.11.4. ML tree from combined (ITS, *MatK* and *rbcL*) data matrices

The ML analysis of combined (ITS, *MatK* and *rbcL*) data matrices resulted in a bootstrap consensus tree with the log likelihood of -10247.60 [SBL (Sum of Branch Length) = 1.188] (Fig. 139). A well defined clades were constructed after this analysis for six wild *Corchorus* species with strong bootstrap support (98%) and no outgroup member (Fig. 139). With 98% bootstrap significance *C. pinnatipartitus* is distant from rest five species make a different sub-clade. Two terminal sub-clade one includes *C. hirtus* and *C. urticifolius* and another includes *C. septentrionalis* and *C. brevicornatus* with less bootstrap

significance (Fig. 139). *Corchorus siliquosus* is distant from that four *Corchorus* species with 100% bootstrap and make a different sub-clade (Fig. 139). Percentage (%) of adenine (A) and thymine (T) is higher than guanine (G) and cytosine (C) in case of combined (ITS, *MatK* and *rbcL*) data matrice for the studied six *Corchorus* species (Table 58).

Table 53. DNA sequences obtained with different primers of six wild *Corchorus* L. species.

Species	DNA sequences with different primers		
	ITS	***MatK***	***rbcL***
C.hirtus	ACTAAGCGACACTATAG GGTCATATTCGTTGGTC CACCAAGACGAGGGGC ACACGCGGCCAGCTGAT AGCGTTACCACCGAAGG CCGCGGCACCAAGATCG CCGAGGACTAGATATTT AGGCCAACCGCGAGCCA GAGCGCACGGGAGGCC ATTTTCCGCCCGAAGCA ACCCGGAGCCTTGGAGG GCTCGGGAGTTGGGAGG GCGACGATGCGTGACAC CCAGGCAGACGTGCCCT CGGCCTAATGGCTTGGG GCGCAACTTGCGTTCAA AGACTCGATGGTTCACG GGATTCTGCAATTCACA CCAAGTATCGCATTTCG CTACGTTCTTCATCGATG CGAGAGCCGAGATATCC GTTGCCGAGAGTCGTTA TAGATTCTCAATAAACA AGGCACGGCCGGTCGCC AACACACCGAGACCGGG GCGGCTTCCGTCGTGGT CCTTTAGTTCGAGTTCCT TGGCGCAATTCACGCCG GGGTTCGTTATAAAGCC ACGGGCGACATGGCCTC GCACATCCCATGGCGCG ATGGCCATGGAACGACA CGGGGTCACGAGCACCC GGGCCGAGGAGACTAG AGCACACTAGTGCGCCC TCGCCCCCGAGTGTTGT GTTACTAGTTCGCGGGT CGCTCTGCTA	ACGGTTCTCTCTCTATG AGTATTGTAATTCGCAC AATTTTTTTACTCCAAA GAAATTTATTTCTATCT TTAACCCAAGATTTTTC TGGTTCCCATTTTATTC TCATGTATGTGAATATG AATCCGTTTTCCTTTTT CTCTGTAACCAATCTTC TCATTTACGATCAACAT CCTCTGGAGTCTTTCTT GAAAGAATTTATTTCTA TGGAAAAACGGAGCAT CTTGTAGAAGTCTTTTC TAATGATTTTCCGAACA ACTTCTGGTTGTTCAAA GATCCTTTCACACATTT TATTAGATATCAAGGA AAGGCAATTCTAGCCT CAAAAGATACGTCTCTT CTGATGAATAGGTGGA AATATTACTTTGTCGAT TTATGGCAATATTATTT TTACGTGTGGTCTCAAT CAGGAAAAGTCCGTAT AAATCAATTATCTAAAT ATTCTCTCGACTTTCTG GGCTATCTTTCAAGTGT GCGATTAAATCCTTCAG TGGTACGGAGTCAAAT GCTAGAAAATTCATTTA TAATAGATAATGCTAT GAAGAAGTGGGATACA AGAATTCCAATTATTTC TCTGATTGGATCATTGT GTAAAGCGAAATTTTG TAACACATTGGGGCAT CCCATTAGTAAGGCGA CGTGGGCCGATTCCTCA GATTCTGAGATTATTGA CCGATTTGTGCGTATAT GCAGAAATCTTTCTCAT TATCACAGTGGATCTTT CAAAAAAAAAGAG	AAATTGACTTATTAT ACTCCTGAGTATCAA GTCAAAGATACTGAT ATCTTGGCAGCCTTC CGAGTAACTCCTCAA CCCGGAGTTCCGGCT GAGGAAGCAGGAGC CGCGGTAGCTGCTGA ATCTTCTACTGGTAC ATGGACAACCGTGTG GACCGATGGGCTTAC CAGCCTTGATCGTTA CAAAGGGCGCTGCTA CCACATCGAGCCCGT TGCTGGAGAAGAAGA TCAATATATATGTTAT GTAGCTTACCCCTTA GACCTTTTTGAAGAA GGTTCTGTTACTAAC ATGTTTACTTCCATTG TGGGTAATGTATTTG GGTTCAAAGCCCTGC GCGCTCTACGTCTAG AGGATCTGCGAATCC CTCCTGCTTATATTAA AACTTTCCAAGGCCC GCCTCATGGGATCCA GGTCGAAAGAGATAA ATTGAACAAGTATGG TCGTCCCCTATTGGG ATGTACTATTAAACC TAAATTGGGGTTATC CGCTAAGAACTACGG TAGAGCTGTTTATGA ATGTCTACGTGGGGG ACTTGATTTTACCAA AGATGATGAGAATGT GAACTCCCAACCATT TATGCGTTGGAGAGA CCGTTTCTTATTTTGT GCCGAAGCAATTTTT

Species	DNA sequences with different primers		
	ITS	***MatK***	***rbcL***
C.siliquosus	CAGGAATCCCGCCTGAC TGGGGTCGCATTGCAGC GTCGACTAAGCGCATAT AGGGTCATATTCGTTGG TCCACCAAGACGAGGGG CACACGCGCCAGTGATA GCGTTACCACCGAAGGC CGCGGCACCAAGATCGC CGAGGACAGATATTTAG CCAACCGCGAGCCGAGC CACGGAGGCCATTTTCC GCCCGAAGCAACCCGGA GCCTTGGAGGGCTCGGG AGTGGGAGGGCGACGAT GCGTGACACCCAGGCAG ACGTGCCCTCGGCTAAT GGCTGGGGCGCAACGCG TTCAAAGACTCGATGGT TCACGGGATTTGCAATT CACCAAGTATGCATTTC GCTACGTTTTCATCGAT GCGAGAGCCAGATATCG TTGCCGAGAGTCGTTAT AGATTCTCAATAAACAA GGCACGGCCGGTGCAAC ACACCGAGACCGGGGCG GCTTCCGTCGTGGTCCTT TAGTTGAGTTCCTGGCG CAATTCACGCCGGGGTT CGTTATAAAGCACGGCG ACATGCCTCGCACATCC CATGGCGGATGCCAGGA GACACGGGTCACGAGCA CCCGGCCGAGGAGACTA GAGCACACTAGTGCGCC CTCGCCCCCGAGTGTTG TGTTACTATTCGCGGGT CGCTCTGCTAGGCAGGT TCGACATGACCTTCCGG TTACAAA	TTTTATTACGGTTCTCT CTCTATGAGTATTGTAA TTTGTAGAGTTTTTTTA CTCCAAAGAAATTTATT TCTATTTTTAACCCAAG ATTTTTCTGGTTCCTAT ATAATTCTCATGTAGGA ATATGAATCCATTTTCC TTTTTTCGTAACCAATC TTTCATTTCGATCAACA TCCTCTGGAGTCTTTCT GAAAGAATTTATTTCTT GGAAAAACGGAGCATC TTGTAGAAGTCTTTTTA ATGATTTTCCGAACAAC TTCTGGTTGTTCAAAGA CCTTTCACACATTTTAT TAGATATCAAGGAAAG GCAATTCTAGCCTCAA AAGATACGTCTCTTCTG ATGAATAGGTGGAAAT ATTACTTTGTCGATTTA TGGCAATATTATTTTTA CGTGTGGTCTCATCAGG AAAGTCCGTATAAATC AATTATCTAAATATTCT CTCGACTTTCTGGGCTA TCTTTCAAGTGTGCGAT TAAATCCTTCAGTGGTA CGAGTCAAATGCTAGA AAATTCATTTTAATAGA TAATGCTATGAAGAAG TTGGATACAAGAATTC CAATTATTTCCTGATTG GATCATTGTCTAAAGC GAAATTTTGTAACACAT TGGGTCATCCCATTAGT AAGGCGACGTGGGCCG ATTCCTCAGATTCTGAG ATTAATGACCGATTTGT GCGTATATGCAGAAAT CTTTCTCATTACCA CAGTGGATCTTCAAAA AAAAAG	AAATTGACTTATTAT ACTCCTGAGTATCAA GTCAAAGATACTGAT ATCTTGGCAGCCTTC CGAGTAACTCCTCAA CCCGGAGTTCCGGCT GAGGAAGCAGGAGC CGCGGTAGCTGCTGA ATCTTCTACTGGTAC ATGGACAACCGTGTG GACCGATGGGCTTAC CAGCCTTGATCGTTA CAAAGGGCGCTGCTA CCACATCGAGCCCGT TGCTGGAGAAGAAGA TCAATATATATGTTAT GTAGCTTACCCCTTA GACCTTTTTGAAGAA GGTTCTGTTACTAAC ATGTTTACTTCCATTG TGGGTAATGTATTTG GGTTCAAAGCCCTGC GCGCTCTACGTCTAG AGGATCTGCGAATCC CTCTTCTTATATTAAA ACTTTCCAAGGCCCG CCTCATGGGATCCAG GTCGAAAGAGATAAA TTGAACAAGTATGGT CGTCCCCTATTGGGA TGTACTATTAAACCT AAATTGGGGTTATCC GCTAAGAACTACGGT AGAGCTGTTTATGAA TGTCTACGTGGGGGA CTTGATTTTACCAAA GATGATGAGAATGTG AACTCCCAACCATTT ATGCGTTGGAGAGAC CGTTTCTTATTTTGTG CCGAAGCAATTT

Species	DNA sequences with different primers		
	ITS	***MatK***	***rbcL***
C.brevicornatus	CGACACTATAGGGTCAT ATTCGTTGGTCCACCAA GACGAGGGGCACACGC GGCCAGCTGATAGCGTT ACCACCGAAGGCCGCGG CACCAAGATCGCCGAGG ACTAGATATTTAGGCCA ACCGCGAGCCAGAGCGC ACGGGAGGCCATTTTCC GCCGAAGCAACCCGGAG CCTTGGAGGGCTCGGGA GTTGGGAGGGCGACGAT GCGTGACACCCAGGCAG ACGTGCCCTCGGCCTAA TGGCTTGGGGCGCAACT TGCGTTCAAAGACTCGA TGGTTCACGGGATTCTG CAATTCACACCAAGTAT CGCATTTCGCTACGTTCT TCATCGATGCGAGAGCC GAGATATCCGTTGCCGA GAGTCGTTATAGATTCT CAATAAACAAGGCACGG CCGGTCGCCAACACACC GAGACCGGGGCGGCTTC CGTCGTGGTCCTTTAGTT CGAGTTCCTTGGCGCAA TTCACGCCGGGGTTCGT TATAAAGCCACGGGCGA CATGGCCTCGCACATCC CATGGCGCGATGGCCAT GGAACGACACGGGGTCA CGAGCACCCGGGCCGAG GAGACTAGAGCACATAG TGCGCCCTCGCCCCCGA GTGTTGTGTTACTAGTTC GCGGGTCGCTCTGCTAG G	CCTCCTCTTTGCATTTA TTACGGTTCTCTCTCTA TGAGTATTGTAATTTG AAGAGTTTTTTTACTCC AAAGAAATTTATTTCTA TTTTTAACCCAAGATTT TCTGGTTCCTATATAAT TCTCATGTATGTGAATA TGAATCCATTTTCCTTT TTCTCTGTAACCAATCT TCTCATTTACGATCAAC ATCCTCTGGAGTCTTTC TTGAAAGAATTTATTTC TATGGAAAAACGGAGC ATCTGTAGAAGTCTTTT CTAATGATTTTCCGAAC AACTTCTGGTTGTTCAA AGATCCTTTCACACATT TTATTAGATATCAAGG AAAGGCAATTCTAGCC TCAAAAGATACGTCTCT TCTGATGAATAGGTGG AAATATTACTTTGTCGA TTTATGGCAATATTATT TTTACGTGTGGTCTCAA TCAGGAAAAGTCCGTA TAAATCAATTATCTAAA TATTCTCTCGACTTTCT GGGCTATCTTTCAAGTG TGCGATTAAATCCTTCA GTGGTACGGAGTCAAA TGCTAGAAAATTCATTT ATAATAGATAATGCTA TGAAGAAGTTGGATAC AAGAATTCCAATTATTT CTCTGATTGGATCATTG TCTAAAGCGAAATTTTG TAACACATTGGGGCAT CCCATTAGTAAGGCGA CGTGGGCCGATTCCTCA GATTCTGAGATTATTGA CCGATTTGTGCGTATAT GCAGAAATCTTTCTCAT TATCACAGTGGATCTTC AAAAAAAAAGAGTTTG TATCGAATAAAA	TAAATTGACTTATTAT ACTCCTGAGTATCAA GTCAAAGATACTGAT ATCTTGGCAGCCTTC CGAGTAACTCCTCAA CCCGGAGTTCCGGCT GAGGAAGCAGGAGC CGCGGTAGCTGCTGA ATCTTCTACTGGTAC ATGGACAACCGTGTG GACCGATGGGCTTAC CAGCCTTGATCGTTA CAAAGGGCGCTGCTA CCACATCGAGCCCGT TGCTGGAGAAGAAGA TCAATATATATGTTAT GTAGCTTACCCCTTA GACCTTTTTGAAGAA GGTTCTGTTACTAAC ATGTTTACTTCCATTG TGGGTAATGTATTTG GGTTCAAAGCCCTGC GCGCTCTACGTCTAG AGGATCTGCGAATCC CTCCTGCTTATATTAA AACTTTCCAAGGCCC GCCTCATGGGATCCA GGTCGAAAGAGATAA ATTGAACAAGTATGG TCGTCCCCTATTGGG ATGTACTATTAAACC TAAATTGGGGTTATC CGCTAAGAACTACGG TAGAGCTGTTTATGA ATGTCTACGGGGGGA CTTGATTTTACCAAA GATGATGAGAATGTG AACTCCCAACATTTA TGCGTTGGAGAGACC GTTTCTTATTTTGTGC GAGCAATTTTT

