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I. Materials

Seeds of 2 cultivated (C. capsularis L. - JRC 321, C. olitorius L. - JRO 524) and 6 wild (C. aestuans L. - WCIJ 088, C. fascicularis Lamk. - WCIJ 150, C. pseudocapsularis L. - CIM 036, C. pseudooolitorius I. and Z. - OIN 507, C. tridens L. - WCIJ 149 and C. trilocularis L. - KBA 222) species of jute belonging to the family Tiliaceae were used as germplasms source (obtained from Central Research Institute for Jute and Allied Fibres - CRIJAF, Nilganj, Kolkata, West Bengal, India) for morphological, palynological, cytological, biochemical and molecular characterization. Further, induced mutant lines (‘viridis’, ‘lax branching’ and ‘pigmented stem’) and a hybrid line (C. trilocularis × C. capsularis) were also analyzed in advance generations (F4 to F6) for stability and trueness.

II. Methods

1. Morphological analysis

Plants of individual species of Corchorus were raised in the experimental field plots of Department of Botany, University of Kalyani (West Bengal plain - latitude 22°50´ N to 24°11´ N, longitude 88°09´ E to 88°48´ E, altitude 9.75 m; sandy loamy soil, soil pH 6.85) during 2010 to 2012 from March to October each year. Morphological traits namely, stem (nature, color), leaf (shape, serration, venation, color), buds (shape, color), sepals (number, shape, color, bracts), petals (number, shape, color), stamens (number, anther shape, color, nature of filament), ovary (shape, color, nature of style, stigma), fruit (color and shape) and seeds were ascertained in Corchorus spp. from identical condition(s). Further, morphological (qualitative and quantitative traits) attributes of hybrid and macromutant lines were also studied.

The specimens were examined under Olympus binocular dissecting microscope (4X, 6X and 10X). The study includes detailed description of every part of the specimens. The flowers were dissected and examined. Measurements of the leaves and floral parts were made. Measurement of seeds was made for an average of 15 seeds per sample using camera lucida in a simple light microscope. Seed moisture content, 100-seed weight and seed viability were also determined.

2. Determination of moisture content
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Moisture content of seeds of 8 *Corchorus* spp. was estimated by drying the seeds (3 replicas for each plant species) in a hot air oven at 100°C for nearly 3 hours. The dried seeds were repeatedly weighed at certain intervals till a constant weight was obtained and the loss in weight represented the weight of water loss due to drying. The moisture content was determined from the following formula:

\[
\text{Moisture content (\%) } = \frac{W_1 - W_2}{W_1} \times 100
\]

Where, \( W_1 \) is the weight of seed sample before drying and \( W_2 \) is the weight of seed sample after drying.

3. Determination of viability of seeds

Viability of seeds was tested following 1% tetrazolium chloride (Patil and Dadlani 2009). This method determines the percentage of viable seeds which may be expected to germinate. The chemical tetrazolium chloride is colorless and it develops intense red color when it is reduced by living cells. This phenomenon is used to determine the percentage of viable seeds in seed sample. Seed viability was tested in 8 species of jute, hybrid and in macromutant lines of *C. olitorius*. Test seeds were soaked in tap water for overnight and were split longitudinally with the help of a blade so that a portion of embryo was attached with each half of the seeds. One half of each seed was placed in a crucible (20 half seeds for each plant type) and dipped in 1% aqueous solution of tetrazolium chloride for overnight. The seeds were then washed in tap water and the number of seeds in which the embryo was stained red was determined. The per cent of viable seed was calculated as follows:

\[
\text{Viable seeds (\%) } = \frac{\text{Number of half seeds stained red}}{\text{Total number of half seeds}} \times 100
\]

4. Cultivation

The *Corchorus* spp., hybrid and the mutant lines were grown in lines (direct seed sowing) keeping 30 cm between lines and between plants. The plants were raised
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during the year 2010, 2011 and 2012. No fertilizer application was made during the growth periods of plants.

Seeds were sown during mid-March and the plants were harvested during late October to early November. Three to 4 weeding were given during early growth periods. Adequate irrigation facilities were provided to the plants during vegetative growth of the plants. On harvest, the seeds were collected, dried and kept in dessicator for further uses.

