3.1 Materials

3.1.1 Source of chemicals

All the chemicals used were of analytical grade and molecular biology grade (free from DNase and RNase). The list of chemicals, fine chemicals and molecular biology kits used in the present study along with the respective firms are listed in **Table 3.1**.

Table 3.1 List of chemicals/kits

Chemicals/Kits	**Source/Firm**
Tris base, HCl, NaCl, Sodium acetate, NaOH, Bacteriological Agar, Chloroform, Glacial acetic acid, PCI	SRL, India
EDTA disodium salt, SDS, Agarose, CTAB, HEPES	Sigma, USA
Isopropanol, Glycerol, Isoamyl alcohol, Ethanol (absolute)	Merck, India
Taq DNA Polymerase, dNTPs	Bangalore Genei, India
Yeast extract, Tryptone	Hi Media, India
100 bp ladder, 1kb ladder, λ DNA *Hin*dIII/*Eco*RI double digested marker, λ DNA *Hind*III digested marker,	Fermentas, Canada
Restriction enzymes, CIP, Klenow fragments, T4-DNA ligase, Vent polymerase, dNTPs mix	New England Biolabs, USA
pGEM-T Easy vector system I	Promega, USA
Liquid nitrogen	Indian made
QIA quick Gel extraction kit, Plasmid mini prep kit	Qiagen, USA
DNA sequencing Kits (ABI PRISM 310 with dye termination cycle sequencing ready reaction kit)	Perkin Elmer, USA

3.1.2 Glasswares, plasticwares and equipments

Glass wares used in the present investigation were procured from Borosil, India and Schott Duran, Germany. Plasticwares comprising of microfuge tubes, PCR tubes, petriplates, micro tips and tip boxes were procured from Tarson, India and Axygen. Micropipettes of different ranges were obtained from Eppendorf, Germany. The lists of instruments used in the present study are listed in **Table 3.2**.

Table 3.2 List of instruments

S. No.	**Name of the instrument**	**Company/Model**
1	Laminar Air Flow	Lab Companion,BC-11, Korea
2	PCR machine	Eppendorf, Germany
3	Refrigerated Centrifuge	SIGMA (3-18K), Germany

4	Refrigerated Microfuge	Eppendorf, Germany
5	Water bath	Lab Companion,BW-20G, Korea
6	UV-Visible Spectrophotometer	SHIMANDZU, Japan, UV-1700
7	Microwave oven	LG (GRILL), India
8	Magnetic Stirrer	BIOSAN, PV-2400, Latvia
9	Refrigerator	LG, GL-406ΔMQ/ India
10	Deep Freezer	Vestfrost, BSF-345, Denmark
11	pH meter	EUTECH, Singapore
12	Digital balance	Precisa, Switzerland
13	Gel Documentation System	Alphalmager, AT126SL, USA
14	Dry bath	BIOSAN, CH-100, Latwia
15	Agarose Gel Electrophoresis system	SCIE PLAS. CHU25. UK
16	Power pack of Agarose Gel Electrophoresis system	Consort, EV 215.
17	Incubator	Lab Companion, Korea
18	Shaker	Lab Companion, Korea
19	Nanodrop Spectrophotometer	Thermo scientific, USA
20	Micropulser	BIORAD

3.1.3 Seed collection of different crops

In the present study, seeds of different cereals (rice, wheat, oat, sorghum, barley, maize) and millets (finger millet, barnyard millet, proso millet, little millet, kodo millet, foxtail millet) were used. The seeds of these crops were collected from the Crop Research Center, Pantnagar and Ranichauri Hill Campus, G. B. Pant University of Agriculture and Technology, India. The names of cultivar and sub-family of crops are provided in **Table 3.3**.

Table 3.3 List of different crops and their cultivars

Crops (Scientific name)	**Cultivar**	**Sub-family**
Rice (*Oryza sativa*)	Pusa sughandha	Oryzoideae
Wheat (*Triticum aestivum*)	Punjab PBW 373	Pooideae
Sorghum (*Sorghum bicolor*)	Pant chari 5	Panicoideae
Barley (*Hordeum vulgare*)	VLB56	Pooideae
Oat (*Avena sativa*)	UP0212	Pooideae
Maize (*Zea mays*)	DQPMC4W	Panicoideae
Finger millet (*Eleusine coracana*)	VL315	Chloridoideae
Barnyard millet (*Echinochloa frumentacea*)	PRB401	Panicoideae
Proso millet (*Panicum*	405	Panicoideae

milliaceum Linn.)		
Little millet (*Panicum milliare* Lam.)	Local	Panicoideae
Kodo millet (*Paspalum scrobiculatum* Linn.)	Local	Panicoideae
Foxtail millet (*Setaria italica* Beauv.)	PRK1	Panicoideae

3.1.4 Plasmids and bacterial strain

The plasmid pGEM-T Easy vector (Promega, USA) and pBSK (pBlueScript) *E. coli* cloning vector (Stratagene) were used for cloning of PCR product. Bacterial strain *E. coli* DH5α (Genei, India) was used as the host cell for transformation.

3.1.5 Sterilization

All the media, buffers and stock solutions were prepared using Millipore elix 3 deionized water unless otherwise mentioned. These were sterilized, as recommended, either by autoclaving at 15 lbs/inch^2 (psi) pressure at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2 µm-0.45 µm (for heat labile compounds).

3.2 Methods

3.2.1 Germination of seeds

Healthy seeds of different crops as mentioned in **Table 3.3** were selected and germinated on the water soaked filter paper in the dark after surface sterilization by 0.1 per cent $HgCl_2$. The etiolated seedlings were harvested for DNA extraction after one week of the growth.

3.2.2 Isolation of genomic DNA by CTAB method

The genomic DNAs of different crops were isolated by CTAB method **(Murray & Thompson, 1980)**. The isolation was based on principle that CTAB precipitates carbohydrates at high temperature and at high salt concentration while it precipitates nucleic acids at low temperature and at low salt concentration.

Stock Solutions

Following stock solutions listed below were prepared prior to genomic DNA extraction.

I. 1M Tris Chloride

For 100 ml

Tris base	12.11 g
Distilled water	80 ml

Adjusted the pH to 8.0 with 1N HCl. The final volume was made up to 100 ml with distilled water. Autoclaved this buffer and store at room temperature (RT).

