Genetic diversity of the genus *Curcuma* in Bangladesh and further biotechnological approaches for *in vitro* regeneration and long-term conservation of *C. longa* germplasm

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Dedicated to

my late parents
who are still alive in my heart

SUMMARY

The genus *Curcuma* is well known for its multivarious uses as spices, medicines, cosmetics, dyes, flavourings, starch, and ornamentals. Species that belong to the genus are currently being threatened due to high anthropogenic interference and habitat destruction. In this study, substantial research on genetic diversity, 2C DNA and genome size, chromosome analysis, *in vitro* regeneration and cryopresevation were conducted.

Randomly Amplified Polymorphic DNA (RAPD) was used to determine inter- and intra-specific genetic diversity. Estimated Shannon's Index values of genetic diversity were ranged from 0.018 ± 0.028 to 0.335 ± 0.117, which inferred that considerable amounts of genetic diversity still exist in some species while the rest of the species presented low genetic variability. Analysis of molecular variance (AMOVA) revealed significant partitioning (Φ_{CT} value 0.265, $P<0.003$) between the wild and cultivated species. A large cluster in the presented dendogram contained morphologically similar species that were found to be triploid (2n=63).

In case of *C. zedoaria*, high intrapopulational genetic diversity (0.717 ± 0.090) and low interpopulational diversity (0.283 ± 0.089) were estimated. The highest genetic variability was observed in the hilly population (Chittagong; 0.349 ± 0.128) and the lowest in the plateau lands (Birganj; 0.149 ± 1.04). Diversity values of the populations were positively correlated to the mean 2C DNA values. AMOVA results inferred that the zedoary populations are moderately partitioned into regional (Φ_{CT} value 0.153, $P<0.001$) and edaphic (Φ_{CT} value 0.142, $P<0.001$) levels.

Cytology and flow cytometry analyses presented various significant results that were not reported so far. Chromosomal investigations revealed that the basic chromosome number n = 21 is more frequent in the genus *Curcuma* with 2n = 42, 63 and 84. Flow cytometry data illustrated that *Curcuma* species covered a range of 2C DNA values and genome sizes ranged from 2.10 ± 0.018 – 5.30 ± 0.025 pg. These values were corresponding to different ploidy levels of diploid, triploid, and tetraploid.

Furthermore, a high frequency *in vitro* regeneration for *C. longa* was achieved. On an average 6.73 ± 0.48 shoots with 5.13 ± 0.31 roots were obtained within four weeks from a single explant of axillary buds. Almost 100% of the transferred plantlets survived and grew up to maturity. RAPD analyses of *in vitro* plants revealed that the *C. longa* var. Surma is likely to be genetically unstable. A proficient protocol for microrhizome induction was also established. A mean number of 8.3 ±0.32 microrhizomes were obtained from a single culture that can be transferred to the soil directly without acclimatisation.

Finally, an efficient cryopreservation system for *C. longa* was established for the first time through vitrification procedure. Under optimum freezing conditions about 80% of the meristems were found to be capable to recover and develop intact plants. The presented cryopreservation protocol seems to be promising for long-term conservation of *Curcuma* germplasm.

***Keywords*: *Curcuma* species, genetic diversity, RAPD, cytology, flow cytometry, *in vitro* regeneration and cryopreservation

ZUSAMMENFASSUNG

Die Gattung *Curcuma* ist aufgrund ihrer vielfältigen Verwendung als Gewürz, Arznei, Kosmetikum, Färbemittel, Aromastoff, Stärke und Zierpflanze sehr bekannt. Die Arten dieser Gattung sind jedoch in neuerer Zeit immer stärker durch den Eingriff des Menschen und die Zerstörung ihres natürlichen Lebensraumes bedroht. In der vorliegenden Studie wurden umfangreiche Untersuchungen zur genetischen Diversität, 2C DNA, Genomgröße, Chromosomenzusammensetzung, *in vitro* Regeneration und Kryo-konservierung durchgeführt.

Zur Bestimmung der inter- und intraspezifischen genetischen Diversität wurde die Methode der Randomly Amplified Polymorphic DNA (RAPD) verwendet. Der berechnete Shannon-Index der genetischen Diversität reicht von 0,335 ± 0,117 bis 0,018 ± 0,028. Dies zeigt, dass in einigen Spezies eine beträchtliche genetische Diversität, in wenigen anderen aber auch nur eine sehr geringe genetische Variabilität besteht. Die Analyse der molekularen Varianz (AMOVA) ergab, dass die kultivierten Spezies und der Wildtyp signifikant voneinander getrennt sind ($\mathbf{\Phi}_{CT}$ Wert 0,265, P<0,003). Ein großes Cluster im vorgestellte Dendogramm umfasst morphologisch ähnliche Spezies, die alle triploid sind (2n=63).