5. Retting process

Conventional method for whole plant retting in mutant lines was performed. For the purpose, defoliated plant types were dipped in stagnant pond water. Brick bats tied in cement bags were used as jak materials. Retting of jute was completed within 15-20 days and fibre was extracted by “beat-break-jerk” method or in some case by single fibre extraction method. The raw fibres were air dried and weighted.

6. Palynological studies

Pollen morphology of 8 Corchorus spp., mutants of C. olitorius and that of hybrid in relation to parents were studied.

i. Acetolysis technique: Pollen grains (obtained from fully opened flower buds) of the germplasms were transferred to eppendorf tubes containing (1.5 ml in each) glacial acetic acid and acetolyzed as per Erdtman (1952). Concentrated HCl (8N; about 35%) was added on each tube (3-4 drops) and the mixture was stirred with a glass rod, heated in a boiling water bath for 3 minutes and centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and glacial acetic acid was added (1 ml in each tube) and the mixture was thoroughly mixed by votex and centrifuged at 5000 rpm for 5 minutes. The supernatant was removed and the acetolysis mixture (9:1 acetic anhydride:conc. H$_2$SO$_4$) was added slowly. The mixture was heated to 100°C for approximately 7 minutes, stirring every 2 minutes. Centrifugation was performed as earlier and the supernatant was discarded. Glacial acetic acid was added (1 ml), stirred and centrifuged as before. Glycine (1 ml) was then added to the samples to form a suspension. A drop of suspension was suspended in a slide and covered with a cover glass slip and observed under the light microscope.
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ii. SEM analysis: For the SEM (Scanning Electron Microscopy) study, the pollen grains from each sample were placed into 70% ethanol for 2 days in 2 ml micro centrifuge tubes (TARSONS, India) and cleaned in an ultrasonic (50/60 Hz, 80 W, 240 V) vibrator (Branson ® Ultrasonics Corporation, Danbury, Connecticut, U.S.A.) for 6 min. Pollen grains from each sample were fixed on glass plates and then mounted on specimen stubs with double-sided adhesive tape and painted with silver. Pollen grains mounted on specimen stubs were placed on a revolving disc and coated with a 200-300 Å thick layer of gold in a vacuum evaporator (Polaron, East Sussex, UK) sputter coating system. The specimen stubs were then observed under SEM (Zeiss EVO® HD, Oberkochen, Germany) at 15 kV accelerating voltage at GSI (Geological Survey of India, Kolkata). On average, 20 pollen grains were analyzed for each sample to assess their morphological parameters. Pollen shape and size were determined as per Erdtman (1952). Photomicrographs were taken from suitable preparations.

7. Meiotic analysis

Meiotic studies were performed in 8 Corchorus spp., mutants and in hybrid plants.

Flower buds of suitable sizes from 3-5 randomly selected plants of each germplasm (studied over the years) were fixed (6 am to 7 am) in Carnoy’s solution (6 ethanol : 3 chloroform : 1 acetic acid) and 2 to 3 changes were given in the fixative at an interval of 24 hours and preserved in 70% alcohol. Anthers were squashed and PMCs were stained in 2% propinocarmine solution. The slides were warmed slightly on a spirit lamp and pressure was applied over the cover glass with thumb through several folds of blotting paper to scatter and flatten the chromosomes in pollen mother cells. Bivalent configurations, chiasma per cell from diplotene, metaphase I (MI), chromosome associations and anaphase I (AI) distribution were studied from well scattered meiocytes of the germplasms. Results obtained were statistically analyzed. Cytological data was pooled over the plants for each year in each germplasm. Photomicrographs were taken from temporary squash preparations.

8. Determination of pollen fertility

Pollen fertility of 8 Corchorus species, mutants and hybrid plants were estimated. Pollen grains from mature anthers were squeezed out in a drop of 1% propinocarmine solution on a glass slide. The debris was removed and a cover glass was placed on it.
The excess stain was removed by a blotting paper. After 10 to 15 minutes the slides were observed under the microscope. Fully stained pollen grains with regular shape were considered fertile; while, shrunken, deformed and partially or unstained pollen grains were noted sterile as was suggested by Marks (1954).