II. 5M NaCl solution

For 100 ml

NaCl	29 g
Distilled water	80 ml

The volume was made up to 100ml with distilled water, autoclaved and stored at RT.

III. 10 % (w/v) SDS

For 100 ml

SDS	10 g
Distilled water	80 ml

The SDS was dissolved by heating the container at 60^{o}C. The final volume was made up to 100 ml with distilled water and stored at RT.

IV. CTAB solution

For 100 ml

CTAB (10 %)	10 g
NaCl (0.7 M)	0.82 g

The volume was made up to 100 ml with distilled water, autoclaved and stored at RT.

V. DNA extraction buffer

For 100 ml

1M Tris-Cl (pH=8)	10ml
0.5M EDTA (pH=8)	4ml
5M NaCl	28ml
10% CTAB	20ml

The volume was adjusted to 100 ml with distilled water, autoclaved and stored at RT. β-mercaptoethanol at final concentration of 0.2% was added prior to use.

VI. Chloroform: Isoamyl alcohol (24:1)

For 25ml

Chloroform	24ml
Isoamyl alcohol	1 ml

These were mixed in the ratio of 24:1.

VII. Isopropanol

Stored at -20^{o}C in 100 ml dark colored bottle.

VIII. 70 % ethanol

For 100 ml

Absolute ethyl Alcohol	70 ml
Distilled water	30 ml

XI. TE buffer (pH 8.0)

For 100 ml

Tris-Cl (1M)	1 ml
EDTA (0.5M)	200 µl

The volume was made up to 100 ml with distilled water, autoclaved and stored at RT.

3.2.2.1 DNA extraction procedure

i. One gram etiolated seedlings were ground in liquid nitrogen to a fine powder in a pre-chilled mortar.

ii. The powder was transferred into a 30 ml oakridge tube containing 10 ml of DNA extraction buffer and incubated at 65ºC for 60 min.

iii. The tubes were cooled at room temperature and equal amount of chloroform: isoamyl alcohol (24:1) was added, mixed vigorously and finally centrifuged at 10,000 rpm for 15 min at 20ºC.

iv. Supernatant was collected and transferred to a fresh sterile oakridge tube. 0.7 (v/v) of chilled isopropanol was added to the fresh tube and incubated for 4 hours at -20ºC.

v. The tubes were then further centrifuged at 10,000 rpm for 15 min at 4ºC to pellet down the DNA.

vi. The pellet was washed with 70% ethanol, air dried and dissolved in 100 µl of TE buffer.

3.2.2.2 DNA purification

i. RNase A at final concentration of 50µg/µl was added to the Eppendorf tube containing DNA solution.

ii. The tube was then incubated at 37ºC for 1 h.

iii. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added in the tube and mixed thoroughly by gentle inversion.

iv. The contents were centrifuged at 10000 rpm at 4ºC for 10 min.

v. Aqueous phase was transferred in to fresh Eppendorf tube and 1/10th volume of 3 M sodium acetate (pH 5.2) and double volume of ethanol was added and finally kept at -20ºC for 4 h.

vi. The tube was then centrifuged at 10000 rpm for 15 min to pellet down the precipitated DNA.

vii. Pellet was washed with 70% ethanol, air dried and dissolved in 100 µl of TE buffer.

3.2.3 Spectrophotometric quantification of genomic DNA

For quantification of DNA the absorbance of DNA samples was measured at 260 nm in UV-visible spectrophotometer. The amount of DNA in the sample was estimated by the fact that 1.0 absorbance at 260 nm is equivalent to 50 µg DNA per ml.

Concentration of DNA (µg/ml) = $A_{260} \times 50 \times$ dilution factor

Purity of genomic DNA can also be analyzed based on value of A_{260}/A_{280}

If the ratio of $\frac{A_{260\,nm}}{A_{280\,nm}} = 1.8 \rightarrow$ indicates pure DNA

$> 1.8 \rightarrow$ RNA contamination

$< 1.8 \rightarrow$ Protein contamination

3.2.4 Qualitative analysis of DNA by agarose gel electrophoresis

Agarose gel electrophoresis is the standard method used to check the quality of DNA fragments. The technique is simple, rapid to perform and capable of resolving fragments of DNA. For resolving the DNA fragments on the gel the DNA samples were loaded with loading dye in 5:1 ratio. The DNA was subjected to electrophoresis in 1X TAE buffer **(Sambrook & Russell, 2001)**.

Stock solutions for gel electrophoresis

I. DNA loading dye (6X)

For 10 ml

Bromophenol blue (0.25 % w/v)	0.025 g
Glycerol (40 %)	4 ml

Dissolved properly and the volume was made up to 10 ml by 1X TAE and stored at 4ºC.

II. Electrophoresis buffer (TAE 50X)

For 100 ml

Tris base	24.2 g
Glacial acetic acid	5.7 ml
0.5 M EDTA	10 ml

The volume was made up to 100 ml by distilled water, autoclaved and stored at RT.

III. Ethidium bromide

Ethidium bromide	10 mg
Distilled water	1 ml

Dissolved properly and stored at 4ºC

Note: Ethidium bromide is highly mutagenic and carcinogenic so gloves should always be used while handling.

3.2.5 Primer designing for PCR amplification of Dof domain and *Dof* genes

Nucleotide sequences of different *Dof* genes of cereals (wheat, maize, rice and barley) reported in the literature were retrieved from NCBI (**http://www.ncbi.nlm.nih.gov**) and DRTF (**http://drtf.cbi.pku.edu.cn**) database. Multiple sequence alignment of different *Dof* domain and genes were done by ClustalW program and primers were designed from resulting consensus sequences using Gene Runner, Primer3 **(Untergasser *et al.*, 2007)** and DNASTAR software **(Burland, 2000)**.

Strategy for designing the primer specific for Dof domain

- The *Dof* gene sequences of rice from DRTF database were taken in FASTA format.
- Alignment of the retrieved *Dof* gene sequences was done using ClustalW and the most consensus sequence was taken.
- To obtain the Dof domain, the sequence was first translated into protein and CPRC motif that is the most conserved motif at the start sequence was identified and from it up to 150 residues were taken.
- The sequence was put in primer3 online primer designing tool. Primer conditions (primer size (20-24bp), Tm value (55-65ºC), GC% (50-60) and product size) were manually set and pick the primer.