Im Fall von *C. zedoria* wurde eine hohe genetische Diversität innerhalb der Population (0,717 ± 0,090) und eine vergleichsweise geringe zwischen den Populationen (0,283 ± 0,089) gemessen. Die höchste genetische Variabilität wurde für die Hügel-Population (Chittagong; 0,349 ± 0,128), die niedrigste für die Population der Hochebenen (Birganj; 0,149 ± 1,04) bestimmt. Es hat sich gezeigt, dass die Werte der Diversität der Populationen positiv mit dem mittleren 2C DNA-Wert korreliert sind. Die AMOVA-Ergebnisse zeigten für die *Zedora*-Population eine mäßige Trennung in regionale ($\mathbf{\Phi}_{CT}$ Wert 0,153, P<0,001) und edaphische Level ($\mathbf{\Phi}_{CT}$ Wert 0,142, P<0,001).

Zytologische und durchflusszytometrische Untersuchungen ergaben verschiedene wichtige Ergebnisse, die bisher noch nicht beschrieben sind. Chromosomale Analysen zeigten, dass die Gattung oft ein Vielfaches des haploiden Satzes (n = 21) aufweist 2n = 42, 63 und 84. Durchflusscytometrische Daten wiederum ergaben eine Überdeckung eines 2C DNA-Bereichs verschiedener *Curcuma*-Spezies und eine Schwankung der Genomgröße von 2,10 ± 0,018 bis 5,30 ± 0,025 pg. Diese Werte entsprechen den verschiedenen ploidie-Ebenen von diploid, triploid und tetraploid.

Darüber hinaus wurde bei *in vitro*-Regenerationsversuchen von *C. longa* eine hohe Regenerationszahl erreicht. Aus dem Explantat eines Achselsprosses konnten im Durchschnitt 6,73 ± 0,48 Sprosse und 5,13 ± 0,31 Wurzeln gezogen werden. Fast 100 % der Pflänzchen überlebten und wuchsen bis zu ganzen Pflänzen. RAPD-Analysen der *in vitro*-Pflanzen ergaben jedoch, dass die Varietät *C. longa* var. Surma wahrscheinlich genetisch instabil ist. Auch wurde ein geeignetes Protokoll zur Induktion von Mikrorhizomen etabliert. Im Mittel konnten mit dieser Methode 8,3 ± 0,32 Mikrorhizome aus einer einzelnen Kultur erhalten werden, die ohne Akklimatisierung direkt in Erde gepflanzt werden konnten.

Schließlich wurde für *C. longa* zum ersten Mal ein effizientes Kryokonservierungs-System mit Hilfe der Vitrifikations-Methode etabliert. Unter optimalen Gefrierbedingungen sind 80 % der Meristeme nach der Konservierung in der Lage sich wieder zu erholen und intakte Pflanzen zu bilden. Daher bietet dieses vorgestellte Protokoll eine viel versprechende Möglichkeit zur Langzeit-Konservierung von *Curcuma* Germplasmen.

Stichwörter: *Curcuma*-Spezies, genetische Diversität, RAPD, Zytologie, Durchflusszytometrie, *in vitro*-Regeneration und Kryokonservierung.