9. **Determination of pollen grain viability**

Pollen grain viability analysis was performed in *Corchorus* spp., mutants and in hybrid plants. Pollen grains viability (lugol’s iodine - detects the presence of starch, viable pollen turns black – Bengtsson 2006; aniline blue in lacto phenol - detects the presence of callose on pollen wall, viable pollen turns blue - Bengtsson 2006; x-gal- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside - detects the presence of β-galactosidase, viable pollen turns green – Atiaksheva et al. 2000; amido black - detects the presence of protein in pollen wall, viable pollen turns black – Regan and Moffatt 1990; FDA – fluorescein diacetate – detects presence of cytoplasmic starch that cleaves moieties from lipid soluble non-fluorescent probe to yield fluorescent product – Coder 1997) was analyzed using different stain tests. FDA analysis was made under fluorescence microscope (Olympus, model: MLXi with Micro-LED fluorescence attachment; excitation maximum 475 nm, emission maximum 535 nm).

10. **Assessment of pollen grain nuclei composition**

Pollen grain nuclei composition was studied in different plant types following DAPI (4’, 6-diamidino-2-phenylindole, a fluorescent vital stain binding DNA – Willemse and Keijzer 1990; and RNA – Hard et al. 1990) staining as per the methodology described by Johnson and McCormick (2001) to assess pollen nuclei (3 nucleate stage; v - vegetative nuclei, g - generative nuclei) per pollen grain. Observations were recorded under fluorescence microscope (Carl Zeiss Axio fluor 900EX, Carl Zeiss MagAnalytic 10.1; DAPI excitation range 350-360 nm, emission maximum 460 nm).

11. **Quantification of pollen grains**

Quantification of pollen grains was made in 8 jute species following direct count of pollen grains from pollen suspension (10 anthers squashed in 50 µl of distilled water for each species) using a improved Neubauer Hemocytometer (3 replicas/species, 10 observations/replica) and observed under light microscope (Olympus CH 20i; 10x ×
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40x). Pollen count was calculated from the formula \( c = n \times 10^4 / \text{ml} \) (\( c \) = cell concentration in cells/ml, \( n \) = average number of cells/mm²) as per Mather and Roberts (1998).

12. **In vitro germination for pollen viability**

Fresh harvested pollen grains were cultured in the medium containing sucrose (5%), agar (0.5%), boric acid (5 ppm) and calcium nitrate (0.03%) and the medium was incubated at 37°C for 72 hours. Pollen grain germination in hybrid and in parent plants was evaluated from pollen tube development.

13. **Crossing experiments**

At anthesis (9 am to 11 am) reciprocal crossings between control and mutants (5 crosses in each set) were performed following all necessary precautionary measures to avoid genetic contaminations. Hybrid seeds obtained from crossings were sown to raise F₁ plant population. The F₁’s were selfed (5 floral buds at the onset of anthesis were bagged in each category) and seeds obtained were sown to raise F₂ plants. The F₂ plants segregated in normal and mutant phenotypes and \( \chi^2 \) test analysis was performed to assess segregation patterns.

14. **Stomatal studies**

Stomatal studies were made in hybrid and its parents. For the study of stomata, quick fix image impression technique (Nayeem and Dalvi 1989) was followed. The leaf prints (3 leaves near to apex from 3 plants in each type were scored) were taken at bud initiation stage. Considering that the shape of the stomata to be elliptical its area was calculated as per Ghosh *et al.* (2004) using the formula \[ \frac{\pi}{4} (L \times W) \], where, \( L \) and \( W \) are the length and width of stomata respectively. Stomatal frequency was recorded in light microscope (10x×40x).

15. **Biochemical analysis**

i. **Quantitative analysis of protein content**

Eight *Corchorus* spp., mutant lines (M₇) and F₆ hybrid plants were assessed in the year 2012.

a. **Extraction of total soluble seed protein:** Extraction of soluble seed protein was done following Osborne (1962). For extraction of total soluble protein, seeds (1 gm seeds from each germplasm were taken, 3 replicas were kept, imbibed in water for
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overnight at room temperature) samples were crushed in liquid nitrogen in a chilled mortar and pestle. The powdery crushed samples were homogenized in a chilled mortar and pestle using suitable volumes of 5% TCA (trichloroacetic acid, E. Merck, Germany) at 0°C to 4°C. The homogenate was centrifuged at 2000 rpm for 45 minutes in a cold (-4°C) refrigerated centrifuge (REMI; Model No: M-12). The supernatant was discarded and the process was repeated twice. The residues were then extracted once with cold absolute alcohol and twice with ethanol: diethyl ether (3:1). Supernatant was discarded each time and the residue was diluted 20 times with 1(N) NaOH.