Strategy for designing the primer for *Dof* genes

The retrieved nucleotide sequences of *Dof* genes were taken in edit sequence format of DNASTAR followed by open the edit sequences in primer select module of DNASTAR. Primers were designed by setting primer characteristic i.e. primer length (20-24bp), Tm value (55-65ºC), GC% (50-60) and product size range (according to gene size). A total of 25 sets of primers were designed for PCR amplification of *Dof* domain and genes of different cereals and millets as shown in **Table 3.4**. For PCR amplification of *Dof* genes of sorghum, the primers were designed by using Gene Runner V3.05 with deliberate incorporation of restriction sites *Eco*RI, *Hin*dIII and *Sma*I sites to assist in cloning as shown in **Table 3.5**.

Table 3.4 List of primers for the PCR amplification of *Dof* domains and genes from different cereals and millets

S. No.	Primer code	Source (Accession number)	Primer sequence (5'----3')	Amplicon size (bp)
1	Dof-1	Rice (OsIBCD001074)	F-GAAGCCAGATAAGATTCTCCCTTG R-ATCAGTAAGTGACGGTAGTGCAAC	210
2	Dof-4	Rice (OsIBCD016217)	F-CCACCAACACCAAGTTCTGCTAC R-AGCTCTGCATCTGCTGTAGTCTC	477
3	Dof-6	Rice (OsIBCD044075)	F-CGGGACACCAAATTCTGCTACT R-GACGAAGACGAGGACTTCTTGC	149
4	Dof-8	Rice (OsIBCD001074 &OsIBCD024636)	F- AGATTCTGCCTTGTCCTCGTTG R-GAGCTCTTACTCTTGCGCCTAC	172
5	Dof-9	Rice (OsIBCD005722 &OsIBCD038179)	F-GTGCGAGTCGATGCAGAC R-ACGAGCGCTTGCTCTTGC	155
6	Dof-10	Rice (OsIBCD036003)	F-CAGGTGGTGGAGGATTGGGAGGAG R-CAGCAGGAAGCCGGTGGTGAAGTC	479
7	Dof-11	Rice (OsIBCD001074)	F-GCCCTCGGTGCAACAGTATGGACA R-CTGCTTGGCCTTCGCTCTCACCTT	1065
8	Dof-12	Rice (OsIBCD036857)	F-TGGCCCGGGAGAAGGTTGA R-CGGGGAGGTTGGGGAGGAC	467
9	Dof-13	Rice (OsIBCD005722)	F-GCCCCCGGTGCAACTCCATC R-GCCGCCCTGCTGCTCGTCTG	638
10	Dof-15	Rice (OsIBCD039459)	F-GCAGGCGACGCAGCAGAGGT R-GACAGCGCCGACGGGAGTGAG	777
11	Dof-16	Rice (OsIBCD012444)	F-GCACCACCACCACCACCACCAG R-CGTTGCCACCGCCCCAGAAT	962
12	Dof-17	Rice (OsIBCD016020)	F=CACGGGGCAGCAGGAGAAGAAGC R=GGCGATGTCGGCGAAGTTGTCC	
13	Dof-18	Rice (OsIBCD009642)	F-GCGCGGCCGAACTCAAT R-AGCCGCCATGTACTGCCTCTG	852
14	Dof-19	Rice (OsIBCD024636)	F-AGGGTGGTGGATCGAGTGAAGAGA R-GGAGAAATGAAGGGCCAAACTGAA	781
15	Dof-20	Rice	F-CGGAGGCGCAAGAAGACGAA	916

		(OsIBCD030524)	R-CGCGATCCCCGAGGTGTAGTAG	
16	Dof-21	Rice (OsIBCD030524)	F-AGAGGAGGAGGAGATGGGCTGAT R-ATGATGATGAGGAGGAGGAGGATG	792
17	Dof-22	Rice (OsIBCD045436)	F-GCAGGGGCAGGGATACG R-TGCCACGCGGTCAGGTC	477
18	Dof-23	Rice (OsIBCD009003)	F-GATACGGCCAACCAGAAGGAGAA R-AGCAGTTGGTCATTGTGGATACGA	437
19	Dof-24	Wheat (AJ012284)	F-GCCCTCGGTGCAAGTCTGG R-ATCACCGCCGCTGTCGTTATT	785
20	Dof-25	Maize (U82230)	F-GTGGCAGTGGGGAGCGTAAG R-TGAGGCCCCACCAGTTGTCT	715
21	Dof-28	Wheat (AY955493)	F-GAGGCGGCCGTCAAGTGC R-GCTCCCGCCGTTCCAGTAATC	702
22	Dof-31	Barley (AJ000991)	F-GCCCTCGGTGCAAGTCTGGTA R-CGCCGCCGTTATTGTTGTTCT	783
23	Dof-34	Barley (AJ312326)	F-ATGGCGGCGGGAGTAGGATT R-TGCATTGCGGCCATTGTGAT	628
24	Dof-35	Barley (AM084356)	F-CACCCACAAAGCCACTGATAACAA R-CACAGAAATGGGACGGAAACAAAT	1120
25	Dof-36	Wheat (AY496057)	F-CCCTTCATTCACCTGATG R-ATGACCTCCATTTCCCATTT	1039
26	Dof-37	Wheat (AY496057)	F-AAACTCCAAGAGCAAGGCAG R-AGCGAAATAAGTCAAGCAAG	1083
27	Dof-38	Rice	F- TCCACAAGGGGACACTGAA R- CATGTCAGAGCCAAAGCTGA	427
28	Dof-D1	Rice	F-GAGAAGGCGCTCAAGTG R-ACGAGCGCTTGTTCTTG	178
29	Dof-D2	Rice	F-CGCGGCCGCAGAAGGAGAAG R-GACGGCCCACGGAGGACGAC	210
30	Dof-D3	Rice	F-GGAGAAGGCGCTCAAGT R-GACGACGGCTTGTTCTT	180
31	Dof-D4	Rice	F-CGCGGCCGCAGAAGGAGA R-GACGGCCCACGGAGGACGAC	210

Table 3.5 List of primers for PCR amplification of *SbDof* genes from sorghum

S.No.	**Primer code**	**Primer sequence 5'---3'**	**Amplicon size (bp)**
1	SbDof1	F- AGTCGTGAATTCAGCAGCAGCTTCTACCTCCATAC R- AGTCGTAAGCTTGTACATCGTCATTCGTCACGTAC	937
2	SbDof19	F-AGTCGTCCCGGGGAGGCGATTGTGTCATCCCCC R-AGTCGTCCCGGGTTACCCCTGCTGCCTCTTGTTG	966
3	SbDof23	F-AGTCGTCCCGGGGTTTCCAAGAGAAGAGAAGCCTAG R- AGTCGTCCCGGGTCAAGATCCCTCCTGGAAGGTCAA	2226
4	SbDof24	F- AGTCGTCCCGGGATGGTGTCATCCCCCAATATTAAG R- AGTCGTCCCGGGTTAAATTGTTTGCAGTAGCAACAC	1043

Note: The restriction sites were underlined.