LIST OF ABBREVIATIONS

μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromole
2D	Two dimension
2ip	6-γ,γ-dimethylallylaminopurine
2n	Diploid
3D	Three dimension
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
BA	6-Benzylaminopurine
BA-R	6-Benzylaminopurine riboside
Bi	Birganj population
bp	Base pair
BSA	Bovine Serum Albumin
Ca	Calcium
Ch	Chittagong population
cm	Centimeter
CTAB	Hexadecyltrimethyl ammonium bromide
DAPI	4'6-diamidino-2-phenylindole
ddH_2O	Double distilled water
Dh	Dhaka population
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
F_{ST}	Write´s F- statistics
g	Gram
G_{ST}	G-statistics (genetic subdivision among population)
h	Hour
H´	Shannon information index
HCl	Hydrochloric acid
H_E	Expected heterozygosity
$HgCl_2$	Mercuric chloride
H_S	Heterozygosity within population
H_T	Heterozygosity of total sample
IAA	Indole acetic acidI
IBA	Indole butyric acid,
IPK	Institut für Pflanzengenetik und Kulturpflanzenforschung
ISSR	Intersimple sequence repeat
ITS2	Internal transcribed spacer 2
JA	Jasmonic acid
kg	Kilogram
km	Kilometer
Kn	Kinetin
Kn-R	Kinetin-riboside
l	Liter
LN	Liquid nitrogen
m	Meter
*mat*K	Maturase-encoding gene located in intron of chloroplast *trn*K gene
Mbp	Mega base pair
MEGA	Molecular evolutionary genetic analysis
mg	Milligram
Mg	Magnesium
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimole
MS	Murashige and Skoog
m-T	Metatopoline
NAA	1- Napthalene acetic acid
ng	Nanogram
NJ	Neighbour joining
*N*m	Number of migrants
NTSYS	Numerical taxonomy and systematics
P	Probability
PCoA	Principal coordinate analysis
PCOORDA	Principal coordinate analysis
PCR	Polymerase chain reaction
pg	Picogram
PGR	Plant growth regulator
PI	Propidium iodide
pop	Population
POPGEN	Population genetics
PVP	Polyvinylpyrrolidone
PVS	Plant vitrification solution
RAPD	Random amplified fragment length polymorphism
rDNA	Ribosomal DNA
RNase	RNA degrading enzyme
rpm	Round per minute
rRNA	Ribosomal RNA
s	Second
SD	Standard deviation
SE	Standard error
SHAN	Sequential, hierarchical, agglomerative and nested
Si	Sitakundu population
SIMQUAL	Similarity of qualitative data
Sp	Species
Sr	Srimangal population
TAE	Tris-acetate-EDTA buffer
TAQ	*Thermophile aquaticus*
TDZ	Thidiazuron
*trn*K	Chloroplast gene for tRNALys
UPGMA	Unweighted pair group method of arithmetic mean
V	Volt
λ DNA	Lambda DNA
Φ_{ST}	Phi statistics
x	Base chromosome number

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1. GENERAL INTRODUCTION

1.1. The genus C*urcuma* L.

The genus *Curcuma* L. belongs to the family Zingiberaceae which is composed of about 70 - 80 species of rhizomatous annual or perennial herbs (Purseglove, 1974; Sirirugsa, 1999). The name *Curcuma* was coined by Linnaeus in his *Species Plantarum* in 1753. Probably the word derives from the Arabic word *'Kurkum'* that means yellow colour. This is due to the prominent yellow colour of the underground rhizome, a major valuable plant part that has been used traditionally from the time immemorial for mankind (Salvi et al., 2000; Shirgurkar et al., 2001). The *Curcuma* is taxonomically a complicated genus and new species are yet to be described. The genus has been divided into two subgenera of *Eucurcuma* and *Paracurcuma* by several taxonomists using different morphological traits. This division is still under question and scientists are till comfortable without this subdivision, hence the taxonomy of the genus is still problematic (Maknoi and Sirirugsa, 2002). Correspondingly, the family Zingiberaceae commonly known as ginger family is a unique plant family comprising of perennial aromatic forest plants. It is one of the economically important flowering plant families of the tropics yields spice, dyes, perfumes, medicines and ornamental flowers (Heywood, 1985). The genus *Curcuma* within the family Zingiberaceae has paramount importance as spice, medicines, dyes, cosmetics, starch and ornamentals.

Many species that belong to the genus *Curcuma* are well known for their significant commercial and medicinal values. One of the most important species is *C. longa* within the genus which yields turmeric, one of the important colouring and aromatic ingredients of curry powders that is enormously used in Asian cuisines (Apavatjrut et al., 1999; Purseglove, 1974) and pharmaceutical industries since it has been considered as an extremely important medicinal plant (Majeed et al., 1995). Among the *Curcuma* species, the chemical composition of *C. longa* has thoroughly been investigated and a number of different active substances have been identified (Yusuf et al., 2001), which exhibit a wide range of medicinal values. The finest Indian arrowroot is derived from *C. angustifolia* Roxb. (Das et al., 1999). In addition to that, many other ginger species are also rich with volatile oils, which are widely used as condiments, herbs, dyes and medicines. Among them the ginger (*Zingiber officinale* Rosc.) is also well known (Das et al., 1999). Three plant species of this family ginger (*Zingiber*

officinale Rosc.), turmeric (*Curcuma longa* L.) and cardamom (*Elettaria cardamomum* (L.) Maton) are the most important economic species produced in great quantities for international trade.