b. **Estimation of soluble protein:** The protein was estimated using the method of Lowry et. al. (1951). Reaction mixture was prepared by adding 2.5 ml of reagent A [2% sodium carbonate in 0.1 (N) NaOH] and reagent B [0.5% CuSO₄, 5 H₂O in 1% Na-K-tartarate] mixed in the proportion of 50:1 (freshly prepared before use) to 0.5 ml of the sample (allow to stand for 20 minutes after thorough mixing, using vortex) followed by addition of 0.25 ml Folin-Ciocalteu phenol reagent. The reaction mixture was mixed thoroughly and kept for 30 minutes in dark for development of color. Similarly a blank was prepared using 0.5 ml of 1(N) NaOH. The optical density of the blue color developed was recorded in a spectro (ELCO, SL171, MINI SPEC) at 660 nm against the blank. Protein content was estimated by referring to a standard curve prepared with known concentrations of Bovin Serum Albumin (BSA) fraction V (Sigma chemical).

ii. **Qualitative estimation of seed protein by SDS-PAGE**

Seed protein profile by SDS-PAGE was prepared in Corchorus spp. and macromutant plant types of C. olitorius in relation to control.

a. **Preparation of seed protein for polyacrylamide gel electrophoresis:** Seeds of germplasms (1 gm for each case) were imbibed in distilled water for overnight at room temperature (30°C ± 1°C). Seeds were then crushed in liquid nitrogen in a chilled mortar and pestle (0°C to 4°C) until it becomes powdery. About 1.5 ml of extraction buffer [for 25 ml volume: 1 M sucrose – 4.279 gm in 12.5 ml dH₂O (volume makeup), 0.2 M Tris-HCl – 0.3025 gm in 12.5 ml distilled H₂O (volume makeup) and 54 µl 2-Mercaptoethanol] at pH 8.5 was added to the samples. The crushed samples were collected in 2 ml eppendrop tubes and kept in dark for overnight. The samples were
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centrifuged (-4°C to 0°C) for 4 times at 12,000 rpm for 30 minutes (each time). The clear supernatant was collected and used as protein source for polyacrylamide gel electrophoresis (SDS-PAGE). To study seed protein polymorphism, one dimensional SDS-PAGE (10%) was carried out following Laemmli (1970) in a vertical gel system (BIOTECH).

iii. SDS-PAGE

For SDS-PAGE following procedures were followed:

a. Assembly of glass plates: Thoroughly cleaned (chromic acid, liquid soap and in running water) and dried (air dried followed by absolute alcohol) glass plates with spacers (1.5 mm) were assembled properly with the help of steel grip tape and clamps.

b. Preparation of separating gel: Separating gel (10.0%) of 53.70 ml was prepared by the addition of following ingredients - stock acrylamide solution (30%) – 17.73 ml, 1.875 M Tris-HCL (pH- 8.8) – 10.67 ml and distilled water – 24.03 ml. The solution was then mixed properly and was degassed on a vacuum pump for 10-15 minutes and then 0.26 µl ammonium persulphate (5%), 0.53 ml 10% SDS and 26.67 µl TEMED (N,N,N',N'-tetramethylethylenediamine) were added to the separating gel. Carefully the separating gel solution (40 ml) was poured in the chamber between the glass plates. Drops of distilled water were given on the top of the gel (to prevent evaporation) and the gel was left to set (polymerization) for 30-45 minutes.

c. Preparation of stacking gel: Stacking gel (4.5%) of about 20 ml was prepared by addition of the following solutions - stock acrylamide solution (30%) – 2.70 ml, 0.6 M Tris–HCL (pH - 6.8) – 3.0 ml, distilled water – 15.0 ml. The solution was mixed properly and degassed for 15 minutes followed by addition of 100 µl ammonium persulphate (5%), 0.2 ml 10% SDS and 20 µl TEMED.