Species	DNA sequences with different primers		
	ITS	***MatK***	***rbcL***
C.urticifolius	CTAAGCGACACTATAGG GTCATATTCGTTGGTCC ACCAAGACGAGGGGCA CACGCGGCCAGTGATAG CGTTACCACCAAGGCCG CGGCACCAAGATCGCCG AGGACTAGATATTTAGG CCAACCGCGAGCCAGAG CGCACGGGAGGCCATTT TCCGCCCGAAGCAACCC GGACTGGAGGGCTCGGG AGTTGGAGGGCGACGAT GCGTGACACCCAGGCAG ACGTGCCCTCGGCCTAA TGGCTTGGGGCGCAACT TGCGTTCAAAGACTCGA TGGTTCACGGGATTCTG CAATTCACACCAAGTAT CGCATTTCGCTACGTTCT TCATCGATGCGAGAGCC GAGATATCCGTTGCCGA GAGTCGTTATAGATTCT CAATAAACAAGGCACGG CCGGTCGCCAACAACCG AGACCGGGGCGGCTTCC GTCGTGGTCCTTTAGTTC GAGTTCCTTGGCGCAAT TCACGCCGGGGTTCGTT ATAAAGCCACGGGCGAC ATGGCCTCGCACATCCC ATGGCGCGATGGCCATG GAACGACACGGGGTCAC GAGCACCCGGGCCGAGG AGATAGAGCACACTAGT GCGCCCTCGCCCCCGAG TGTTGTGTTACTAGTTCG CGGG	TTTATTACGGTTCTCTC TCTATGAGTATTGTAAT TTGCAGAGTTTTTTTAC TCCAAGAAATTATTCTA TTTTTAACCCAAGATTT TTCGGTTCCTATATAAT TCTCAGTATGTGAATAT GAATCCATTTCTTTTCT TAACCAATCTTCTCATT ACGATCAACATCCTCTG AGTCTTTCTTGAAAGAA TTTATTTCTATGGAAAA ACGGAGCATCTTGTAG AAGTCTTTCTAATGATT TTCCGAACAACTTCTGG TTGTTCAAAGATCCTTT CACACATTTTATTAGAT ATCAAGGAAAGGCAAT TCTAGCCTCAAAAGAT ACGTCTCTTCTGATGAA TAGGTGGAAATATTAC TTTGTCGATTTATGGCA ATATTATTTTTACGTGT GGTCTCAATCAGGAAA AGTCCGTATAAATCAA TTATCTAAATATTCTCT CGACTTTCTGGGCTATC TTTCAAGTGTGCGATTA AATCCTTCAGTGGTACG GAGTCAAATGCTAGAA AATTCATTTATAATAGA TAATGCTATGAAGAAG TTGGATACAAGAATTC CAATTATTTCTCTGATT GGATCATTGCTAAAGC GAAATTTTGTAACACAT TGGGGCATCCCTTAGTA AGGGACGTGGGCCGAT TCCTCAGATTCTGAGAT TATTGACCGATTTGTGC GTATATGCAGAAATCTT TCTCATTATCACAGTGG ATCTTCAAAAAAAAGA GTTTGTATCGAAAAAA	AAATTGACTTATTAT ACTCCTGAGTATCAA GTCAAAGATACTGAT ATCTTGGCAGCCTTC CGAGTAACTCCTCAA CCCGGAGTTCCGGCT GAGGAAGCAGGAGC CGCGGTAGCTGCTGA ATCTTCTACTGGTAC ATGGACAACCGTGTG GACCGATGGGCTTAC CAGCCTTGATCGTTA CAAAGGGCGCTGCTA CCACATCGAGCCCGT TGCTGGAGAAGAAGA TCAATATATATGTTAT GTAGCTTACCCCTTA GACCTTTTTGAAGAA GGTTCTGTTACTAAC ATGTTTACTTCCATTG TGGGTAATGTATTTG GGTTCAAAGCCCTGC GCGCTCTACGTCTAG AGGATCTGCGAATCC CTCCTGCTTATATTAA AACTTTCCAAGGCCC GCCTCATGGGATCCA GGTCGAAAGAGATAA ATTGAACAAGTATGG TCGTCCCCTATTGGG ATGTACTATTAAACC TAAATTGGGGTTATC CGCTAAGAACTACGG TAGAGCTGTTTATGA ATGTCTACGTGGGGG ACTTGATTTTACCAA AGATGATGAGAATGT GAACTCCCAACCATT TATGCGTTGGAGAGA CCGTTTCTTATTTTGT GCCGAAGCAATTTTT AAA

Species	DNA sequences with different primers		
	ITS	***MatK***	***rbcL***
C.pinnatipartitus	TGCGAGCGTCGTCCTTG CGACACTTAAAGGGTCA TTTGTGGATTCCGGCCA AGCAAAGGCCGCGACCA ACTAGGTTCGAGGAACA CCTTGAGGCATTACCAC CAAAGTGAAGGTCGCAG CATAATCTTTGCCTGGG AATCAGATTTCGGCCAA CCGCGACAGCAGAGCGC ACGGGAGACCAATATCC GTCCCGCAACACAAACC AAAATGGGGTTGTTGAG GGCGACGATGCGTGACA CCCAGGCAGGCGTGCCC TCGGCCTAATGGCTTGG GGCGCAACTTGCGTTCA AAGACTCGATGGTTCAC GGGATTTGCAATTCACA CCAAGTATCGCATTTCG CTACGTTCTTCATCGATG CGAGAGCCGAGATATCC GTTGCCGAGAGTCGTTA TAGACTACAACAAGAAA GGAGACACTTCGCCCTC CGGCAGCACACCGCAAA CGGGGCAGCTAAGGGAT TCATGCTCATTTTCGTTT CATTTCCTTGGCGCGAC TCGCGCCGTGGGTTGGT TGATGAGCACAAGCGAA GAGGCAGTCCTTTGCAC CCCGCAACTAGGCAAGA TGACTTCGGAAGGCCCC ATACACCCTTAGCTCGA AGGGAAGAACTTGGAGC GGTAAAGCATCCTCGGA ACCCCCCGGGTGTTTTT AA CTTGTTCGCGGGTCGTT	TTCTTCTTTGCATTTATT ACGGTTCTCTCTCTACG AGTATTGTAATTTGAAG AGTTTTATTACTCCAAA TAAATCTATTTTTAATC CAAGATTATTCTTGTTC CTATATAATTCTCATGT ATGTGAATACGAATCG ATTTTCCTTTTTCTCCGT AACCAACCTTCTCATTA CGATCAACATCTTCTGG AGTTTTTCTTGAACGAA TTTATTTCTATGGAAAA ATAGAGTATCTGTAGA AGTCGTTTGTAATGATT TTCAGAACAACCTAGG GTTGTTCAAAGACCCTT TCATACATTTTATTAGG TATCAAGGAAAGGCAA TTCTGGCCTCAAAAGAT ACGCCTTTTCTGATGAA TAAGTGGAAATATTAC TTTGTCGATCTATGGCA ATATTATTTTTACGTAT GGTTTCAATCAGGAAG AGTTCGTATAAATCAAT TATCTAAATATTCTCTC GACTTTCTGGGCTATCT TTCAAGTGTGCGATTAA ATACTTCAGTGGTACG GAGTCAAATGCTAGAA AATTCATTTATAATAGA TAATGCTATGAAGAAG TTGGATACAAGAATTC CAATTATTTCTCTCATT GGATCGTTGTCTAAAG CGAAGTTTTGTAACAC ATTAGGGCATCCCATTA GTAGCCGACGTGGGCC GATTCCCCCGATTCTGA TATTATTGACCGATTTG TGCGTATATGCAGAAA TCTTTCTCATTATCACA GTGGATCTTCAAAAAA AAAGAGTTTGTATCG AATAAAA	AAATTGACTTATTAT ACTCCTGAATATGAA GTCAAAGATACTGAT ATCTTGGCAGCCTTC CGAGTAACTCCTCAA CCCGGAGTTCCACCT GAGGAAGCAGGGGC CGCGGTAGCTGCTGA ATCTTCTACTGGTAC ATGGACAACCGTGTG GACCGATGGGCTTAC CAGCCTTGATCGTTA CAAAGGGCGATGCTA CCACATTGAGCCCGT TGCTGGAGAAGAAAA TCAATATATATGTTAT GTAGCTTACCCTTTA GACCTTTTTGAAGAA GGTTCTGTTACTAAC ATGTTCACTTCCATTG TGGGTAATGTATTTG GATTCAAAGCCCTGC GCGCTCTACGTCTAG AGGATCTGCGAATCC CTAATTCTTATATTAA AACTTTCCAAGGCCC GCCTCATGGTATCCA GGTTGAAAGAGATAA ATTGAACAAGTATGG TCGTCCCCTATTAGG ATGTACTATTAAACC TAAATTGGGGTTATC CGCTAAGAACTACGG TAGAGCAGTTATGAA TGTCTACGTGGTGGA CTTGATTTTACCAAA GATGATGAGAATGTG AACTCCCAACCGTTT ATGCGTTGGAGAACC GTTTCTTTTTTGTGCC GAAGCAATTT

Table 54. NCBI Gene Bank Accession Numbers of taxon incorporated in this study.