The species belonging to the genus *Curcuma* can be grown in diverse tropical conditions, from sea level to a height of 1500 m on the hilly slopes, in the temperature range of 20 to 30^0C. A rainfall of 150 cm or more or an equivalent amount of irrigation is essential for optimum growth and development of *Curcuma* species. Ideal soil requirements for *Curcuma* growing are loose, friable loamy or alluvial suitable for irrigation that should have efficient drainage capacity. The species are naturally found in mixed deciduous tropical forests and tropical broad-leaved evergreen forests of the tropical and subtropical regions. The geographic distribution of the genus reaches from India to Thailand, Indochina, Malaysia, Indonesia and finally to northern Australia (Apavatjrut et al., 1999). Major distribution of the genus *Curcuma* is stated in the Table 1.1. Along with ginger, *C. longa* was probably taken from India, south-east Asia, China and northern Australia to the West Indies and South America by the Spaniards. Subsequently, its cultivation spread over other countries. There is no available documented literature about the origin and distribution of African and South American *Curcuma* species. The members of the genus in these regions are important resources and have great potentials in terms of commercial values as source of spices, medicines and horticultural products (Apavatjrut et al., 1999; Cao et al., 2001; Cao and Komatsu, 2003; Joe et al., 2004; Maciel and Criley, 2003; Majeed et al., 1995; Paisooksantivatana et al., 2001a and 2001b; Purseglove, 1974; Sasaki et al., 2002; Sasaki et al., 2004; Yusuf et al., 2001).

Table 1.1. Distribution of the members of genus *Curcuma* worldwide

Geographic Area	*Curcuma* species (approx.)
Bangladesh	16-20
China	20-25
India	20-25
Indochina	20-25
Malaysia	20-30
Nepal	10-15
The Philippines	12-15
Thailand	30-40
World total	70-80

The members of *Curcuma* are commonly distributed in Bangladesh. The species are more frequent on the slope of the hilly areas spreading over south- to north-eastern part of the country and also scatteredly distributed throughout the Gangetic floodplain and Pleistocene plateau lands. The species thrive in a well-watered soil with plentiful rain and in light shade or in open areas of forest margins. Unfortunately, injudicious use together with habitat destruction has critically been threatening the species, especially in the densely populated non-hilly areas of plain and plateau lands where agricultural practices are very intensive. The decreasing number of natural populations and the rapid fragmentation of natural habitats have a severe impact on genetic diversity of the genus *Curcuma* as reported by Paisooksantivatana et al. (2001a). Yusuf (2000) described a total twenty species from Bangladesh. Among the species recorded from Bangladesh, ten species were described as new species, which were entirely based on morphological characters. It is assumed that some species are still unidentified. In addition to that in some cases the taxonomic identity of the species is confusing. Their potential uses, genetic diversity at species and population levels are also mostly unexplored. However, the taxonomic identity of the species is important to search and confirm the origins of different potential uses as herbal drugs (Cao et al., 2001; Sasaki et al., 2002). Extending to that the knowledge of genetic diversity will greatly help to utilize and conserve the *Curcuma* genetic resources of the country.

In broadly, the members of the family Zingiberaceae are mostly rhizomatous herbs, naturally occurred in the Indo-Malaysian subkingdom, comprise highly evolved monocotyledons with floral characters that appear to converge with those of orchids, though they are not homologous. They mostly grow in damp of humid shady places of forest floor. Some species can expose to the sun, and well adapted on high elevation. The member of Zingiberaceae distributed mostly in tropical and subtropical areas with the centre of distribution is in south-east Asia. The greatest concentration of genera and species is in the regions of Indonesia, Malaysia, Singapore, Brunei, Papua New Guinea, and the Philippines (Sirirugsa, 1999). The pantropical Zingiberaceae is the largest family in the order Zingiberales with about 53 genera and more than 1,200 species (Kress et al., 2002). The worldwide distribution of the family Zingiberaceae is presented in Table 1.2.

1.1.1. Morphology and taxonomy of the genus *Curcuma*

Morphologically the genus *Curcuma* is highly variable in different taxonomically important traits (Apavatjrut et al., 1999). The rhizomes of *Curcuma* are branched, fleshy and aromatic.