After decanting of the water layer from the top of the separating gel and washing with a little stacking gel solution, the stacking gel mixture was poured on the top of the separating gel and immediately the combs (1.5 mm) was placed carefully in the stacking gel and the gel was allowed to set for 30 to 45 minutes until complete polymerization.
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d. Installation of the gel in the electrophoresis apparatus: After the stacking gel had polymerized, the comb was removed carefully without distorting the shape of the well. Carefully the steel grip tape and clamps were removed and the gel was installed in the electrophoresis apparatus.

e. Loading of protein samples: The extracted protein samples prepared for electrophoresis were adjusted by using one strength (1X) sample buffer (0.5 ml Tris-HCl buffer of pH 6.8, 500 mg sucrose, 50 gm SDS, 0.1 ml bromphenol blue and water up to 1 ml) in each lot of sample in such a way that the same amount of protein (0.0725 µg/µl) is present per unit volume. The protein samples were dipped in boiling water 10 times (each dip – 8 seconds) to ensure complete interaction between proteins and SDS. The samples were cooled to 0°C to 4°C for 5 minutes and 12 µl of each sample was loaded in each well (marker protein 12 µl was also added in the same way) after the wells were thoroughly washed with distilled water and running buffer. The sequence of the samples loaded in the wells was maintained through specific adjustments.

f. Preparation of running buffer (pH- 8.2-8.4): Running buffer for SDS-PAGE was prepared by adding the followings- Tris – 2.4 gm, glycine – 11.52 gm, SDS – 800 mg and distilled water up to – 800 ml.

g. Electrophoresis: The gel apparatus was filled with running buffer and any trapped air bubbles at the bottom of the gel was removed. The cathode and the anode were connected to the gel apparatus and to the DC – power pack to complete the electric circuit. The entire gel unit (gel apparatus and the glass plates) was kept in cooling condition so that heat generated during the run of the protein sample is dissipated and does not affect the gel. A constant current of 3 mA/slot (voltage depends on the total current applied) for 3 to 4 hours was given for complete run of the protein samples as indicated by the bromphenol blue dye marker (current was stopped when dye marker reaches almost at the bottom of the gel).

h. Staining of the gel: After complete run of the protein samples in the gel, the gel was carefully removed from between the plates and immersed in staining solution (250 mg Coomassie Brilliant Blue R250 in 40 ml methanol followed by addition of 10 ml glacial acetic acid and 50 ml water) for overnight with uniform shaking. The proteins absorb the Coomassie Brilliant Blue.
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i. **Destaining of the gel:** The gel was then transferred in a suitable container with at least 50ml to 70 ml destaining solution (methanol:glacial acetic acid:water – 40:10:50). The dye that was not bounded to proteins was removed. Frequent changes in the destainer were made until the background of the gel was colorless. The proteins fractioned were observed as blue colored bands. Destaining process was carefully conducted so that all faint bands could be appropriately visualized. The gel was then stored in fixative (7% acetic acid solution).

j. **Photography:** The gel with stained protein bands was placed in glow box to take appropriate photographs with digital camera.

k. **Recording and computer analysis of data:** The position of the protein bands in the gel was detected and expressed as relative mobility (Rm) by measuring the distance migrated by the particular protein band to that of bromophenol blue. The protein bands were evaluated against the protein molecular weight marker for SDS gel electrophoresis (molecular weight range: 14,300 Da to 97,400 Da) obtained from Bangalore Genei Pvt. Ltd., India (Cat No. PMW - M). Molecular weight of different bands was studied using the software (TotalLab™ Quant v 11; website: http://www.totallab.com/).

16. **Extraction and estimation of chlorophyll content**

Chlorophyll was extracted and estimated from leaf tissues (of identical maturity) of control and ‘viridis’ (3 replicas in each case) following the method of Arnon (1949). For chlorophyll extraction, samples of 1 gm fresh lamina (excluding midrib and veins) were dipped (in each case) in 25 ml of 80% acetone for overnight, filtered and the supernatants were collected and volumes were made up to 100 ml by addition of 80% acetone.

For densitometric readings, 5 ml of the extract was transferred to a 50 ml volumetric flask and volume was made up with 80% acetone. Readings of optical density values were recorded in a spectro (ELCO, SL171, MINI SPEC) at 663 nm and 645 nm for chlorophyll-a and chlorophyll-b respectively against 80% acetone as blank. Estimation of chlorophyll-a, b and total chlorophyll was made by using the following formulae:
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- mg chlorophyll-a/gm of tissue = \[12.7(A663)-2.69(A645)\] \times \frac{V}{(1000 \times W)}
- mg chlorophyll-b/gm of tissue = \[22.9(A645)-4.68(A663)\] \times \frac{V}{(1000 \times W)}
- mg total chlorophyll/gm of tissue = \[20.2(A645)+8.02(A663)\] \times \frac{V}{(1000 \times W)}

Where, A = absorbance at specific wavelength, V = final volume of chlorophyll extract in 80% acetone and W = fresh weight of tissue extracted. Final quantitative determination of chlorophyll content was performed by averaging the values of the replicas in each plant type.