3.2.6 PCR amplification of Dof domain and *Dof* genes from different cereals and millets

3.2.6.1 Basic requirements for PCR

I. Oligonucleotide primer

The Dof domain and *Dof* genes specific primers were commercially synthesized by Life Technology and Sigma Aldrich. Primers were supplied in lyophilized form and were dissolved in 1X TE buffer as recommended by the company to obtain the final concentration of 100µM primer stock. Stock solution was then diluted 100 times to achieve a final primer concentration of about 6ng/µl. This served as a working solution for preparation of reaction mixture.

II. Template DNA

50 ng of template DNA was used in each PCR reaction.

III. *Taq* DNA polymerase

Taq DNA polymerase was used at a final concentration of 1 unit per PCR reaction.

IV. dNTPs

1.0 µl of dNTPs mix (10mM) was used in the reaction mixture.

V. 10 X *Taq* buffer

10X assay buffer was used to the final concentration of 1X in each reaction mixture with 1.5mM $MgCl_2$.

3.2.6.2 PCR reaction set up

PCR reaction set for 25 µl reaction is shown in **Table 3.6**. In first step all the PCR tubes are labelled properly, and then sterile water followed by 2.5 µl 10X *Taq* buffer were added. A master mix of primers (F-R) and dNTPs were prepared to reduce pipetting error. The master mix was then redistributed in each PCR tube and 50 ng of template DNA was added in each tube. Finally *Taq* DNA polymerase was added in each tube. The contents were gently mixed by centrifugation for one minute.

Table 3.6 Concentrations of reaction mixture in PCR amplification

Components	**Final concentration**
DNA template	50 ng
dNTPs mix (10mM mix)	10 mM
Taq DNA polymerase (5U/µl)	1 U
Taq buffer (10 X)	1 X

Forward primer (F)	30 ng
Reverse primer (R)	30 ng
Sterile water (autoclaved)	According to primer and template concentration.

3.2.6.3 Cycling conditions

The PCR reaction set up were used in Eppendorf thermocycler for carrying out amplifications under following defined cycling conditions.

Step 1 (Initial denaturation): 95ºC for 5 min

Step 2 (Denaturation): 95ºC for 1 min

Step 3 (Annealing): Temp based on Tm value of primer for 1 min

Step 4 (Extension): 72ºC, time based on product size (1 min for 1kbp fragment)

Step 5 Go to step 2 for 30 more times

Step 6 (Final extension): 72ºC for 10 min

3.2.6.4 Analysis of PCR amplicons using agarose gel electrophoresis

PCR amplified DNA fragments were resolved on 1.5% agarose gel by standard electrophoresis technique as described in section 3.2.5.

3.2.6.5 Scoring of amplification data points and construction of a dendrogram

The PCR products amplified using Dof domain and gene-specific primers were scored for the presence (1) or absence (0) of bands of various sizes across the twelve crops to generate a binary matrix. Data were analyzed using the NTSYS-pc version 2.11w software to calculate the similarity values and generate the phenogram **(Rohlf, 2001)**. The SIMQUAL program was used to calculate the Jaccard's coefficients. Jaccard's similarity coefficient was utilized for estimating the pairwise similarity between the operational taxonomic units (OTUs) using the following formula **(Jaccard, 1908)**.

$$\text{Jaccard's coefficient} = N_{AB} / (N_{AB} + N_A + N_B)$$

Where,

N_{AB} is the number of bands shared by samples,

N_A represents amplified fragments in sample A, and

N_B represents fragments in sample B

Similarity matrices were utilized to construct the UPGMA (unweighted pair-group method with arithmetic average) dendrogram, clustering was performed by sequential

agglomerative hierarchical nested clustering, a distance based method, where series of successive mergers were used to group individuals with similar characteristics. To determine robustness of the dendrogram, the data were bootstrapped with 1000 replications along with Jaccard's coefficient by the computer programme WINBOOT **(Yap & Nelson, 1996)**.

3.2.6.6 Gel elution of PCR Product

QIAquick gel extraction kit was used for extraction and purification of PCR amplicons from agarose gel. The protocol provided with the kit was followed for gel extraction.

Protocol:

i. DNA fragment was excised from the agarose gel with a clean and sharp scalpel.
ii. Gel slice was weighed in a micro centrifuge tube and 3 volumes of QG buffer were added to 1 volume of gel (100 mg ~ 300 µl).
iii. The tube was then incubated at 50°C until the gel slice has completely dissolved
iv. One gel volume of isopropanol was added to the sample and mixed.
v. A QIAquick spin column was placed in a provided 2 ml collection tube.
vi. To bind DNA, the sample was applied to the column, and centrifuged for 1 min at 13,000 rpm
vii. The flow-through was discarded and the column placed back in the same collection tube.
viii. Washed the column with 0.75 ml of PE buffer (wash buffer).
ix. The flow-through was discarded and the column was centrifuged at 13,000 rpm for an additional 1 min to completely remove residual ethanol from column.
x. To elute DNA, column was placed into a fresh 1.5 ml micro centrifuge tube and 30-50 µl of elution buffer (10 mM Tris.Cl, pH 8.5) was added to the centre of the QIAquick membrane, the column was left for 2 min and centrifuged for 1 min at 13,000 rpm.
xi. The quality of the elution product was analyzed on 1.2 % agarose gel and quantified by Nanodrop instrument.