Roots often bear conical or ellipsoid tubers. Leaves are basal and the blade is broadly lanceolate or oblong or rarely linear and narrow. The genus can easily be recognised by its large compound spike inflorescence bearing prominent spiral bracts, which laterally fused to form pouches. Each pouch subtends a cincinnus of two to ten flowers that contain a single versatile anther. The terminal bracts form a sterile cluster called a 'coma', very long and often brightly coloured. It has two distinct flowering times as well as habits. Early flowering (April - May) species developed laterally from rhizomes before development of leafy shoots. Late flowering (August–September) species usually developed terminally from the leafy shoots (Sirirugsa, 1999). The plants vary from 50 – 200 cm in height. *Curcuma* species are mostly triploid and do not produce seeds. They reproduce asexually by means of rhizomes. Members of the genus are enriching the biodiversity of native forests in maintaining interesting as well as important assemblages of many rare and threatened insects through their colourful inflorescences. The identification of *Curcuma* has traditionally been achieved using morphological data. However, *Curcuma* species exhibit large morphological variations both intra- and inter species, but in some cases, especially early flowering group shows a very similar pattern of morphology between them which led to confusion in their identification (Apavatjrut et al., 1999).

Table 1.2. Distribution of the members of family Zingiberaceae worldwide

Geographic Area	Genera (approx.)	Species (approx.)
Bangladesh	15	80
China	21	200
India	18	120
Indochina	14	120
Malaysia	25	650
Nepal	11	35
The Philippines	15	103
Thailand	20	200
World total	52	1,500

The genus *Curcuma* has been placed in the tribe Zingibereae within the subfamily Zingiberoideae as proposed by Kress et al. (2002). However, according to the previous classification the genus belongs to the tribe Hedychieae as stated by Purseglove (1974). Classifications from the time of Roxburgh in 1812 to the recent advancement in this genus are largely based on morphological description. Recently a preliminary molecular research on the genus has been carried out in The Royal Botanic Garden Edinburgh, UK. This revealed that

the percentage of sequence divergence in the nuclear DNA internal transcribed spacer 2 (ITS2) among species of subgenus *Curcuma* was very low. The result also showed that *Roscoea* Sm., which was chosen as an out group is closer in terms of sequence similarity to subgenus *Paracurcuma* than to *Eucurcuma*. Subgeneric divisions of *Eucurcuma* and *Paracurcuma* have been accepted by several taxonomists but this division is still under argumentation. Apavatjrut et al. (1999) studied on isozyme polymorphism to identify some early flowering *Curcuma* species that includes only seven species. Chen et al. (1999) performed RAPD analysis to investigate genetic variations of two Chinese *Curcuma* species of *C. wenyujin* and *C. sichuanensis* and suggested that these two species are not genetically distinct and therefore should be combined into one species. To establish a rapid and simple molecular identification method for six medicinal *Curcuma* species of *C. longa, C. phaeocaulis, C. sichuanensis, C. chuanyujin, C. chuanhuangjiang,* and *C. chuanezhu* in Sichuan Province, the *trn*K nucleotide sequencing was used by Cao and Komatsu (2003) and they stated that the sequence data were potentially informative in the identification for these six *Curcuma* species at the DNA level. Molecular analysis of medicinally-used Chinese and Japanese *Curcuma* based on 18S rRNA gene and *trn*K gene sequences were used by Cao et al. (2001) and they stated that the molecular data can be used to confirm the *Curcuma* species and their derived drugs. Very recently Sasaki et al. (2004) investigated the single-nucleotide sequence of the *trn*K gene to identify *Curcuma* species of *C. longa*, *C. phaeocaulis*, *C. zedoaria*, and *C. aromatica*. Previously they showed that drugs derived from these species can be identified by using sequence data of *trn*K gene (Sasaki et al., 2002).