17. Analysis of plant pigments using thin layer chromatography (TLC)

Leaves (1 gm for each sample) of identical maturity from ‘viridis’ and control (C. olitorius) plant types (3 replicas in each case) were chopped into pieces (excluding midrib) and soaked in acetone:pet. ether (22:3 ratio) mixture for overnight (pet. ether – boiling range 60°-80°C). To the mixture one spatula of CaCO₃ (secondary metabolite adsorption) was added, crushed and filtered in Whatman™ No. 42 filter paper. The filtrate was collected and put into a separating funnel containing 20 ml of pet. ether and 20 ml of 10% NaCl for elimination of primary metabolites. The separating funnel was shaken vigorously and the solution was allowed to stand. The aqueous layer was dipped out, washed with 5 ml of distilled water and the process was repeated for 4 to 5 times. Anhydrous sodium sulphate (one spatula) was added to the solution and shaken, allowed to settle, solution decanted off and reduced to 3 ml. TLC plates were prepared in triplicates as suggested by Fried and Sherma (1999) and 10 µl volume was loaded uniformly for each sample (control and ‘viridis’ in each plate). The mobile phase was pet. ether:isopropanyl alcohol:water (9:1:5 drops). After complete run, the TLC plates were taken out of the chambers, air dried and the spots (uniformly among the plants was observed and the best plate was considered) showing different colorations were observed and photographed immediately. Rf values were calculated and compared with reference chart provided by Pavia et al. (1999) for preliminary quantitative plant pigment analysis.

18. Molecular analysis

i. Extraction: Total genomic DNA (seeds of 8 species, F₆ hybrid plants along with parents and M₇ mutant plants) was isolated from 0.2 gm of germinated seedlings
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of each sample using DNeasy Plant Mini Kit of Qiagen, USA. The seedlings were raised in Petri plates lined with moist filter papers (27±1°C). The mucilage and polyphenolic compounds were removed by passing DNA through an Au-Prep purification column (Life Technologies, Rockville, MD, USA). Subsequently, quality and quantity of extracted DNA were checked by running the dissolved DNA in 0.8% agarose gel by comparison with standard lambda DNA marker of known concentration. The DNA was diluted to 30 ng/μl for RAPD and ISSR analysis.

ii. RAPD and ISSR analyses: Ten-base 30 RAPD primers (OPA 01-10, OPB 01-10, OPC 01-10; Operon Technologies, Alameda, USA) and 7 non anchored oligonucleotide ISSR primers (synthesized by Isogen) were used for Polymerase Chain Reaction (PCR). Amplification reactions were performed in volumes 25 μl containing 2.5 μl of 10X assay buffer (100 mM Tris-HCl; pH 8.3, 500 mM KCl, 15 mM MgCl2), 100 mM (For RAPD) / 200 mM (for ISSR) of each dNTPs (dATP, dCTP, dGTP and dTTP) (MB1 Ferment Inc., Maryland, USA) 5 pg of RAPD primer / 15 ng of ISSR primer, 1.0 unit of Taq DNA polymerase (Bangalore Genei, India) and 30 ng of template DNA from each germplasm source.

The amplification reactions were carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 44 cycles (for RAPD) and it follows as: 1st cycle of 5 min at 94°C followed by 43 cycles each of 1 min at 92°C, 1 min at 37°C, 2 min at 72°C. For ISSR, the amplification reactions were programmed for 27 cycles: 1st cycle of 2 min at 94°C followed by 26 cycles each of 1 min at 92°C, 1 min at 52°C, and 4 min at 72°C. For both RAPD and ISSR, the final step consisted of one cycle of 7 min at 72°C for complete polymerization. After completion of the PCR, 2.5 μl of 6X loading dye (MB1 Ferment Inc., Maryland, USA) was added to the amplified products and was electrophorized in a 1.5% (m/v) agarose (Bangalore Genei Pvt. Ltd., Bangalore, India) gels with 1X TAE buffer, stained with EtBr (ethidium bromide) and documented by a gel documentation system (Syngene, Cambridge, UK). Three repeat runs were made to see the consistency of the band positions for each sample and with each primer. The primers documenting uniformity were considered for analysis.