Taxon	Accession Numbers for Specific Regions		
	ITS	*MatK*	*rbcL*
Triumfetta lappula	FR875084.1	JQ589357.*1*	JQ594279.*1*
Grewia occidentalis	KY754191.1	JX517699.*1*	JX572642.*1*
Pterospermum acerifolium	KC981072.1	KJ510943.1	KJ440043.1
Gossypium barbadense	U12715.1	JN201360.1	JN243157.1
Gossypium robinsonii	AF057755.1	JN201498.1	L13186.2
Triumfetta pilosa var. tomentosa	MG730183.1	JF270979.1	JF265638.1
Hibiscus sabdariffa var. sabdariffa	FJ527608.1	JQ693596.1	KU568464.1
Hibiscus cannabinus	FJ527607.1	JQ693597.1	HQ235601.1
Hibiscus surattensis	FJ527609.1	JQ693598.1	KU567993.1
Sparrmannia ricinocarpa	FR875088.1	-	-
Tilia amurensis	KF445432.1	-	-
Tilia heterophylla	AF174639.1	-	-
Grewia biloba	MH710784.1	-	-
Dombeya mandenensis	JF939251.1	-	-
Dombeya calantha	AY083656.1	-	-
Dombeya x cayeuxii	KF587908.1	-	-
Pentapetes phoenicea	JF939204.1	-	-
Triumfetta procumbens	MH768335.1	-	-
Sparrmannia palmata	FR875087.1	-	-
Gossypium hirsutum	-	JN201459.1	-
Malva moschata	-	MG220686.1	-
Malva parviflora	-	JQ412263.1	-
Dombeya tiliacea	-	JX495704.1	-
Dombeya cymosa	-	JF270747.1	-
Trochetiopsis ebenus	-	LT746101.1	-
Grewia occidentalis	-	JX517699.1	-
Sparrmannia africana	-	MF350141.1	-
Heliocarpus americanus	-	JQ589797.1	AJ233153.1
Triumfetta rhomboidea	-	-	KX527080.1
Sparrmannia ricinocarpa	-	-	AJ233128.1
Grewia orientalis	-	-	GU981731.1
Microcos paniculata	-	-	AY328196.1
Trochetiopsis erythroxylon	-	-	AJ233143.1
Dombeya calantha	-	-	AY082354.1
Pterospermum menglunense	-	-	KR529958.1

Table 55. Percentage (%) nucleotide composition generated based on the ITS sequence analysis.

ITS	T	C	A	G
Corchorus hirtus	17.6	29.9	21.7	30.8
Corchorus siliquosus	17.3	29.4	22.4	30.8
Corchorus septentrionalis	17.6	29.8	21.7	30.9
Corchorus brevicornatus	17.6	29.8	21.7	30.9
Corchorus urticifolius	17.6	29.7	22.0	30.7
Corchorus pinnatipartitus	19.3	27.8	25.3	27.6
Pterospermum acerifolium	26.0	27.0	20.7	26.3
Dombeya calantha	23.8	28.6	20.2	27.3
Dombeya mandenensis	23.7	29.1	19.7	27.5
Dombeya x cayeuxii	24.5	28.2	19.9	27.3
Pentapetes phoenicea	25.2	27.6	20.1	27.1
Triumfetta lappula	23.5	28.1	20.6	27.7
Triumfetta procumbens	23.2	27.8	20.8	28.3
Parrmannia palmata	15.9	34.7	18.9	30.5
Sparrmannia ricinocarpa	16.1	34.5	19.3	30.1
Grewia occidentalis	19.0	32.6	19.3	29.1
Grewia biloba	19.4	32.2	19.4	29.1
Tilia amurensis	19.9	32.3	18.6	29.2
Tilia heterophylla	18.8	33.0	18.8	29.5

Table 56. Percentage (%) nucleotide composition generated based on the *MatK* sequence analysis.

MatK	**T**	**C**	**A**	**G**
Corchorus hirtus	37.0	17.7	28.5	16.8
Corchorus siliquosus	37.1	17.3	28.9	16.7
Corchorus septentrionalis	37.1	17.4	28.5	17.0
Corchorus brevicornatus	37.0	17.2	29.1	16.7
Corchorus urticifolius	36.6	17.3	29.3	16.8
Corchorus pinnatipartitus	36.5	17.3	29.4	16.9
Trochetiopsis ebenus	37.0	16.9	29.3	16.8
Dombeya cymosa	36.9	17.2	29.0	16.9
Dombeya tiliacea	36.9	17.2	28.8	17.1
Triumfetta lappula	37.0	17.5	29.0	16.5
Triumfetta pilosa var. *tomentosa*	37.4	17.1	29.1	16.4
Gossypium barbadense	36.6	18.4	28.6	16.4
Gossypium hirsutum	36.6	18.4	28.6	16.4
Gossypium robinsonii	36.9	18.3	28.6	16.2
Grewia occidentalis	36.3	18.2	28.4	17.1
Heliocarpus americanus	36.9	17.5	29.0	16.6
Malva moschata	37.0	17.8	29.1	16.1
Malva parviflora	36.9	17.9	29.0	16.2
Sparrmannia africana	36.8	17.8	29.1	16.4

Table 57. Percentage (%) nucleotide composition generated based on the *rbcL* sequence analysis.

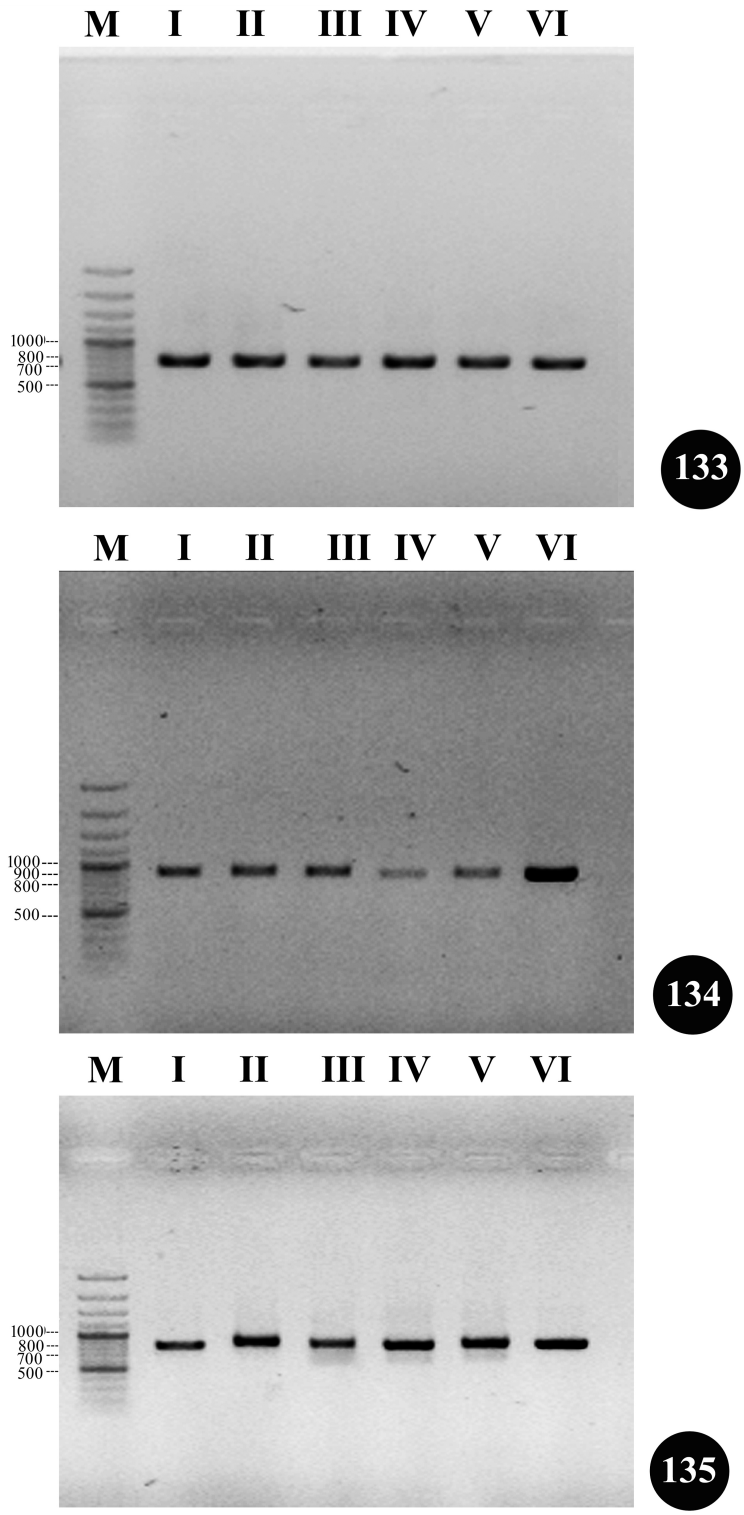
rbcL	**T**	**C**	**A**	**G**
Corchorus hirtus	28.6	21.8	26.0	23.6
Corchorus siliquosus	28.9	21.6	26.1	23.4
Corchorus septentrionalis	28.6	21.8	26.0	23.6
Corchorus brevicornatus	28.7	21.5	26.1	23.6
Corchorus urticifolius	28.6	21.8	26.0	23.6
Corchorus pinnatipartitus	29.2	21.0	27.1	22.7
Dombeya calantha	29.3	20.9	26.5	23.2
Pterospermum acerifolium	28.8	21.4	27.0	22.7
Pterospermum menglunense	28.8	21.4	27.0	22.7
Trochetiopsis erythroxylon	29.5	20.9	26.8	22.7
Triumfetta rhomboidea	29.0	21.4	26.2	23.4
Sparrmannia ricinocarpa	28.6	21.6	27.0	22.7
Grewia occidentalis	28.8	21.6	26.7	22.9
Heliocarpus americanus	28.5	21.9	26.7	22.9
Hibiscus sabdariffa var. *sabdariffa*	29.1	21.6	26.5	22.7
Microcos paniculata	28.3	21.9	26.5	23.2

Table 58. Percentage (%) nucleotide composition generated based on the combined sequence (ITS, *MatK* and *rbcL*) analysis.

ITS, *MatK* and *rbcL*	**T**	**C**	**A**	**G**
Corchorus hirtus	29.6	22.1	25.8	22.5
Corchorus siliquosus	29.9	21.5	26.2	22.3
Corchorus septentrionalis	30.0	21.8	26.0	22.3
Corchorus brevicornatus	30.0	21.8	26.0	22.3
Corchorus urticifolius	29.6	21.6	26.4	22.4
Corchorus pinnatipartitus	30.4	20.8	27.6	21.2
Triumfetta lappula	30.5	21.4	26.5	21.5
Triumfetta pilosa var. *tomentosa*	30.2	21.8	26.7	21.3
Grewia occidentalis	29.3	23.0	25.9	21.8
Gossypium barbadense	29.9	22.2	26.4	21.5
Gossypium robinsonii	30.1	22.1	26.5	21.3
Hibiscus cannabinus	29.4	22.7	25.8	22.1
Hibiscus sabdariffa var. *sabdariffa*	29.5	23.0	25.6	21.9
Hibiscus surattensis	29.4	22.6	25.9	22.1

Table 59. Properties of the data matrices generated based on the sequence analysis.

Name of the data matrices	Number of taxa	Total number of characters	Number of conserved characters	Number of variable characters	Number of parsimony-informative characters
ITS	19	648	114	530	474
MatK	19	738	566	156	101
rbcL	16	636	563	48	30
Combined (ITS, *MatK* and *rbcL*)	14	1864	862	1030	816



Figs 133-35. PCR amplification of six wild *Corchorus* L. species with three bar code primer, 133. ITS, 134. *Mat*K and 135. *rbcL*. Here, M = 100 bp DNA ladder, I = *C. hirtus*, II = *C. siliquosus*, III = *C. septentrionalis*, IV = *C. brevicornutus*, V = *C. urticifolius* and VI = *C. pinnatipartitus.*