Similarly, the classification of the family Zingiberaceae first projected in 1889 and gradually developed afterwards. During that time four tribes were recognised such as Globbeae, Hedychieae, Alpinieae and Zingibereae based on morphological features such as number of locules and placentation in the ovary, development of staminodia, modifications of the fertile anther, and rhizome-shoot-leaf orientation. Phylogenetic analyses based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *matK* regions suggest that at least some of these morphological traits are homoplasious and three of the tribes are paraphyletic. The former Alpinieae and Hedychieae for the most part are monophyletic taxa with the Globbeae and Zingibereae included within the later. Kress et al. (2002) proposed a new classification of the Zingiberaceae on the basis of molecular phylogenetic investigation that recognizes four subfamilies and six tribes. Subfamilies are Siphonochiloideae (Siphonochileae), Tamijiodideae (Tamijieae), Alpinioideae (Alpinieae and Riedelieae) and

Zingiberoideae (Zingibereae and Globbeae). Morphological features are congruent with this newly proposed classification. The members of this family are chiefly related to bananas, Canna lilies and bird-of-paradise flowers.

1.1.2. Taxonomic hierarchy of the genus *Curcuma*

The taxonomic hierarchy of the genus *Curcuma* is presented in the following box.

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Zingiberidae
Order	Zingiberales
Family	Zingiberaceae
Subfamily	Zingiberoideae
Tribe	Zingibereae
Genus	*Curcuma*

1.1.3. Importance of the genus *Curcuma*

The significance of *Curcuma* in health and nutrition has greatly been recognised since the discovery of the antioxidant properties of naturally occurring phenolic compounds. The dried rhizome of *C. longa* L. has been found to be a rich source of beneficial phenolic compounds known as the curcuminoids (Lechtenberg et al., 2004; Srinivasan, 1953). The most important species *C. longa* is commercially known as turmeric plant. Turmeric is the processed underground rhizome used as spice, herbal medicines, dyeing agents and cosmetics since Vedic age (Salvi et al., 2000; Shirgurkar et al., 2001). Turmeric illustrates its clinical applications over time, which was partly overshadowed in the past by its common use as commercial dyestuff and ingredients of curries. Its medicinal values have long been recognised in traditional cultures of south east Asia. Most likely the recent development in medical research on turmeric is based on this traditional knowledge. The most important components of turmeric are curcuminoids, which refer to a group of phenolic compounds, which chemically related to its principal ingredient of curcumin. Three main curcuminoids

were isolated from turmeric are curcumin, demethoxycurcumin and bisdemethoxycurcumin (Jayaprakasha et al., 2002; Lechtenberg et al., 2004; Majeed et al., 1995). All of these components characteristically develop yellow pigmentation to turmeric rhizome while tetrahydrocurcuminoids is a colourless component derived from curcuminoids by hydrogenation. It is therefore used in achromatic foods and cosmetics (Majeed et al., 1995). A vast majority of the studies were carried out on curcumin, which is the major curcuminoid. The detailed studies using curcumin include anitoxidative, anti-inflammatory, anticarcinogenic, antiviral, and antiinfectious activities. In addition, very recently the wound healing and detoxifying properties of curcumin have also received considerable attention (Joe et al., 2004).

Among the *Curcuma* species, the chemical composition of *C. longa* has extensively been studied. A number of different biologically active substances have been identified by which demonstrate germicidal, aromatic, carminative, antihelmentic, antioxidant, anti-tumour, cholesterol lowering and neuroprotective activities (Cao et al., 2001; Cao and Komatsu, 2003; Jitoe et al., 1992; Joe et al., 2004; Kikuzaki and Nakatani, 1993; Majeed et al., 1995; Masuda et al., 1993; Sasaki et al., 2002; Sasaki et al., 2004; Purseglove, 1974). These drugs were originally used in traditional Asian medicines and in Chinese medicine for treating various syndromes due to obstruction of blood circulation and retention of blood stasis such as arthralgia, psychataxia, and dysmenorrhea (Cao et al., 2001; Sasaki et al., 2002). In Thai traditional medicine, turmeric is used as a carminative, for dyspepsia and also externally for itching and infected wounds (Saralamp et al., 1996). Pharmacological and clinical studies have indicated the effectiveness of turmeric for the treatment of dyspepsia, peptic ulcers (Prucksunand et al., 2001) and gastric ulcers (Masuda et al., 1993).

The finest Indian arrowroot derived from *C. angustifolia* Roxb. (Das et al., 1999) has been used as a source of starchy food for centuries. A number of *Curcuma* species have beautiful inflorescences and luxurious foliages that have an immense commercial value in floriculture as a versatile ornamental crop used as cut flower, pot and landscape plant (Maciel and Criley, 2003; Paisooksantivatana et al., 2001a and 2001b). Among them *C. alismatifolia* is recognized and popular in international trade as cut flower (Paisooksantivatana et al., 2001b). Some other species such as *C. aeruginosa, C. amada, C. angustifolia, C. caesia, C. elata, C. petiolata, C. rubescens, C. zanthorrhiza and C. zedoaria* have also received considerable attention as cut flowers and tropical glasshouse ornamentals.