19. Statistical analyses
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i. $\chi^2$ test of heterogeneity: This statistical test was performed to assess variations, if any, for quantitative morphological traits, cytological and pollen parameters among the species, mutants and hybrid across the years. Further, $\chi^2$ test of heterogeneity was also performed at 7 DF (degree of freedom) for different parameters namely, pollen fertility, pollen viability (pooled over the stain tests for a species), pollen grains/flower, anther/flower, pollen area, pollen nuclei composition, capsule length, capsule/plant and seed set/fruit among the species to assess significant variations, if any, as well as in each species for different stain tests.

ii. T-test: Student t-test analysis was performed between control (C. olitorius) and ‘viridis’ mutant in relation to chlorophyll contents.

iii. Correlation analysis: Pearson’s correlation coefficient analysis (DF=7) between pollen productive parameters (pollen fertility, pollen viability, pollen grains/flower, anther/flower, pollen area, pollen nuclei composition, capsule length, capsule/plant and seed set/fruit) were conducted to study interrelationship considering each of the jute species as independent variables.

iv. Cluster analysis by UPGMA: Upon considering 492 discrete variables (94 - morphological parameters: nature and color of stem, leaf shape, serration, venation and color, bud number, color and shape, sepal number, shape, color and bract, petal number, shape and color, stamen number, shape, color and nature of filament, ovary shape, color, style and stigma, fruit shape and color; 4 - cytological attributes: bivalent configurations, occurrence of cytomixis and aneuploid PMCs; 28 - pollen morphological data: polar axis, equatorial diameter, shape, colpus length, colpus shape, pore diameter, exine thickness, muri diameter, lumen shape and lumen area; 81 - pollen and productive parameters: pollen fertility, viability, anther number/flower, pollen quantity/flower, pollen size, pollen nuclei composition, capsule/plant, capsule length, seed set/fruit and seed size; 42 - biochemical: band profile; 243 - molecular data: band profiles of RAPD and ISSR) in the species a data sheet has been prepared separately (in Corchorus spp.: pollen morphology, pollen attributes and reproductive parameters, SDS-PAGE and RAPD and ISSR markers; in hybrid: molecular data) as well as taken together. The presence (1) and absence (0) of the parameters in the germplasms were scored and entered in a binary data
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matrix. Based on correlation analysis, proximity matrix was generated for all possible pairs from Euclidean Distance and a dendrogram was construct by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for pollen and pollen productive parameters, biochemical and molecular attributes as well as all variables taken together using the software IBM SPSS statistics (v.19, 2010) as well as STATISTICA version 7.1 (www.statsoft.com).

v. Unrooted phyllogenetic tree: Using pollen morphological parameters and molecular data unrooted phyllogenetic tree was constructed upon considering 8 Corchorus spp. using the software DendroUPGMA (website: www.genomes.urv.cat/UPGMA/ - Garcia-Vallve et al. 1999).

vi. Principal component analysis (PCA): PCA is the simplest of the true eigenvector based multivariate analysis and used on the basis of the net merit of the species constructed by taking together the scores of each character (pollen morphological characters and band analysis from molecular data) as was proposed by Jain (1982). Based on correlation matrix, PCA was performed to judge the factor score of each species due to highest eigen value(s) as described by Dillon and Goldstein (1984). Percentage variation explained by the highest eigen value(s) was also calculated. Plot of factor coordinates was made.

vii. Efficiency of genetic parameters: RAPD and ISSR bands were designated based on their molecular weight and calculated using the kilo base (kb) ladder used as marker. Molecular data processed in MS excel for calculating polymorphic band(s) of individual primer, average polymorphic band per primer and percentage polymorphism. Analysis of informative potential of molecular markers and genetic diversity of the genotypes under assessment including the effective number of allele per locus (Weir 1996), Shannon’s diversity index (Martynov et al. 2003), genetic diversity/diversity index (Weir 1996), marker index and polymorphism information content (Anderson et al. 1993) were computed for each primer across the species based on frequency of alleles of each locus. AMOVA (Analysis of molecular variance) was conducted for RAPD and ISSR markers to assess variation among species as well as among the primers.