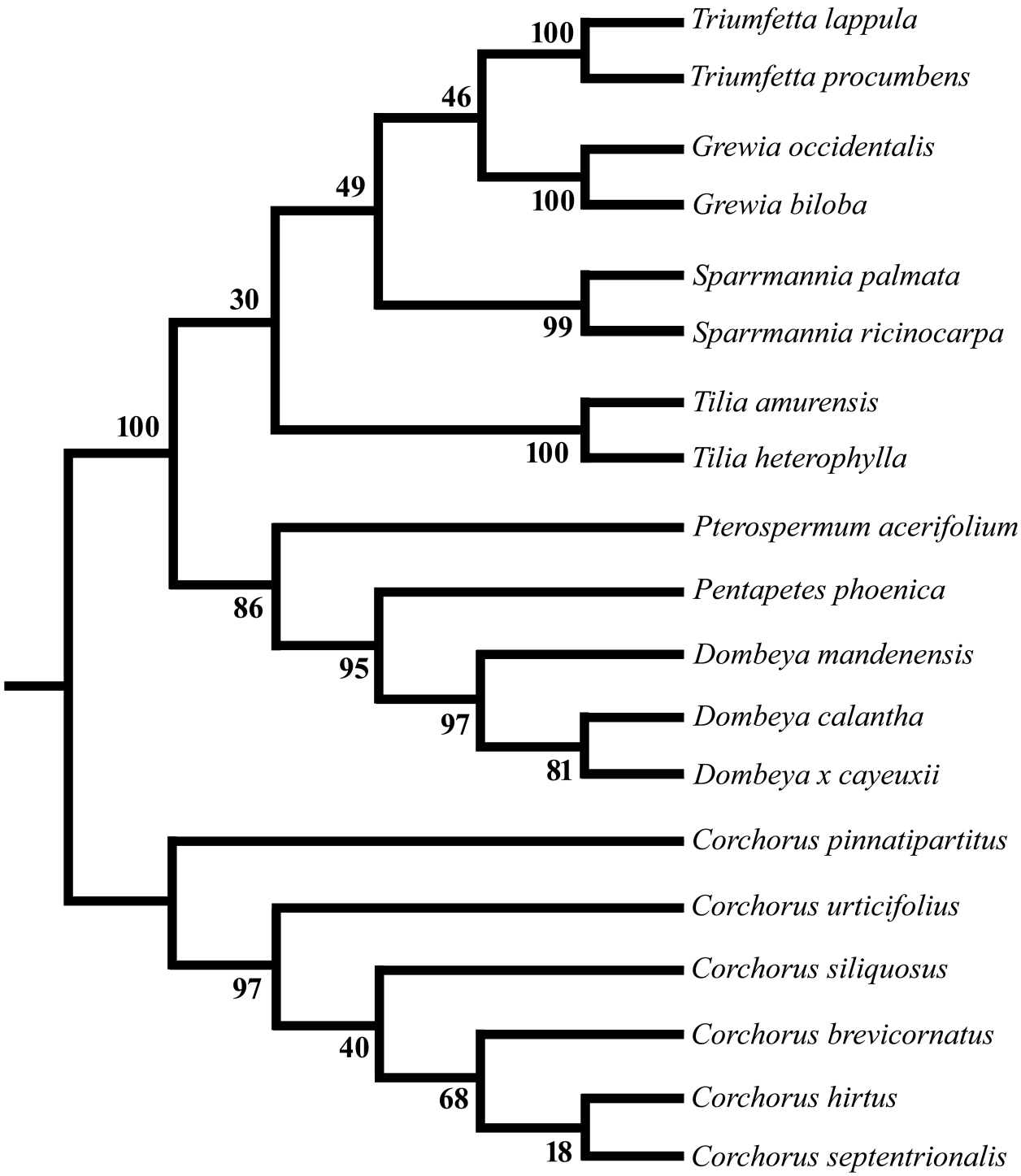


Fig. 136. Maximum Likelihood (ML) tree based on ITS data showing evolutionary relationships in six wild *Corchorus* species.

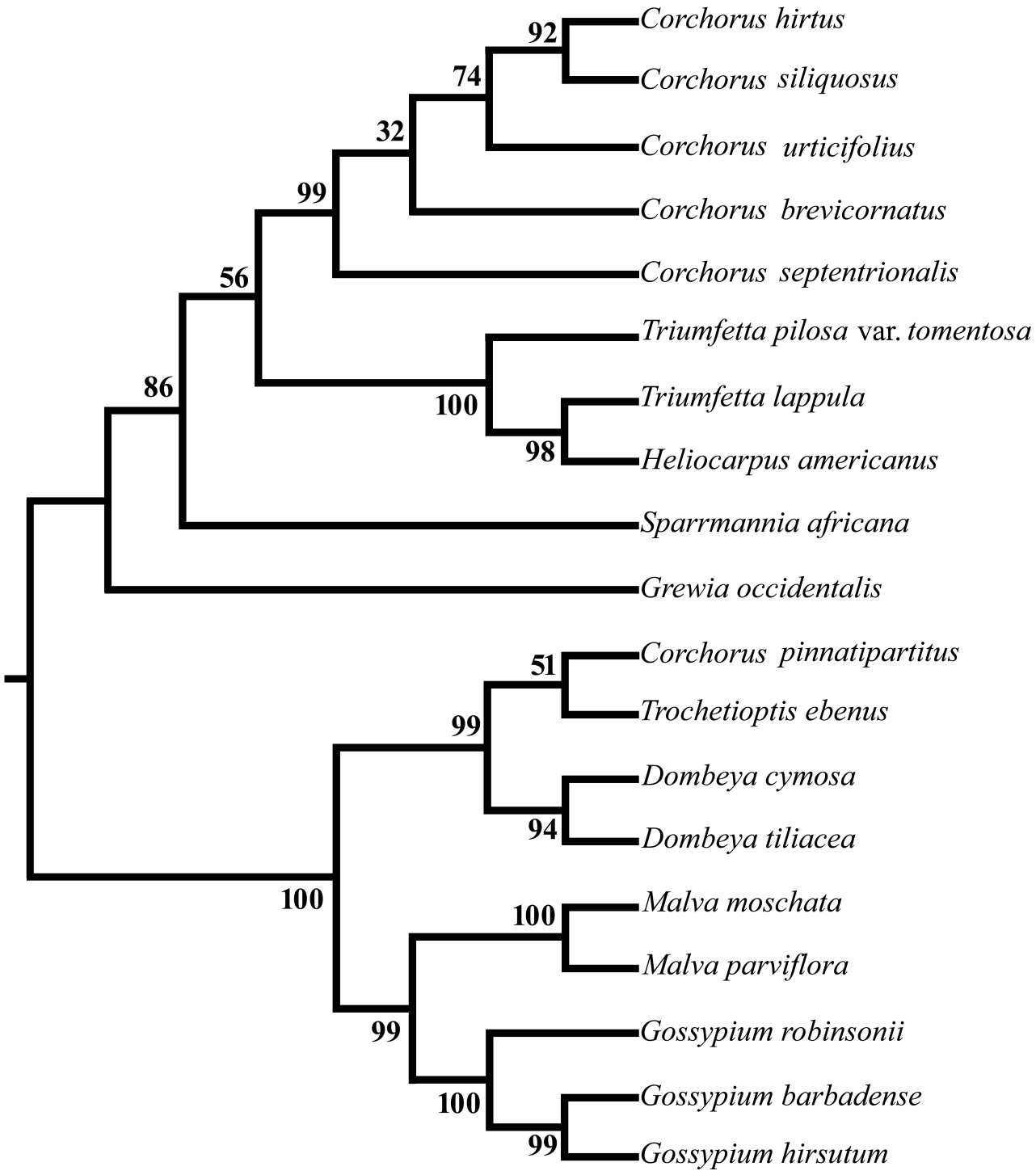


Fig. 137. Maximum Likelihood (ML) tree based on *mat*K data showing evolutionary relationships in six wild *Corchorus* species.

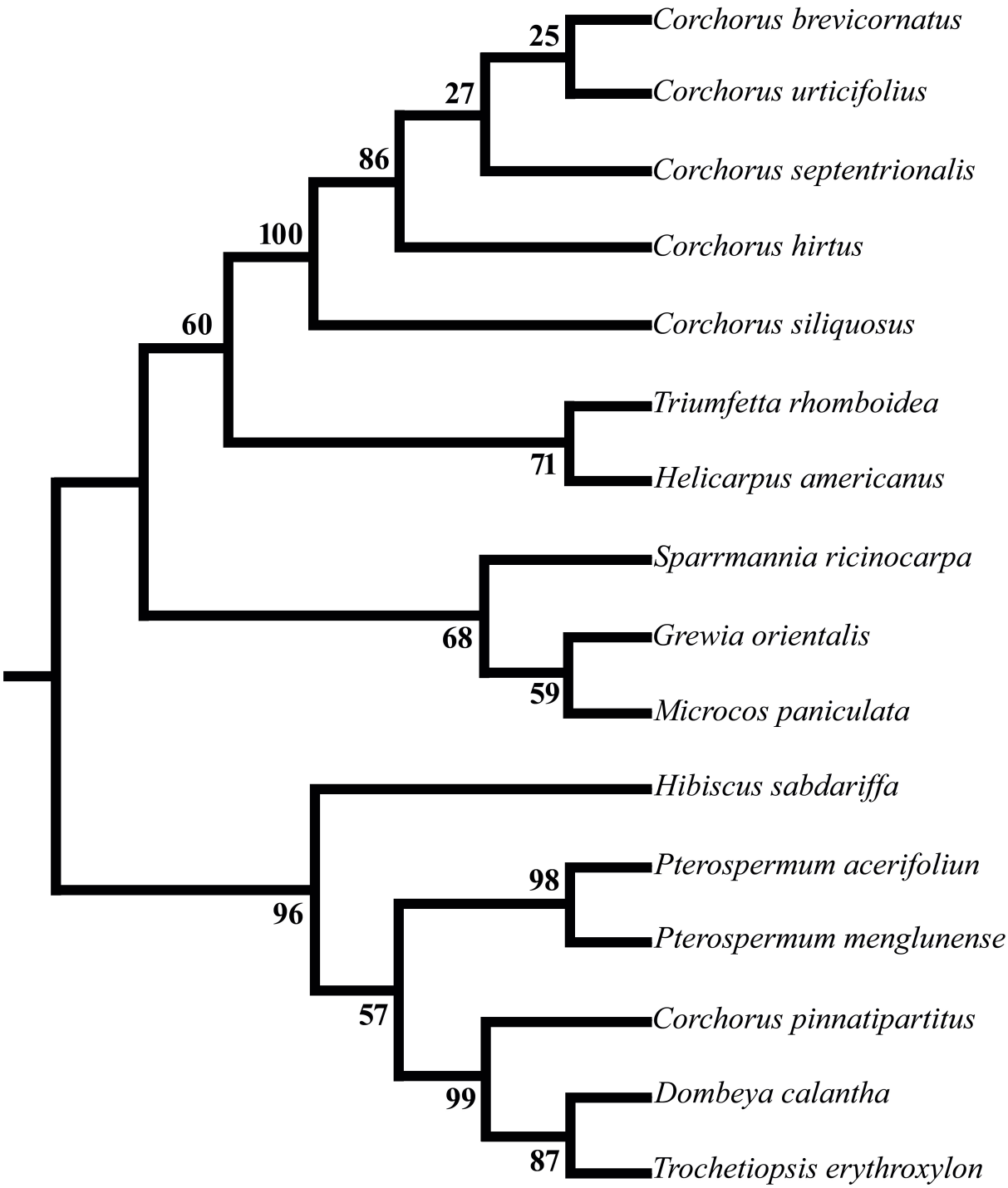


Fig. 138. Maximum Likelihood (ML) tree based on *rbcL* data showing evolutionary relationships in six wild *Corchorus* species.

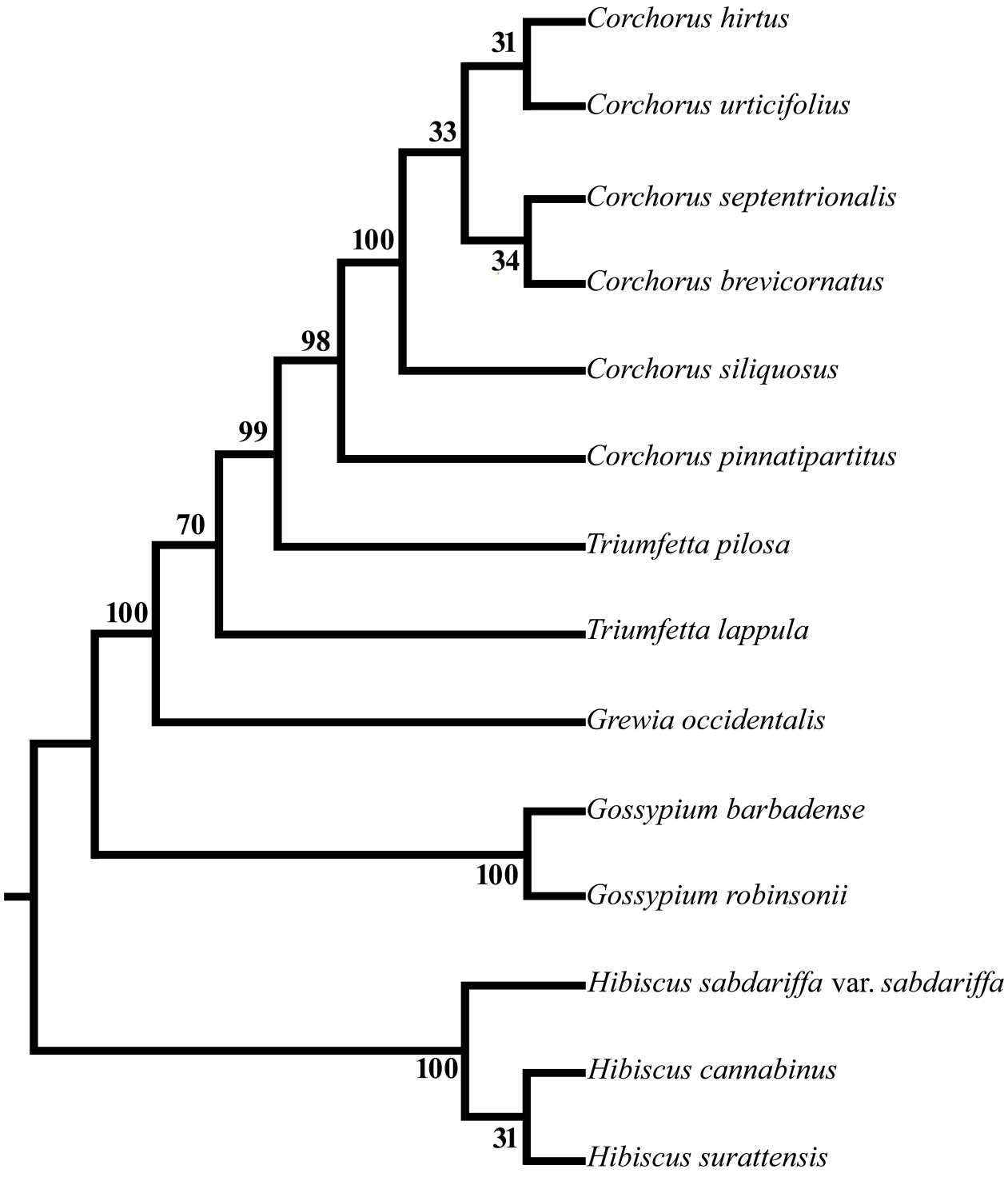


Fig. 139. Combined Maximum Likelihood (ML) tree based on ITS, *mat*K and *rbcL* data showing evolutionary relationships in six wild *Corchorus* species.