3.2.7 Cloning of gel eluted PCR products

3.2.7.1 Ligation of eluted PCR product in pGEM-T Easy vector

PCR amplified genes were cloned in pGEM-T Easy vector through TA cloning method (Promega). pGEM-T Easy vector is a convenient system for cloning PCR products. The vector was prepared by cutting pGEM-T Easy plasmid with *Eco*RI and adding 3'terminal

thymidin on both ends as shown in **Fig. 3.1**. This single 3'adenine overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmid by preventing re-circularization of vector and providing compatible overhangs for PCR products generated by certain thermostable polymerase. The ligation reaction was set up as described below. Ligation buffer (2X) was vortexed vigorously before each use. Tubes (0.5 ml) known to have low DNA binding capacity were used. To calculate the appropriate amount of PCR products (insert) to include in the ligation reaction, the following equation was used:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{Kb size of insert}}{\text{Kb size of vector}} \times \text{insert:vector molar ratio}$$

Reagents	**Standard reaction**	**Positive control**	**Background control**
2X rapid ligation buffer	5 µl	5 µl	5 µl
pGEM-T Easy vector (50 ng)	1 µl	1 µl	1 µl
PCR product	2 µl	-	-
Control insert DNA	-	2 µl	-
T4ligase (2 unit)	1 µl	1 µl	1 µl
Deionized water to final volume of	10 µl	10 µl	10 µl

The contents of reaction were mixed by pipetting and incubated overnight at 4°C for the maximum number of transformants.

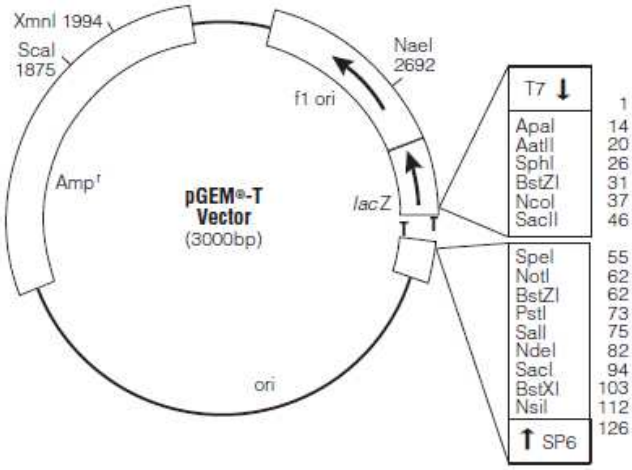

Fig. 3.1 pGEM-T Easy vector map (Source: Promega technical manual).

3.2.7.2 Ligation of eluted PCR product in pBSK vector

The gel eluted and quantified PCR products were cloned at multiple cloning sites (MCS) of pBSK vector (**Fig. 3.2**). The MCS region of pBSK plasmid was digested with suitable restriction enzymes followed by calf intestinal phosphatase (CIP) treatment to prevent self ligation of digested plasmid. The eluted products were also digested with suitable restriction enzymes, purified and were ligated at MCS region of pBSK cloning vector. Ligated products were then transformed in *E. coli* host (DH5α) using electroporation method.

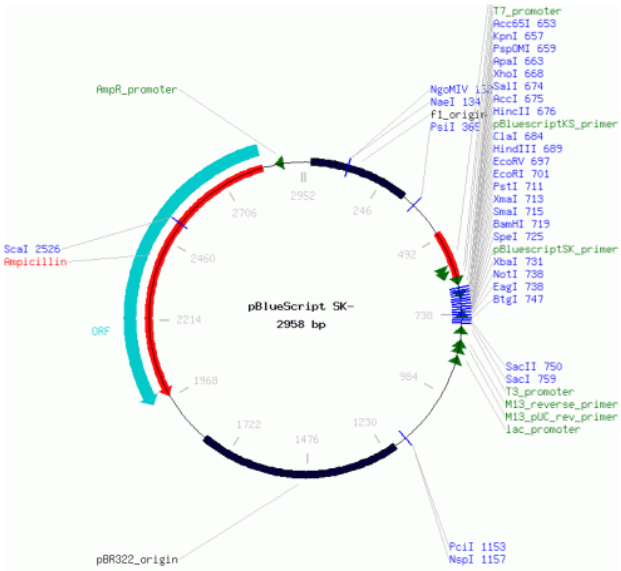

Fig. 3.2 pBlueScript SK (-) vector map (Source: Lab life vector database).

3.2.7.2.1 Restriction digestion

Restriction digestion reaction was performed in suitable 10X NEB buffer using different restriction enzymes of New England BioLabs (as per the company's instructions). 10X bovine serum albumin (BSA) was added in reaction for stabilizing enzyme and incubated the reaction mixture for 2 hours at 37^{o}C.

3.2.7.2.2 Dephosphorylation of vector

Phosphatase treatment was given to vector DNA molecule after digestion with restriction enzymes to decrease the frequency of self ligation and hence the vector background, by dephosphorylating its 5' ends. The digested vector was treated with CIP in suitable NEB buffer of New England BioLabs. The reaction sample was incubated at 37ºC for 1 hour (for blunt end ligation, the sample was incubated at 37ºC for 1 h and then at 55ºC for another 1 h).

3.2.7.2.3 Ligation reactions

For ligation, T4 DNA ligase was used with 1X T4 DNA ligase reaction buffer (50mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25 µg/ml BSA) of New England BioLabs. Relative vector: insert ratio was kept 1:3 to 1:5 for ligation. If the absolute concentration of vector is 100 ng, then insert concentration can be kept 200-300 ng for ligation. Ligation mixture was incubated at 16^{o}C for 16 h in a water bath. The sample was stored at -20ºC or continued with transformation.

3.2.7.3 Transformation of ligated product in chemically competent *E. coli* host cells (DH5α strain)

The $CaCl_2$ mediated transformation was performed as per the standard protocol **(Hanahan, 1983)** with slight modifications. Following stock solutions were prepared.

Stock Solutions:

I. Luria Bertani (LB) Medium

For 1000 ml

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

All the components were dissolved in 950ml of distilled water. pH was adjusted to 7.0 with NaOH and final volume was made up to 1 litre. Autoclaved and stored at RT.

II. LB agar

It comprised of LB medium with 20 g of agar per 1000 ml. Autoclaved and stored at RT.