Besides the wide medicinal and commercial utilities, turmeric is also used in Indian cuisine as a colouring and flavouring agent. Finely diced fresh rhizomes are often added as a punch to salads in many regions of India. *Curcuma* powder was greatly appreciated in distant past as a food additive in curries to improve storage conditions. *C. amada* rhizomes that smell of fresh green mango is also used as a flavouring spice in Asian cooking, besides producing a fine inflorescence in summer season. Zedoary oil derived from the rhizomes of *C. zedoaria* is used as a spice, tonic, and perfume. The terminal bracts form a sterile cluster called a coma, often brightly coloured and in the case of some species these coma bract are being rich in volatile oils that are also used to produce perfumes and cosmetics. Recently, leaf essential oils of *C. longa* and *C. aromatica* have been analysed by Behura et al. (2002). They found several important essential oils such as α-phellandrene, 1,8-cineole, C_8-aldehyde and Linalol. These essential oils are valuable for pharmaceutical as well as cosmetic industries.

1.1.4. Turmeric is one of the ancient spice and dye yielding plants

Turmeric have been started to be used since dates back nearly 4000 years, to the Vedic culture in India where it was used as a culinary spice and had some religious significance (Salvi et al., 2000; Shirgurkar et al., 2001). Since then turmeric was being established as a popular spice and gradually distributed throughout the East and Middle East as a condiment and culinary dye. In India, it has been used to colour many sweet dishes. It has also been used in many fish and meat curries, possibly because it successfully masks fleshy odours. Up till now, it is one of the main ingredients providing the associated yellow colour in different commercially available curry powders. Turmeric have also become of special importance to man with discovery that its powdered rhizomes when added to various food preparations preserved their freshness and nutritive value. Turmeric belongs to a group of aromatic spices that was originally used as a food additive in curries to improve storage conditions, palatability and presentation of food. Long before the time of cheaper synthetic food preservatives, spices like turmeric played a vital role as food additives and were valued more than gold and precious stones (Majeed et al., 1995). Consequently, the turmeric was highly esteemed by the ancient Indo-European people for its golden-yellow dye (Majeed et al., 1995; Srimal, 1997).

Turmeric has also been popular from distant past to the region of south-east Asia as dyes and condiments. It is cultivated principally in south-east Asia including Bangladesh, China, Java, India, Malaysia, Sri Lanka, Taiwan and Thailand. Some other countries such as Australia, Japan, Peru, West Indies and some regions of Africa also cultivate *Curcuma* to some extent.

The brilliant-yellow colour of turmeric, which is resistant to very high dilutions, established it in the way to commercial use as a colouring agent for a range of items including cotton, silk, paper, wood, foodstuffs and cosmetics. It is still used in customs of the Hindu religion as a holly dye. Turmeric is in fact one of the cheapest ancient spices that encompasses diverse commercial and medicinal values. As a dye it is used similarly to saffron, however, the culinary uses of the two spices should not be confused.

1.1.5. Chromosome research and polyploidy in *Curcuma*

A variety of facts of chromosomal research are gaining importance for analysis of genetic and chromosomal variations of different taxa (Das et al., 1999). Chromosome numbers and karyomorphology data are also excellent tools in studies that search taxonomic relationships and evolutionary patterns inside the groups (Joseph et al., 1999). The somatic chromosome numbers of *Curcuma* species comprising 2n = 20, 24, 28, 32, 34, 36, 42, 56, 62, 63 and 84 which were previously reported by different workers (Apavatjrut et al., 1996; Beltran and Kiew, 1984; Darlington and Wylie, 1955; Das et al., 1999; Eksomtramage et al., 1996 and 2002; Weerapakdee and Krasaechai, 1997). The variation in chromosome numbers in *Curcuma* demonstrates that this genus comprises both polyploidy and aneuploidy (Eksomtramage et al., 2002).