4. Discussion

Cytogenetical and molecular analysis was done to characterize six wild species *Corchorus* L. *viz. C. brevicornatus* Vollesen, *C. septentrionalis* Planch, *C. urticifolius* Wight & Arn., *C. hirtus* L., *C. siliquosus* L. and *C. pinnatipartitus* Wild following orcein, CMA- and DAPI- staining. For determining the genetic distance and understanding relationship and evolutionary trends among them molecular investigation with Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR) and barcode primers were used.

4.1. Pattern of heterochromatin distribution

The nature of heterochromatin distribution in interphase nuclei and prophase chromosomes considerd as a karyomorphological features to characterize different germplasm. Characterization of germplasm using these criteria was first proposed by Tanaka in 1971. According to Tanaka (1971) the nature and distribution of heterochromatins varies in different species following orcein staining. However, interphase nuclei and prophase chromosomes were classified into five categories according to the staining property (Tanaka 1971). Further, this technique was applied to characterize different plant materials by several researchers (Vosa 1973, Alam *et al.* 1993, Begum and Alam 2004, Hossain *et al.* 2016, Sultana and Alam 2016a, Bonna *et al*. 2018).

In this study, the interphase nuclei of diploid *Corchorus* species *viz. C. brevicornatus, C. septentrionalis* and *C. urticifolius* stained homogenously after orcein staining which could be classified as “Diffuse Type” (Tanaka 1971) (Figs 25-27, Table 60). On the other hand, the polyploid *Corchorus* species *viz. C. hirtus, C. siliquosus* and *C. pinnatipartitus* had small heterochromatic blocks distributed scatterdly in the interphase nuclei after orcein staining. According to Tanaka (1971) it could be considered as “Simple chromocenter type” (Figs 28-30, Table 60).

Uniformly stained prophase chromosomes were found in *C. brevicornatus* and *C. hirtus* after orcein staining (Figs 43 and 46, Table 60). According to Tanaka 1971, this type of prophase chromosomes could be regarded as "Continuous type". On the other hand, "Interstitial type" prophase chromosomes were observed in *C. siliquosus* and *C. pinnatipartitus* as the prophase chromosomes stained at the interstitial region with orcein (Figs 47 and 48, Table 60). However, the prophase chromosomes of *C. septentrionalis* and *C. urticifoilus* possessed "Gradient type" because the chromosomes were darkly stained at one end and gradually become faint towards another end (Figs 44 and 45, Table 60).

Usually, the equivalently distributed heterochromatin of interphase nuclei further located homogeneously in prophase chromosomes and localized heterochromatin of interphase nuclei firmly aggregated at the different region of prophase chromosomes. In this study, *C. brevicornatus* followed the above general rule having "Diffuse Type" interphase nuclei and "Continuous type" of prophase chromosome (Figs 25 and 43, Table 60). In addition, *C. siliquosus* and *C. pinnatipartitus* also followed the rule having "Simple chromocenter type" of interphase nuclei and "Gradient type" of prophase chromosomes (Figs 29, 30, 47 and 48, Table 60). However, homogeously distributed heterochromatin of interphase nuclei aggregated in different position of prophase chromosomes in *C. septentrionalis* and *C. urticifolius* (Figs 26, 27, 44 and 45, Table 60). In contrast, heterochromatins are condensed to form block whether in interphase nuclei these are homogeneously distributed along the prophase chromosomal length of *C. hirtus* (Figs 28 and 46, Table 60). This different type of observation might be due to presence of facultative heterochromatin in *C. septentrionalis*, *C. urticifolius* and *C. hirtus*. Whatever the reason is, six wild *Corchorus* species could be characterized on the basis of these characters of interphase nuclei and prophase chromosomes.

Table 60. Types of interphase nuclei and prophase chromosomes of six wild *Corchorus* L. species after staining with orcein.

Species	Type of interphase nuclei	Type of prophase chromosomes
C. brevicornatus	Diffuse	Continuous
C. septentrionalis	Diffuse	Gradient
C. urticifolius	Diffuse	Gradient
C. hirtus	Simple Chromocenter	Continuous
C. siliquosus	Simple Chromocenter	Interstitial
C. pinnatipartitus	Simple Chromocenter	Interstitial

4.2. Multibasic chromosome number in *Corchorus*

The six wild *Corchorus* species *viz. C. brevicornatus*, *C. septentrionalis*, *C. urticifolius*, *C. hirtus*, *C. siliquosus* and *C. pinnatipartitus* had variation in somatic chromosome numbers having different basic chromosome number. In this study, 2n = 2x = 14 chromosomes were observed in *C. brevicornatus*, *C. septentrionalis* and *C. urticifolius* with basic chromosome number x = 7 (Figs 73 - 75). In earlier studies identical somatic chromosome number was recorded only for *C. urticifolius* (Mandal and Datta 2011, Saha *et al.* 2014) and there was no record for rest two species. Therefore, the 2n chromosome number has been newly recorded for *C. brevicornatus* and *C. septentrionalis*. In this present investigation, *C. hirtus* and *C. siliquosus* were found to posses 2n = 4x = 28 somatic chromosome with basic chromosome number of x = 7 (Figs 76 and 77). Many researchers confirmed similar 2n chromosome number for these two species (Rao and Datta 1953, Sharma and Roy 1958, Islam and Qaiyum 1961, Cristobal 1967, Haque and Ahmad 1972, Coleman 1982, Wilkins and Chappill 2002). On the other hand, 2n = 78 chromosomes were found in somatic body of *C. pinnatipartitus* with different basic chromosome number except x = 7 (Fig. 78). Moreover, there is no such chromosomal report for this species. Thus this has been the first 2n chromosome number report for *C. pinnatipartitus*. The basic chromosome number of *C. pinnatipartitus* might be originated in many

ways such as hybridization of wild *Corchorus* species with genetically close species of other genus of Malvaceae or secondary modification of polyploid wild *Corchorus* species (Figs 140-142). Therefore, this finding indicates *Corchorus* as multibasic genus. Earlier report on the chromosome number of studied six wild *Corchorus* species and others genus of Malvaceae which is incorporated in this study to make molecular phylogeny has been given in Table 61.

Table 61. List of chromosome count database of studied *Corchorus* species and genus of Malvaceae.

Name of species	2n chromosome number	References
C. brevicornatus	No previous report	No previous report
C. septentrionalis	No previous report	No previous report
C. urticifolius	2n=14	Datta *et al.* 1975 Mandal and Datta 2011 Saha *et al*. 2014
C. hirtus	2n=28	Islam and Qaiyum 1961 Cristobal 1967 Coleman 1982
C. siliquosus	2n=28	Rao and Datta 1953 Sharma and Roy 1958 Islam and Qaiyum 1961 Brizicky 1965b Haque and Ahmad 1972 Wilkins and Chappill 2002
C. pinnatipartitus	No previous report	No previous report
Triumfetta pilosa	2n=64	Krishnappa 1980 Kumar and Subramaniam 1988
Dombeya spp.	2n=64	Seyani 1991
Grewia spp.	2n=18	Derman 1932 Gajapathy 1962a Nanda 1962 Moussel 1966 Singhal *et al*. 1980a

4.3. Primitive and advance *Corchorus* species

Out of six wild *Corchorus* species, karyotype of five species contained all the metacentric chromosomes (Figs 85 - 89). Only 2 pairs of submetacentric chromosomes were detected in *C. pinnatipartitus* (Fig. 90). The distance between small and large chromosomes was < 2 µm in *C. septentrionalis*, *C. urticifolius*, *C. hirtus* and *C. siliquosus*. In contrast, range of chromosomal length was more than 2 µm in case of *C. brevicornatus* and *C. pinnatipartitus* (Table 24). Assessment of karyotype asymmetry was analyzed with conventional and "KaryoType" software data. After analysis in both cases, satellite, mean centromeric asymmetry (M_{CA}, Peruzzi and Eroglu 2013), coefficient of variation of the chromosome length (CV_{CL}; Paszko 2006), percentage of karyotype asymmetry index (AsK%; Arano 1963), intrachromosomal asymmetry (A_1), interchromosomal asymmetry index (A_2), degree of asymmetry of karyotypes (A), dispersion index (DI), was highest in *C. brevicornatus* (Table 25). In contrast, the highest total haploid chromosome length (THL, µm), coefficient of variation of centromeric index ($CV_{CI,}$ Paszko 2006) and asymmetry index (AI) was observed in *C. pinnatipartitus*. On the other hand, all the above values were lowest in *C. septentrionalis* except DI value (Table 25). According to Altmordu *et al.* (2016) and Begum *et al.* (2020) lowest value of the above parameter considered as primitive character and highest as advanced character as well as highest total form value (TF%), karyotype symmetry index (Syi%) considered as primitive nature and lowest value considered as advance nature. Considering these parameter *C. brevicornatus* and *C. pinnatipartitus* could be suggested as most advance and *C. septentrionalis* most primitive in nature (Table 25). According to Stebbins (1971), presence of all metacentric chromosomes is a feature of symmetric karyotype. In this study except *C. pinnatipartutus* rest five *Corchorus* species possessed all metacentric chromosomes. However, few submetacentric chromosomes made slightly asymmetric karyotype for *C. pinnatipartutus* (Tables 24, 25, Figs 85 - 90 and 97 – 102, Tables 24, 25). Many scientists

reported that, evolution of genome size (also co-related with haploid chromatin length) in angiosperm is in upward direction *i. e.* primitive karyotype posses small genome and advanced karyotype posses large genome (Leitch *et al.* 2007, Hawkins *et al.* 2008, Peruzzi *et al.* 2009, Bennett and Leitch 2011, Saha *et al*. 2014). In the present study, the total haploid chromatin length was largest in *C. pinnatipartitus* and smallest in *C. septentrionalis* (Table 25). Therefore, based on the above criteria *C. pinnatipartitus* could be considered as most advanced and *C. septentrionalis* most primitive among six wild *Corchorus* species studied.

4.4. Fluorescent banding

In this investigation, two base specific fluorochromes *i. e*. Chromomycin A_3 (CMA) and 4´-6-Diamidino-2-Phenylindole (DAPI) were used which was confined to GC- and AT-rich base segments (Schweizer 1976), respectively. With this technique, a detail characterization of chromosome can be conducted even having similar morphology and other conventional karyotypic features. Reversible chromosome banding with CMA- and DAPI- was conducted for all the studied materials except *C. pinnatipartitus*. The details of critical karyotype analysis of six wild *Corchorus* species has been described in the following sections.