III. 20 % Glucose solution

For 100 ml solution

Glucose	20 g

Dissolved in 80 ml of distilled water and the final volume was made up to 100 ml with distilled water, filter sterilized and stored at 4ºC.

IV. SOB medium

For 50 ml

Tryptone	1 g
Yeast extract	0.25 g
NaCl	0.025 g

The components were dissolved in 49 ml of distilled water and autoclaved. 0.5 ml each of 1 M $MgSO_4$ and $MgCl_2$ were added prior to use.

V. 0.1M $CaCl_2$

For 100 ml solution

$CaCl_2$. 6 H_2O	2.70 g

It was dissolved in 80 ml of distilled water and the final volume was made up to 100 ml with distilled water. Autoclaved, aliquoted and stored at -20ºC.

VI. 1M $MgCl_2$

For 10 ml solution

$MgCl_2$. $6H_2O$	2.03 g

Dissolved in 8 ml of distilled water and final volume was made up to 10 ml. Aliquoted, autoclaved and stored at 4°C.

VII. 1M $MgSO_4$

For 10 ml Solution

$MgSO_4$	1.204 g

Dissolved in 8 ml of distilled water and final volume was made up to 10 ml. Aliquoted, autoclaved and stored at 4ºC.

VIII. Ampicillin Stock (50 mg/ml)

For 10 ml

Ampicillin hydrochloride	500 mg

Sterile distilled water	6 ml

The final volume was made up to 10 ml and filter sterilized with 0.22 μm filters. It was then aliquoted and stored at -20ºC.

IX. SOC medium (Freshly prepared)

For	100 ml	
	SOB medium	98 ml
	Glucose 20 % (w/v)	2 ml

Procedures:

Preparation of chemical competent cells

i. Inoculated single colony of host *E. coli* (DH5α strain) from the fresh plate in 5 ml of LB medium and incubated overnight at 37°C with shaking (200 rpm).
ii. The overnight grown culture was inoculated at 0.05 OD in 50 ml of LB media.
iii. Culture was incubated at 37°C with vigorous shaking for 3-4 h till it reached at $O.D_{600}$ 0.4 to 0.5.
iv. Culture was transferred aseptically in pre-cooled Oak ridge tube and kept on ice for 10-15 minutes.
v. Cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C.
vi. Supernatant was discarded completely and the pellet was resuspended in 5 ml of 0.1 M chilled $CaCl_2$ and kept on ice for 30 minutes.
vii. Cells were recovered again by centrifugation at 4000 rpm for 10 minute at 4°C.
viii. Supernatant was removed completely and cells were resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$.
ix. It was aliquoted (200 μl per tube) and stored at -20ºC until use.

Transformation procedure

i. The chemically competent cells were thawed on ice and 10μl of ligation mix was added.
ii. Contents were mixed and kept on ice for 10 min.
iii. A heat shock was given at 42°C for exactly 90 seconds in a circulating water bath and tubes were immediately transferred onto ice for 2 min.
iv. Then 200 μl of SOC medium was added to each tube, mixed gently and incubated at 37°C with shaking for 45 min.

v. The transformation mixture was then plated onto LB Agar plates with ampicillin (50μg/ml final concentration).

vi. The plates were incubated at 37°C overnight to obtain transformants**.**

3.2.7.4 Transformation of ligation mixture in electro-competent *E. coli* host cells (DH5α strain)

The electroporation method of transformation was performed as per the standard protocol as described in **(Sambrook & Russell, 2001)**. Following stock solutions were prepared.

Stock Solutions

I. SOB

For 1000 ml

Bactotryptone	20 g
Bacto yeast extracts	0.5 g
NaCl	0.5 g

Above-mentioned components were dissolved in 950ml of water. 10 ml of 250 mM KCl was added and pH was adjusted to 7.0 with 5N NaOH, volume was made up to 995 ml and autoclaved. Just before use, 5ml of filter sterilized 2M $MgCl_2$ was added.

II. SOC

SOB + 20 mM Glucose

III. 1mM HEPES buffer

For 1000 ml

HEPES	238.3 mg

Dissolved in 1000 ml of distilled water, autoclaved and stored at RT.

IV. 10% Glycerol

For 100 ml

Glycerol	10 ml
Distilled water	90 ml

Autoclaved and stored at RT.

Procedure:

Preparation of electro-competent cells

i. Inoculated single colony of host *E. coli* DH5α strain from the fresh plate in 5 ml of LB medium and incubated overnight at 37°C with shaking.

ii. The overnight grown culture was inoculated at 0.05 O.D in 500 ml of LB broth.
iii. Culture was incubated at 37°C with vigorous shaking for 3-4 h till it reached at $O.D._{600}$ 0.4 to 0.5.
iv. Kept the culture on ice for 30 min.
v. Culture was transferred aseptically in pre-cooled Oak ridge tube and cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C.
vi. Washed the harvested cells twice with HEPES buffer, followed by a single wash with 10% glycerol.
vii. Resuspended the cells in 20 ml of 10% glycerol and incubated at 4ºC for 30 min.
viii. Harvested the cells and resuspended the pellet in 200 µl of 10% glycerol.
ix. Aliquoted (50µl per tube) the cell suspension in prechilled micro centrifuge tubes and stored at -80ºC.

Transformation procedure

In electroporation method, electro-competent cells were first thawed on ice. Ligation mixture was added (1 µl), pipette mixed and transferred to pre-chilled electro cuvettes. A single pulse was given using Micropulser (BIORAD) as per company's instructions. LB broth was immediately added to the cell suspension and incubated at 37ºC in shaker for 45 minutes to 1 hour. The cells were plated on LB plates containing ampicillin (50µg/ml) and incubated at 37ºC for 12 to 16 hours.

3.2.7.5 Screening of recombinant *E. coli* clones

Recombinant clones were screened by restriction digestion method as described by **(Sambrook & Russell, 2001)**. Few bacterial transformants were picked up from the selection plate, inoculated in 5ml of LB medium containing ampicillin (50 µg/ml) and incubated at 37^{o}C with shaking (200 rpm) for 14-16 h. Plasmid was isolated from the culture and then digested with suitable restriction enzyme(s).

3.2.7.5.1 Minipreparation of plasmid DNA from transformed colonies

Minipreparation of plasmid DNA was performed by alkali lysis method **(Sambrook & Russell, 2001)**. Following stock solutions were prepared.