4.4.1. Nature of CMA- band

In case of six wild *Corchorus* species, a total number of 35 terminal CMA- bands were generated which revealed the tendency of accumulation of repetitive nucleotide bases at the terminal region of chromosomes (Figs 91a - 96a). Zaman and Alam (2009) reported similar tendency of accumulation at the terminal region of chromosomes. Rather than terminal bands, *C. hirtus* and *C. pinnatipartitus* possessed centromeric CMA- bands suggesting the presence of GC- rich region on those locations. In addition, some entirely fluoresced CMA- chromosomes were found after CMA- banding analysis (Figs 94a and 96a). Tandem duplication of GC- and AT- rich bases may result that entirely

fluoresced CMA- chromosomes. Such results support the earlier findings in different plant species indicating tandem duplication of GC- and AT- rich repeats is a common feature (Schweizer 1976, Hiron *et al.* 2006, Mahbub *et al.* 2007, Khatun and Alam 2010, Sultana and Alam 2016b). The number, location, distribution and intensities of CMA-bands were diverse visualized through karyotype analysis with CMA-stain. The number of CMA-bands ranged from 4 (*C. septentrionalis*) to 23 (*C. pinnatipartitus*) (Figs 92a and 96a, Table 26). Total length of CMA-positive banded region and percentage of GC-rich repeats was lowest in *C. siliquosus* (2.99 ± 0.05 µm and 4.45, respectively) and that was highest in *C. pinnatipartitus* (27.72 ± 0.10 µm and 12.58, respectively) (Table 26). Therefore, the studied six wild *Corchorus* species had different CMA- banding pattern from each other.

4.4.2. Heteromorphic CMA-band

In respect of CMA-staining heteromorphicity (different in number and position of bands in homologue member of chromosome) was recorded among these materials. In *C. hirtus* one member of the chromosome pair 2 showed terminal short bands while its homologue member had no such band (Fig. 94a). In contrast, a number of heteromorphicity was observed in *C. pinnatipartitus*. Terminal band was found in a member of chromosome pair 5 and 39 while no band was observed in their homologue (Fig. 96a). In addition, a member of pair 23 had centromeric CMA- positive band while there was no such GC- rich repetitive segment was found in its homologue (Fig. 96a). The above findings may be the result of probable deletion of the banded region from the respective chromosomes. Such type structural aberration is common for *Corchorus* species (Khatun and Alam 2010). Therefore, the result of the present investigation indicates similarity with previous findings using jute germplasm.

4.4.3. Nature of DAPI-bands

A total number of 32 terminal bands were observed after DAPI- banding analysis indicating the tendency of accumulation of AT- rich bases at the

terminal position of chromosomes (Figs 91b-96b). Zaman and Alam (2009) reported similar tendency of accumulation at the terminal region of chromosomes. Rather than terminal bands, *C. urticifolius, C. siliquosus* possessed centromeric DAPI- bands revealing the presence of AT- rich region on that location (Figs 93b and 95b). In addition, some entirely fluoresced DAPI- chromosomes were found with DAPI- stain. Tandem duplication of GC- and AT- rich bases may result that entirely fluoresced DAPI- chromosomes. Such results correspond with earlier findings of different plant species (Schweizer 1976, Hiron *et al.* 2006, Mahbub *et al.* 2007, Khatun and Alam 2010, Sultana and Alam 2016b).

The karyotype of six wild *Corchorus* species were compared critically using DAPI-staining which represents a considerable variation in respect of number, location, distribution and intensities of DAPI-bands. The number of DAPI-bands varied from 3 (*C. brevicornatus* and *C. siliquosus*) to 25 (*C. pinnatipartitus*) among six wild *Corchorus* species (Figs 91b, 95b and 96b, Table 27). Total length of DAPI-positive banded region was highest (38.64 ± 0.32µm) in *C. pinnatipartitus* and that was lowest (2.19 ± 0.10µm) in *C. siliquosus*. The percentage of GC-rich repeats ranged from 3.26% (*C. siliquosus*) to 17.54% (*C. pinnatipartitus*) (Table 27). On the basis of number and location of DAPI-bands, all these species showed diversity in their karyotype.

4.4.4. Heteromorphic DAPI-band

In respect of DAPI-staining heteromorphicity (difference in number and position of bands in homologue member of chromosome) was found. This was detected in *C. brevicornatus, C. siliquosus* and *C. pinnatipartitus* after DAPI-staining (Figs 91b, 95b and 96b). In *C. brevicornatus*, a member of chromosome pair 5 was entirely fluoresced while its homologue member had no DAPI- band (Fig. 91b). In case of *C. siliquosus*, a centromeric band was observed in a member of pair 1 while there was no such AT- rich repetitive

segment was found in its homologue. In addition, a homologue member of pair 6 had centromeric band while its correspondent member was fluoresced entirely (Fig. 95b). On the other hand, heteromorphicity was observed in chromosome pair 9 of *C. pinnatipartitus* where only one chromosome was fluoresced entirely (Fig. 96b). The above findings may be the result of probable deletion of the banded region from the respective chromosomes. This type of structural aberration was previously reported in other *Corchorus* species (Khatun and Alam 2010). Therefore, the result of the present investigation was identical to the previous findings of wild jute species.

4.5. Reversible fluorescent banding and Marker chromosomes

When a segment of chromosome fluoresces with a fluorochrome (positive staining) and does not take another counter fluorochrome (negative staining) known as reversible banding. This type of banding pattern was observed in *C. brevicornatus, C. urticifolius* and *C. siliquosus* (Figs 91a and b, 93a and b, 95a and b, arrow). After CMA-staining, one and two pairs of satellites were observed in *C. brevicornatus* (pair 6) and *C. urticifolius* (pair 4 and 6), respectively revealing extremely GC- rich repeated nature (Figs 91a and 93a, arrow). However, fully GC- rich satellites was reported previously by a number of workers (Hizumi *et al.* 1992, Alam and Rahman 2000). In addition two terminal CMA-bands were found on short arm in pair 7 of *C. urticifolius* (93a, arrow). In *C. siliquosus,* both members of pair 7 possess chromosome having bands at terminal end of both long and short arm (Fig. 95a). However, there were no visible chromosomal segments at the above mentioned sites after DAPI-staining representing DAPI-negative of banding nature. As a consequence, the DAPI-negative chromosomes looked smaller (something missing at the CMA-fluoresced region) when compared to the CMA-positive chromosomes (Figs 91b, 93b and 95b). Similarly pair 1 of *C. siliquosus* possess smaller chromosome in DAPI than that of CMA after counter staining might be due to the presence of mainly GC-rich repetitive sequence. Reversible fluorescent banding (CMA-positive and DAPI-negative) indicated the presence

of complete GC-rich repeats in those regions of chromosomes reported earlier by Schweizer (1976), Alam and Kondo (1995), Alam *et al*. (1996). The reversible banding was so unique that these chromosomes could easily be used as the marker chromosomes for the respective karyotypes.

On the other hand, terminal region of long and short arm of both the homologue member of pair 14 and 30 showed DAPI- positive bands indicating AT- rich repeated terminals in *C. pinnatipartitus* (Fig. 96b). These chromosomes of *C. pinnatipartitus* are unique since they are absent in the other species studied and thus could be used as a marker for this species.

4.6. Nature of Satellites

Satellite is an important parameter for karyotype analysis of jute. After orcein staining, satellites were detected in both the member of chromosome pair 4 in all studied species of wild *Corchorus* except C. *pinnatipartitus* (Figs 85-90). In addition to above satellites in chromosome pair 4, two satellites were also appeared in pair 6 of C. *brevicornatus* and C. *urticifolius*. Saha *et al.* (2014) reported, satellites confined to pair 1 and 2 in ten asian *Corchorus* species including two cultivated species. Besides this, there are numerous articles reported on satellites for both the cultivated and wild *Corchorus* species (Alam and Rahman 2000, Khatun and Alam 2011, Mandal and Datta 2014, Saha *et al.* 2014). In the present investigation four satellites were observed in pair number 4 and 6 of C. *urticifolius* (Collected from Kenya) whereas only one pair of satellites was reported in pair number 1 of asian species of C. *urticifolius* (Saha *et al.* 2014). So, it could be stated that the studied *Corchorus* species may be originated from a common ancestor except *C. pinnatipartitus* and chromosome reshuffling occurred during dispersion in course of evolution.

The nature of satellites was detected by using base specific flurochrome. In CMA- staining, satellites were extended in some species while contracted at the terminal region of short arm in the rest. It was also observed that 2 CMA-positive round, similar sized bands were observed in pro-metaphase that

located away from respective chromosomes of five wild *Corchorus* species (Figs 61 - 65). Satellites contracted to terminal region were reported earlier (Alam and Rahman 2000). From this point of view, *C. brevicornatus* and *C. urticifolius* had four satellites after CMA- staining while there was no such a satellite or terminal band on short arm after DAPI- staining (Figs 91a and b, 93a and b). Moreover, two contracted satellites were found in *C. siliquosus* with CMA- and absent in DAPI- stain (Figs 95a and b). Therefore, these satellites could be considered as stain specific and DAPI- negative in nature. Alam and Rahman (2000) also observed stain specific and DAPI- negative satellites for three *Corchorus* species. After the reversible banding pattern, it was revealed that the satellites were fully made of GC- rich base sequences. The GC- rich satellites were reported in many plants (Hizume *et al.* 1992, Alam and Kondo 1995). Thus, the present findings correspond with the results of Hizume *et al.* (1992), Alam and Kondo (1995) and Alam and Rahaman (2000). In contrast, two contracted satellites were found in *C. septentrionalis* after CMA- and DAPI- banding technique (Figs 92a and b). In addition, the number and position of satellites were same with both CMA- and DAPI- staining of *C. hirtus* and *C. pinnatipartius* (94a and b, 96a and b). This result suggesting the co-existence of GC- and AT- rich sequences in that location. Co-localization of GC- and AT- rich repeats were reported earlier in many plant species (Alam and Rahaman 2000, Afroz *et al*. 2013, Sultana and Alam 2016b).

So, the number and nature of satellites could be considered as an important parameter to characterize the six wild studied *Corchorus* species.

4.7. DNA fingerprinting

RAPD and SSR markers were used to know the relationship among six wild *Corchorus* species at molecular level. For this purpose thirteen oligonucleotide primers and eight microsatellite primer pairs were utilized for RAPD and SSR analysis, respectively.

4.7.1 DNA fingerprinting by Random Amplified Polymorphic DNA (RAPD)

According to Esmail *et al.* (2008) RAPD is a PCR based marker technique that has been used for estimation of genetic diversity of populations and for studying the genetic relationships among different genotypes.

4.7.1.1. Polymorphism as detected by RAPD analysis

For RAPD analysis, thirteen primers exhibited good quality banding patterns with sufficient variability on genomic DNA of six wild *Corchorus* species. The primer sequence, band size and banding pattern of six wild *Corchorus* species are shown in Tables 7, 28-41. A total number of 69 RAPD loci were observed. All the bands were polymorphic in nature due to absence of common band. So, the polymorphism was hundred percent (100%) among them indicating the highest level of polymorphism. The size of bands ranged from 150-5000 bp following amplification with all primers. Two types of bands were detected after amplification *i. e.* light band and dark band. These light and dark bands were produced due to low and high homology between the primer and the pairing site on the DNA strand, respectively (Thormann *et al.* 1994).

Diverse polymorphism was reported previously only for two cultivated *Corchorus* spp. Heikal *et al*. (2015) scored 28.3% of polymorphism in three genotypes of *C. olitorius* L. While Mir *et al.* (2008) reported 96.03% polymorphism among 16 accessions of two cultivated jute species. Haque *et al.* (2007) found 50.72% polymorphism in 18 genotypes of two cultivated jute species. Hossain *et al.* (2002) scored 26% polymorphism in nine jute varieties of two cultivated species.

In this study, 100% polymorphism was recorded among six wild *Corchorus* species. On the other hand, most of the previous report showed low level of DNA polymorphism which was confined in two cultivated jute species. Therefore, high polymorphism indicated high genetic diversity in six wild

Corchorus species. The diverse nature of the wild species would be useful for breeding programme of jute.

4.7.1.2. Unique RAPD markers

After RAPD analysis, 46 unique bands were observed in six wild *Corchorus* species with thirteen primer combinations. The band found with a specific primer of a germplasm but absent in other germplasm considered as unique band (Figs 110 - 122, Tables 28 - 41). These bands were stable, reproducible and distinct for the particular germplasm. Highest (20) number of unique bands were found in *C. septentrionalis* and lowest (3) in *C. brevicornatus*, *C. urticifolius* and *C. pinnatipartitus* (Tables 28 - 41). Many scientists used unique band as a parameter to characterize germplsms (Sultana and Alam 2016, Hossain *et al.* 2017). Therefore, unique band could be utilized as a useful tool for characterization of six wild *Corchorus* species used in this investigation.

4.7.1.3. Genetic relationships among six wild *Corchorus* species

Genetic distances were used to find out the genetic relationship among six wild *Corchorus* species. By using computer software "popgene32" the values of pair-wise Nei's (1972) genetic distances were obtained from data of thirteen RAPD primers among the studied species. The values of genetic distance were ranging from 0.2638 to 1.141 (Table 42). In this study, *C. septentrionalis* is distantly related from other 5 wild species and formed a separate cluster at highest genetic distance with *C. pinnatipartitus* (1.1431) (Fig. 123, Table 42). On the other hand, *C. hirtus* and *C. pinnatipartitus* are close to each other at lowest genetic distance 0.2638 (Fig. 123, Table 42). These results made diverse genetic variability among six wild *Corchorus* species. Wide genetic diversity in different crops has been reported earlier (Molla *et al.* 2010, Sultana and Alam 2016c). The highest genetic distance between *C. septentrionalis* and *C. pinnatipartitus* might be obtained due to the differences in genetic constituent and the lowest genetic distance between *C. hirtus* and *C. pinnatipartitus* due to highest homology in their genetic constituent. Therefore, the germplasm having

lowest genetic distance can be considered as potential parents for the improvement of *Corchorus* species through breeding program.

4.7.2. DNA fingerprinting by Simple Sequence Repeats (SSRs)

Simple sequence repeats (SSR) is an excellent tool for germplasm identification, the evaluation of genetic distances, genetic ralationship due to having abundance, multiallelic and co-dominant nature, high level of reproducibility and cross-species transfer-ability (Hokanson *et al.* 1998, Priolli *et al.* 2002, Satya *et al.* 2016).

4.7.2.1. Polymorphism as detected by SSR analysis

After SSR analysis, a total number of 27 loci were generated from eight primer pair combinations (Table 51). A common locus (200 bp) was detected with primer pair HK-30 indicating the sharing of similar DNA fragments among six wild *Corchorus* species. As a result, 96.30% polymorphism was recorded for six wild *Corchorus* species (Table 51). These results indicating the high level of polymorphisms among wild *Corchorus* species. After amplification with all primers the size of bands ranged from 100-1500 bp. Two type's intensities of bands were detected after amplification *i. e.* light band and dark band. These light and dark bands were produced due to low and high homology between the primer and the pairing site on the DNA strand, respectively (Thormann *et al.* 1994). Highest (7) number of polymorphic bands were observed with primer pair HK- 18 and HK- 30 and lowest (1) with HK-12 (Figs 128, 130 and 131, Table 51).

Wide range of SSR polymorphisms were reported both intra-specific and inter-specific levels. Zhang *et al.* (2015) reported 78.2% polymorphism among 58 accessions of two cultivated jute species. Ghosh *et al.* (2014) scored 83.42% of polymorphism in 63 genotypes of the two cultivated jute species. Huq *et al.* (2009) scored 92.20% polymorphism in 16 elite jute genotypes of cultivated

species. Mir *et al.* (2009) observed 98% polymorphism in four inbred lines of two cultivated jute.

Therefore, high polymorphism was recorded for the first time for wild *Corchorus* species. On the other hand, most of the previous report was confined in two cultivated jute species. This remarkable feature of the wild jute species would be used as a source of variation to improve in the genetic constituents' jute.

4.7.2.2. Unique SSR markers

Eleven unique bands were detected with eight SSR primer pair combinations in six wild *Corchorus* species (Table 51). The band found with a specific primer of a germplasm but absent in other germplasm considered as unique band (Figs 124-131, Tables 43-51). These bands were stable, reproducible and distinct for the particular germplasm. The highest (08) number of unique bands were in *C. urticifolius* that made its distant relationship from the rest 5 species (Tables 43-51). In addition, three unique bands were found in *C. septentrionalis* (Tables 42-50). Primer pair CEDG, HK-7, HK-18 and HK-30 generated 3, 1, 2, 3 and 5, respectively (Tables 43 - 51). On the other hand, no unique band was found in case of primer pair AW-584539, CEDG-56, HK-12 and HK-16 (Table 51). The earlier literature reported only polymorphic SSR bands for *Corchorus* species (Zhang *et al.* 2015, Ghosh *et al.* 2014, Huq *et al.* 2009). Unique SSR bands are also considered as a parameter for characterization of germplasm (Sultana and Alam 2016c). Therefore, unique SSR bands obtained through this investigation could be utilized as a useful tool for characterization of six wild *Corchorus* species.

4.7.2.3. Genetic relationships by SSR analysis

Genetic distances were used to find out the genetic relationship among six wild *Corchorus* species. By using computer software "popgene32" the values of pair-wise Nei's (1972) genetic distances and UPGMA dendrogram were

obtained from data of eight primer pair combinations in this study. The values of genetic distance were ranging from 0.0770 to 1.3499 (Table 52). This high genetic diversity among six wild *Corchorus* species made SSR data highly informative. Wide genetic diversity in different crops including jute has been reported earlier (Huq *et al.* 2009, Molla *et al.* 2010, Ghosh *et al.* 2014, Zhang *et al.* 2015, Sultana and Alam 2016c). In the dendrogram, *Corchorus urticifolius* is distantly related from other 5 wild species and formed a separate cluster at highest genetic distance with *C. septentrionalis* (1.3499) (Fig. 132, Table 52). On the other hand, *C. siliquosus* and *C. pinnatipartitus* are close to each other at lowest genetic distance 0.0770 (Fig. 132, Table 52). The highest genetic distance between *C. urticifolius* and *C. septentrionalis* might be obtained due to the differences in genetic constituent and the lowest genetic distance between *C. siliquosus* and *C. pinnatipartitus* due to highest homology in their genetic constituent. Therefore, the species having lowest genetic distance can be considered as parents during improvement of *Corchorus* species through breeding program.

4.8. Molecular phylogeny and evolutionary relationship using barcode primer analysis

To understand evolutionary and phylogenetic relationship of plants, molecular data are considered more reliable than morphological information (Gottlieb 1988, Donoghue and Sanderson 1992). Now a days, different coding and non coding region of genome are used for identification of species or germplasms and determine the relationship and evolution among and within species (Hasebe *et al*. 1994, Zimmer *et al.* 2002, Hebert *et al*. 2003, Small 2004, Ma *et al*. 2013, Yan *et al*. 2015). Combined phylogenetic data provide more reliable information when variation occurs with different region of the genome (Kress *et al*. 2005, Liu *et al*. 2012, Ma *et al*. 2013). *Corchorus* considered under Malvaceae from Tiliaceae family because of momophyletic nature *i. e.* common ancestral origin. To confirm the monophyletic nature of studied six wild *Corchorus* species, several members of other genera of its respective

family were incorporated as out group member. However, sequences of three regions (ITS, *MatK* and *rbcL*) were not available in the database for all out group members. As a result different regions such as ITS, *MatK* and *rbcL* of different genera were used to make phylogenetic tree with studied *Corchorus* (Table 54).

The phylogeny tree interpreted from ITS and combined (ITS, *Mat*K and *rbcL*) data matrices made six wild *Corchorus* species as monophyletic nature since they make a distinct clade without the other member of the family. Earlier reports supported the present findings of monophyletic nature for the genus *Corchorus* (Belay 2011, Benor *et al.* 2012, Tanmoy *et al*. 2015, Benor 2018). On the other hand, paraphyletic nature of *Corchorus* was observed with *MatK* and *rbcL* data because *C. pinnaipartitus* made a separate cluster with out-group members of Malvaceae.

Close relationship between *C. hirtus* and *C. septentrionalis* (ITS data matrice), *C. hirtus* and *C. siliquosus* (*MatK* data matrice), *C. brevicornatus* and *C. urticifolius* (*rbcL* data matrice) were observed as they made terminal clade indicating their origin from common ancestors (Figs 136-138). In contrast, in case of combined data matrices two terminal clade were made *i.e. C. hirtus* and *C. urticifolius* in one clade and *C. septentrionalis* and *C. brevicornatus* in another clade indicating strong kinship in them (Fig. 139). This finding is significantly important because these plant materials were collected from different continents. In all phylogenetic tree except ITS, the five wild *Corchorus* species *i. e. C. brevicornatus, C. septentrionalis, C. urticifolius, C. hirtus* and *C. siliquosus* were close to the genera of Sparrmanniaceae (most close to *Truimfetta* species) other than those from Malvaceae (Figs 136-139). Tonmay *et al*. (2015) reported similar results for the genus *Corchorus*. Benor (2018) suggested *Truimfetta* as close sister of *Corchorus* which support the present findings. In contrast, present findings in case of *C. pinnatipartitus* are different to Benor's findings. However, in considering the multidimentional data gathered from distinct morphological characters, cytogenetical features,

molecular data and DNA sequence compared with other *Corchorus* species suggesting that further study require clarifying the status of *C. pinnatipartitus*.

4. 9. Species status of most primitive *Corchorus septentrionalis*

After orcein staining *C. septentrionalis* showed most primitive nature among the studied materials. In the literature, there was little information is available about this species. Herbarium specimen is stored at Royal Botanic Garden Kew (K) collected by Riddell JL from Louisiana New Oreleans of USA (Herbarium Hookerianum 1867). Recently Bangladesh Jute Research Institute (BJRI) collected germplasms of this species from USA which has been used in this investigation. Unfortunately, cytogenetical data and other important features of this species are limited all over the world including Bangladesh. Lacking of information made this species very interesting for analysis. Therefore, the findings of this research could be considered as pioneer data for *C. septentrionalis*. Some features of *C. septentrionalis* are distinct from rest five wild *Corchorus* species which are listed below:

(i) It showed most symmetric features in both conventional and using karyoType software (Table 25).

(ii) After Orcein, CMA- and DAPI- staining, 2 satellites were observed in almost each mitotic metaphase stage. Interestingly, 2 small and round CMA- and DAPI- bands which are similar in size of satellite were observed in mitotic interphase, prophase and pro-metaphase stage indicating equi-local distribution of heterochromatin in this species. This equi-locality of heterochromatin could be considered as a salient features for this species among six wild *Corchorus* species.

(iii) *Corchorus septentrionalis* was distant from rest five *Corchorus* species in case of RAPD analysis and distant from other diploid species after SSR analysis (Fig 132, Table 52).

(iv) After sequence analysis with *MatK* primer pair (to see maternal closeness among different germplasms) combinations, *C. septentrionalis* was distant from rest five *Corchorus* species and made a different sub-clade (Fig. 137).

The above discussion clearly suggested separate place of *C. septentrionalis* from other five wild *Corchorus* species. However, it might have connection with primitive other diploid *Corchorus* species which had 2 satellites reported by different scientists (Alam and Rahman 2000, Saha *et al.* 2014).

4. 10. Species status of *Corchorus pinnatipartitus*

According to morphological analysis, *C. pinnatipartitus* showed completely different features (perennial herb with a woody root stock, leaf shape linear lanceolate, sparsely hairy, flower pinkish purple in color, red round capsules with distinct ridges, seeds angular and bigger) from rest five studied *Corchorus* species (Table 3).

In the present study, *C. pinnatipartitus* had 2n = 78 somatic chromosomes. There was no earliar report on chromosome number of this species in the available literature. Therefore, 2n = 78 could be considered as a new report for *C. pinnatipartitus*. Moreover, 2n = 78 is also the first report for the genus *Corchorus*. After conventional and fluorescent banding analysis, *C. pinnatipartitus* showed remarkable differences with other five studied *Corchorus* species (Table 3) which are mention below:

i. Total length of diploid chromosome complements 220.28 ± 20.50 µm was recorded for this species. This length was the highest among six wild *Corchorus* species.
ii. *Corchorus pinnatipartitus* had four sub-metacentric chromosome (74m + 4sm) while no sub-metacentric chromosomes was observed in rest five *Corchorus* species. This feature indicated relatively asymmetric nature of its karyotype.

iii. On the basis of fluorescent banding analysis, *C. pinnatipartitus* showed difference in number and position of bands and satellites, area and percentage occupied with GC- and AT- rich repetitive sequences (Figs 96a and 96b, Tables 26, 27).

iv. According to Maximum Likelihood (ML) tree based ITS data, *C. pinnatipartitus* was distant from rest five *Corchorus* species with strong bootstrap value (97%) and Closely related with *C. urticifolius* (2n = 2x = 14, diploid) (Fig. 136). In case of ML tree based on *Mat*K and *rbcL* data, *C. pinnatipartitus* is placed in different cluster (bootstrap 99%) with out-group members of Malvaceae such as *Trochetioptis ebenus* (2n = 2x = 40, diploid), *Dombeya* spp. (n = 23, 27, 28, 30) (Dombeyoideae sub family of Malvaceae, previous member of Sterculiaceae). Rest five *Corchorus* species are placed in far away (Figs 137 and 138).

v. Combined ML tree based on ITS, *matK* and *rbcL* data, *C. pinnatipartitus* segregated from rest five species with bootstrap value 98%. However, *C. pinnatipartitus* show closeness with *Triumfetta pilosa* (2n = 2x = 64) (Apeibeae, sub family of Malvaceae which also includes *Corchorus* spp.) (Figs 139, Table 61).