Stock solutions

I. Solution I

Glucose	50 mM
Tris base (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

The solution was autoclaved and stored at 4ºC.

II. Solution II (Freshly prepared)

NaOH	0.2 N (freshly diluted from 10 N stock solution)
SDS	1.0 % (diluted from 10 % stock solution)

III. Solution III

For 100 ml

5M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

The solution was autoclaved and stored at 4ºC

Procedure

i. A single bacterial colony was inoculated in to 3 ml LB medium containing ampicillin (50µg/ml) and incubated overnight at 37ºC with shaking (200 rpm).

ii. 1.5 ml of culture was poured in microfuge tube and centrifuged at 6,000 rpm for 5 min at 4ºC.

iii. The pellet was dried and resuspended in 100 µl of ice-cold solution I by vigorous vortexing. The tubes were stored in ice for 5 min.

iv. Freshly prepared solution II (200 µl) was added and mixed by inverting the tubes rapidly five times. The tubes were stored in ice for 2 min.

v. 150 µl of ice-cold solution III was added and mixed by inverting tubes 3-5 times.

vi. The tubes were centrifuged at 10,000 rpm for 10 min.

vii. Supernatant was transferred in fresh microcentrifuge tubes, 0.7 volume of ice-cold isopropanol was added and incubated for 4 h at -20ºC.

viii. Microcentrifuge tubes were then centrifuged at 10000 rpm for 15 min.

ix. Supernatant was decanted and pellet was washed with 70% ethanol, air-dried and dissolved in 50 µl TE buffer.

x. The presence of plasmid DNA was analyzed by agarose gel electrophoresis.

Purification

The procedure as described in section 3.2.2.2 was followed.

3.2.7.5.2 Digestion of Plasmid DNA

The plasmid DNA of transformants was digested with suitable restriction enzyme(s) in 10X NEB buffer according to the company's instructions. The digestion profile was studied by running the reaction sample in 1.2% agarose gel in parallel with a negative control (undigested vector) and a positive control (vector digested with the same restriction enzymes) along with standard DNA molecular weight marker.

3.2.8 Sequencing reaction

Putative cloned *Dof* genes and domain were sequenced with M13 universal primers present on pBSK/pGEM-T Easy vector using Applied Biosystems automated sequencer and some of PCR products were directly sequenced using *Dof* domain/gene specific primers (at DNA sequencing facility provided by National Institute of Plant Genome Research (NIPGR), New Delhi and Institute of Microbial Technology (IMTECH), Chandigarh, India). The template DNAs were prepared using plasmid mini-prep kit from Qiagen (as per company instruction). DNA sequencing was done by using ABI Big Dye v3.1 Cycle sequencing machine and the sequencing reaction was carried out as per the manufacturer's instructions.

3.2.8.1 Sequencing PCR

For each reaction the following reagents (**Table 3.7**) were added in separate tube.

Table 3.7 Concentrations of reaction mixture in sequencing PCR

Reagent	**Quantity**
Terminator Ready Reaction Mix	1 µl
Sequencing Buffer (5X) (200 mM Tris-Cl, 5mM $MgCl_2$, pH 9.0)	1.5 µl
Double stranded DNA (Plasmid) PCR product DNA	150 to 300 ng 1 to 50 ng (1-2ng/100 bases
Primer	3.2 pmol*
Sterile water	According to reaction volume
Total volume	10 µl

* Final conc. of primer is 320 nM.

The contents were mixed well, spun briefly and amplifications were carried out by using Eppendorf thermocycler, as per following cycling conditions.

Step 1: 96ºC for 1 min

Step 2: 96ºC for 10 sec

Step 3: 50ºC for 05 sec

Step 4: 60ºC for 4 min

Step 5: Go to step 2 for 24 more times

Step 6: 4ºC hold

3.2.8.2 Post-reaction clean up

Post-reaction clean up involves the removal of the unincorporated dye terminators from the sequencing reaction. Following step were involved in this process:

i. Added 2 µl of 125 mM EDTA to each reaction tube containing 10µl of reaction.
ii. Added 50 µl of 95 % ethanol to each tube and mixed the contents thoroughly. Incubated at RT for 15 min.
iii. Centrifuged at a speed of 12,000 rpm for 20 min at RT.
iv. Decanted the supernatant (immediately and completely) and added 250 µl of 70 % ethanol to each tube.
v. Centrifuged the tubes again at 12,000 rpm for 10 min and decanted the supernatant (swiftly and completely).
vi. Repeated the 70 % ethanol wash (after adding 70 % ethanol vortexed briefly and then centrifuged).
vii. Aspirated or decanted the supernatant (swiftly and completely) and air-dried the pellet. The dried pellet was stored at -20°C for several weeks until required.

3.2.8.3 Reaction resuspension

i. Added 10 to 12 µl of HiDi Formamide to the dried pellet.
ii. Heated at 95°C for 3 min and immediately chilled on ice for 5 min.
iii. Briefly spun the tube, and transferred the contents to 96 well reaction plates.
iv. Proceeded for capillary electrophoresis (CE).

3.2.9 *In silico* analysis of sequenced Dof domain and *Dof* genes

The chromatogram files were analyzed using the software FinchTV v.1.4.0 (**http://www.geospiza.com/Products/finchtv.shtml**) and DNA sequences were retrieved in FASTA format for further analysis.

Various online and offline Bioinformatics tools and softwares were used for analysis of sequenced products. The sequenced products were subjected to homology search with both general and transcription factor specific databases using BLASTN, TBLASTX and discontinuous MEGABLAST **(Altschul *et al.*, 1990; Altschul *et al.*, 1997)**.

The sequences were further subjected to bioinformatics softwares namely GENESCAN **(Stormo, 2000)** and FGENESH **(Solovyev *et al.*, 2006)**for fishing out the probable genes and the putative coding sequence (CDS) and were translated to protein sequence using translation tool (**http://ca.expasy.org/tools/dna.html**). The translated Dof protein sequences were subjected to protein functional analysis using PFAM version 23.0 **(Finn *et al.*, 2006)**, PROSITE version 20.37 **(de Castro *et al.*, 2006)** and InterProScan version 4.4 **(Quevillon *et al.*, 2005)**.