4.11. Probable way of origin of *Corchorus pinnatipartitus*

Corchorus pinnatipartitus could be originated through different ways such as:

(i) According to Maximum Likelihood (ML) analysis of combined (ITS, *MatK* and *rbcL*) data matrice, *C. pinnatipartitus* is distant from rest five *Corchorus* species and make a different sub-clade with 98% bootstrap (Fig. 139). However, it showed close affinities towards *Triumfetta pilosa* (2n = 2x = 64, n = 32) (Sub-family: Apeibeae of Malvaceae) (Table 61). In addition, in case of ML analysis of ITS sequence data matrice, *C. pinnatipartitus* was close to *C. urticifolius*(2n = 2x = 14, n = 7) (Sub-family: Apeibeae of Malvaceae) (Fig. 136). On the other hand, According to summary of Nei's (1972) genetic distances obtained by RAPD analysis, *C. pinnatipartitus* had close relationship with *C. brevicornatus* (2n = 2x = 14, n = 7) (Sub-family:

Apeibeae of Malvaceae) by genetic distance 0.3840 (Fig. 123, Table 42). The above data indicated *C. pinnatipartitus* as an allopolyploid which might be originated from hybridization of two closely related genus *Triumfetta pilosa* and *C. brevicornatus* or *C. urticifolius* resulted in sterile F_1 hybrid (2n = 2x = 39). Consequently after chromosome doubling, a fertile amphiploid *C. pinnatipartitus* (2n = 4x = 78) could be obtained (Fig. 140).

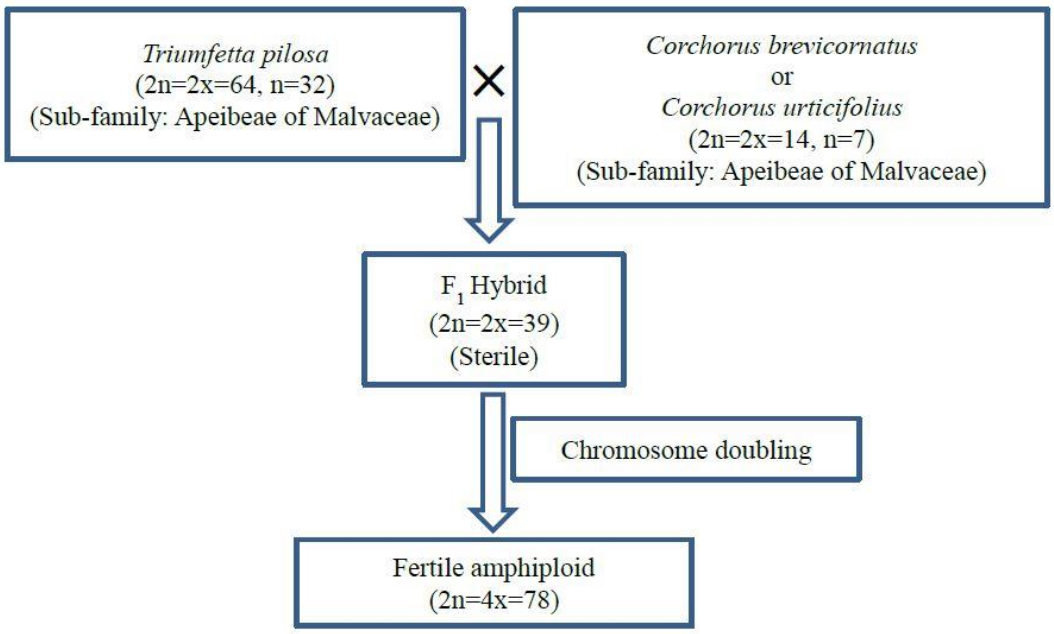


Fig. 140. Probable origin of *C. pinnatipartitus* from hybridization of *Triumfetta pilosa* with *C. brevicornatus* or *C. urticifolius.*

(ii) According to ML analysis of *MatK* and *rbcL* data matrice, *C. pinnatipartitus* showed close affinities towards *Dombeya* spp. (2n = 2x = 60, n = 30) (Sub-family: Dombeyoideae of Malvaceae) with bootstrap value 99% (Figs 137 and 138, Table 61). However, in ML analysis of combined (ITS, *MatK* and *rbcL*) data matrice, *C. pinnatipartitus* is close to *Grewia* spp. (2n = 2x = 18, n = 9) (Sub-family: Grewieae of Malvaceae (Fig. 139, Table 61). The above data suggested that, probably *Dombeya* spp. and *Grewia* spp. hybridized resulting in a sterile F_1 hybrid (2n = 2x = 39) and

after chromosome doubling fertile amphiploid (2n = 4x = 78) had been evolved (Fig. 141).

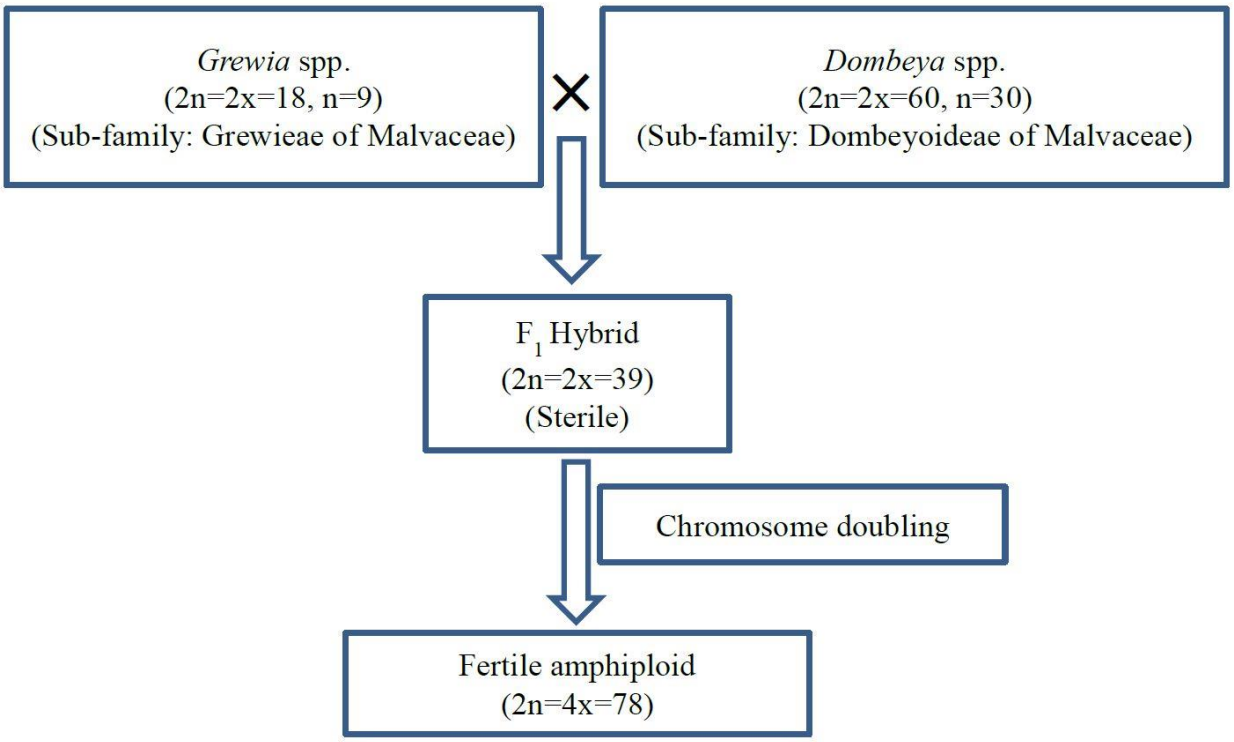


Fig. 141. Probable origin of *C. pinnatipartitus* from hybridization of *Grewia* spp. with *Dombeya* spp.

(iii) On the other hand, 2n = 78 in *C. pinnatipartitus* could be originated through secondary modification (hyper-aneuploidy) of polyploidy as proposed by Stebbins (1971). It is well known that, numerical chromosomal aberration in the polyploidy generation due to addition or deletion of few chromosomes could not be lethal in a polyploidy species because of possessing multiple copies of a basic set of chromosomes (Fig. 142).

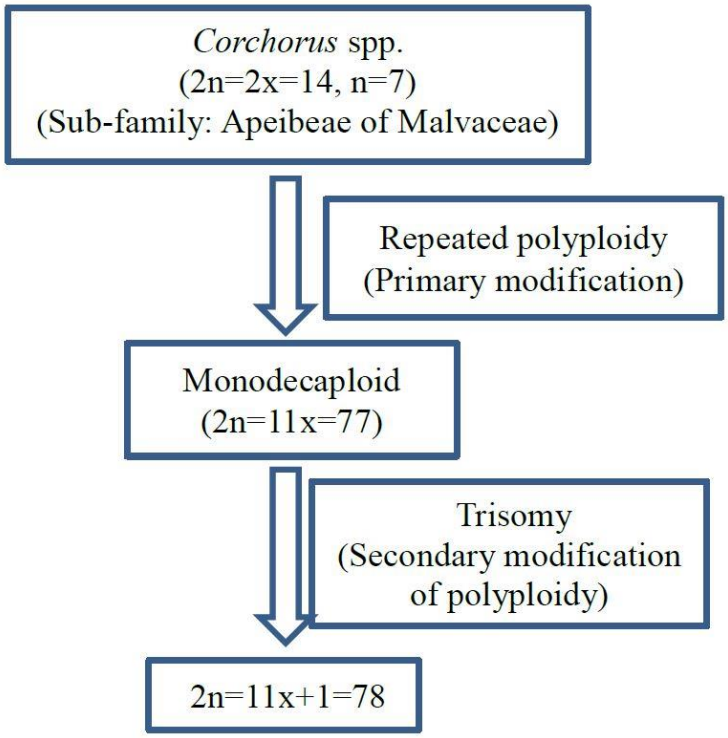


Fig. 142. Probable origin of *C. pinnatipartitus* through secondary modification of polyploidy.

Therefore it could be concluded that, whatever the reason is, *C. pinnatipartitus* is distant from studied rest five *Corchorus* species and closely related to other genus of Malvaceae.

4.12. Conclusions

The information regarding the nature of heterocromatin in mitotic interphase and prophase chromosomes, somatic chromosome number, fluorescent chromosome banding pattern, RAPD and SSR bands and molecular phylogeny with barcode primers of wild *Corchorus* was scare. Therefore, in this study, all the above techniques were used for authentic characterization, find out relationship and evolutionary trends of six wild *Corchorus* species.

The interphase nuclei and prophase chromosomes of these species showed variation in heterochromatin distribution with orcein-staining. The somatic chromosome numbers were 14m for *C. brevicornatus*, *C. septentrionalis* and *C.*

urticifolius, 28m for *C. hirtus* and *C. siliquosus* and 74m + 4sm for *C. pinnatipartitus*. The different basic chromosome number of *C. pinnatipartitus* might be originated by hybridization between wild *Corchorus* species with genetically close other genus of Malvaceae or secondary modification of polyploid wild *Corchorus* species.

In this study, reshuffling of chromosomal segments were detected following CMA- and DAPI- analysis. Each species has its characteristic CMA- and DAPI-banding pattern. The six wild *Corchorus* species had unique RAPD and SSR bands which could be utilized as a useful parameter for characterize them. The phylogenetic tree generated from barcode primers made *C. pinnatipartitus* distinct from the rest studied *Corchorus* species and placed alone in a separate cluster that correlated with its phenotypic and cytogenetical features. Therefore, the six wild *Corchorus* species available in Bangladesh could be characterized authentically by combined cytogenetical and molecular analysis.

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