3.2.10 Multiple sequence alignment and phylogenetic analysis

The sequences showing maximum similarity in BLAST analysis were retrieved and aligned with the sequences of Dof proteins from different cereals and millets using ClustalW **(Thompson *et al.*, 1994)** and ClustalX2.0.10 **(Thompson *et al.*, 1997).** Based on aligned protein sequences, phylogenetic tree were constructed using UPGMA and Neighbor-Joining method **(Saitou & Nei, 1987)**. A tree was inferred by Bootstrap phylogenetic inference using ClustalX2.0.10 and MEGA4.1 **(Tamura *et al.*, 2007)**.

3.2.11 Motif identification

The conserved motifs present in *Dof* domain and gene sequences were analyzed using BLOCKS and MEME (Multiple EM for Motif Elicitation) tool version 3.5.7 **(Bailey & Gribskov, 1998; Bailey *et al.*, 2006)**. For motif analysis of Dof domain, the selection of maximum number of motifs was set to 5 with minimum width of 15 amino acids while for *Dof* gene analysis maximum number of motifs was set to 10 with minimum width of 15 amino acid residues while other factors were of default selections. These selections were made in order to minimize the 'E-value' of the given parameter based on the probability of finding an equally well conserved pattern in set of sequences. Motifs involved with regulatory region of putative *Dof* genes and domain were analysed using PLACE **(Higo *et al.*, 1999)**.

The protein sequences of the 28 *SbDof* genes of sorghum along with 36 AtDof proteins of Arabidopsis and 30 OsDof proteins of rice were also analyzed by means of the MEME tool version 4.4.0 **(Bailey & Gribskov, 1998; Bailey *et al.*, 2006)** for motif analysis.

To identify conserved motifs in these sequences, the selection of maximum number of motif was set to 50 with minimum and maximum width of 20 and 50 amino acids respectively while other factors were of default selections.

3.2.12 *In silico* prediction of *Dof* gene family members in *S. bicolor* (L) Moench

The Dof sequences of *O. sativa* and *A. thaliana* were collected from two different sources, DRTF **(Gao *et al.*, 2006)** and DATF **(Guo *et al.*, 2005)** databases. The nucleotide sequences of *Dof* domain were used to search the potential *Dof* domain homologs hit in the whole genome shotgun sequence of *S. bicolor* through BLAST search at the NCBI database **(Pruitt *et al.*, 2007)**. The 3 kb upstream and 3 kb downstream sequences of *Dof* domain homologs were retrieved from whole genome shotgun sequence of *S. bicolor* for fishing out the putative *SbDof* genes.

The annotated sequences were further subjected to FGENESH **(Solovyev *et al.*, 2006)** server for prediction of full-length genes with putative CDS and protein sequences. The putative Dof protein sequences of *S. bicolor* were subjected to protein functional analysis using PFAM version 24.0 **(Finn *et al.*, 2006)**, PROSITE version 20.58 **(de Castro *et al.*, 2006)**, InterProScan version 24 **(Quevillon *et al.*, 2005)**, and MOTIFSCAN **(Falquet *et al.*, 2002)** databases. The Dof protein sequences were then subjected to NUCPRED server **(Brameier *et al.*, 2007)** for identification of nuclear localization signal (NLS).

3.2.12.1 Mapping of *SbDof* genes on sorghum chromosomes and its intron/exon gene structure prediction

Each of the *SbDof* genes was positioned on sorghum chromosomes by the BLASTN search with NCBI genomes (chromosome) database. The resulting position of *SbDof* genes on sorghum chromosome accession numbers CM000760 - CM000769 were manually marked on bar. The gene structures of predicted *SbDof* genes were carried out using FGENESH server and intron/exon structures were manually designed.

3.2.12.2 *Cis*-regulatory element analysis

For promoter analysis 500 bp upstream sequences from the initiation codon of the putative *SbDof* genes were retrieved. The retrieved sequences were then subjected to search for CARE program (**http://bioinformatics.psb.ugent.be/webtools/plantcare/html/**) of PlantCARE database **(Lescot *et al.*, 2002)** for identification of *cis*-regulatory elements.

3.2.13 Three dimensional structure prediction, refinements and evaluation of Dof proteins

It was ascertained that the three dimensional structure (3D) of Dof protein is not available in PDB (**http://www.rcsb.org/**) database, hence an attempt has been made in the present study to determine the structure of four cloned SbDof genes from sorghum. The three dimensional structure of SbDof protein and Dof domain has been predicted using iterative threading assembly refinement algorithm (I-TASSER) Standalone Package (Version 1.1). I-TASSER server predicts protein structure based on multiple-threading alignments by LOMETS and iterative TASSER assembly simulations **(Roy *et al.*, 2010)**. The rough model generated was subjected to energy minimization using the steepest descent technique to eliminate bad contacts between protein atoms. Computations were carried out in vacuo with the GROMOS96 43B1 parameters set, implemented through Swiss-PdbViewer version 4.0.1 (**http://expasy.org/spdbv/**). The final refined models were submitted to PMDB database (**http://www.caspur.it/PMDB/**).

3.2.13.1 Validations

The backbone conformations of the final models were inspected using the Phi/Psi Ramachandran plot obtained from PROCHECK server (**http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html**). The quality of SbDof proteins models were estimated using Qualitative Model Energy Analysis (QMEAN) server **(Benkert *et al.*, 2009)** (**http://swissmodel.expasy.org/qmean/cgi/index.cgi**).

3.2.13.2 Superposition of predicted structure and template (Dof domain PDB structure)

The structural superposition of Cα trace of the Dof domain (PM0076448) and predicted structure of SbDof proteins (PM0077395 to PM0077398) were performed using combinatorial extension (CE) of Polypeptides (**http://cl.sdsc.edu/**) and Swiss-PdbViewer.

3.2.13.3 Active site prediction and docking study

After obtaining the final model of Dof domain, the possible binding sites were searched using Q-SiteFinder tool (**http://bmbpcu36.leeds.ac.uk/qsitefinder/**). Metal detector studies of Dof domain was performed using metal detector V2.0 (**http://metaldetector.dsi.unifi.it/**). The interaction of zinc metal with Dof domain was analyzed by Molegro Virtual Docker Version 4.3.0 **(Thomsen & Christensen, 2006)**.