Diploid and haploid chromosome numbers from root tip and anther cells of 17 Thai *Curcuma* species were investigated by Apavatjrut et al. (1996) and reported a wide range of 2n chromosome numbers in *Curcuma* species which include 2n=42 (*C. roscoeana* Wall., *C. petiolata* Wall.), 2n = 63 (*C. zedoaria* Rosc., *C. zanthorrhiza* Roxb., *C. elata* Roxb. and *C. aeruginosa* Roxb.), 2n = 84 (*C. attenuata* Wall.), 2n = 32 (*C. alismatifolia* Gagnep.) 2n = 24 (C. *thorelii Gagnep*), 2n = 28, 34, 36 (*C. parviflora* Wall.). Chromosomes sizes of this genus were very small ranging from 0.5-2.0 µm (Apavatjrut et al., 1996; Joseph et al., 1999). Among these wide range of chromosome numbers the basic chromosome number n = 21 is very frequent in *Curcuma* with 2n = 2x = 42, 2n = 3x = 63, 2n = 4x = 84 as described by different authors. However, there are still disagreements among some reports on chromosome numbers of some species, which have to be reconfirmed. Besides, there is no report available on chromosome number of Bangladeshi *Curcuma* species. It is also to be mentioned that the genetic improvement of this important crop through conventional breeding is handicapped due to incompatibility and high pollen sterility resulting in no seed set (Joseph et al., 1999). In addition to that, detailed karyomorphological studies on this genus were not tried so far,

which is may be the reason of very small size of chromosomes, however, this is a prerequisite for *Curcuma* taxonomy as well as for executing genetic improvement programmes in Bangladesh.

1.1.6. 2C DNA values and genome size of *Curcuma*

Nuclear 2C DNA values or genome size are important biodiversity characters with fundamental biological significance utilities (Bennett and Leitch, 1995; Bennett et al., 2000). To determine nuclear DNA amounts and genome size of plants are emphasized and currently being increasing the percentage of species with known C-values from approx. 1% to 1.4% (Obermayer et al., 2002). Analysis of genome size may also support further studies on plant population genetics and conservation programmes. In addition, genome size of the different plant populations may be used to interpret the composition of the communities and other aspects of geobotanical studies (Lysák et al., 2000). Inter- and intraspecific variation in nuclear DNA content among flowering plants has also been well documented in the literature (Bennett and Leitch, 1995; Bennett and Smith, 1976 and 1991; Cavallini and Natali, 1991; Price, 1988).

Determination of genome size and nuclear 2C DNA amounts of *Curcuma* was not comprehensively tried so far. Only the genome size of *C. zanthorrhiza* is available in the Kew database reported by Bharathan et al. (1994). Genome sizes of few other species of *C. amada*, *C. caesia* and *C. longa* have been reported by Das et al. (1999). This inadequate information cannot be based on further studies on the genus *Curcuma* and still a considerable amount of investigation on the genome size estimation is required to facilitate further taxonomic research as well as crop genetic improvement programmes.

1.1.7. Needs of *Curcuma* genetic resources conservation

Most of the species of *Curcuma* including cultivated ones are triploids reproducing vegetatively by means of underground rhizomes, however, some diploids and tetraploids have been reported which also do not produce or rarely produce fertile seeds due to incompatibility and high pollen sterility (Joseph et al., 1999). Clonally propagated plants are thought to be comprised low allelic diversity within the species and are always in risk of extinction. Clonal plants are characterised by the ability to produce genetically identical genet that can produce potential independent ramets. Owing to this life history trait, low genetic diversity and gene flow between populations are expected in clonal plants (Auge et al., 2001; Eckert et al.,

2003). It seems that the biodiversity of the genus *Curcuma* has been depleted in the context of its original stands. Considerable research effort is required before the knowledge on *Curcuma* in Bangladesh can be brought up to the same standard as that of other conservation prioritised taxa, because *Curcuma* resources are undervalued and under-researched, but of high conservation value, significantly serving as a source of spices, medicines, dyes, starches and ornamentals. Understanding the genetic structures of the species is a prerequisite to undertake any successful conservation program, because species that lack adequate genetic variations are at greater risk of extinction and the existing levels of genetic variations and maintenance of these variations are the major issues for plant genetic diversity conservation.

The key information including population structures, species association and eco-physiological phenomena on the entire genetic diversity of the genus and their characterization are essential before establishing any other biotechnological approaches for genetic improvement and long-term conservation. The genus *Curcuma* has not been taken any considerable priority in terms of gene diversity, population structures as well as other qualitative or quantitative studies related to the their genetic improvement and conservation. Only a few reports based on the species of Thailand are available that are relevant to the genetic diversity of several *Curcuma* species. Paisooksantivatana et al. (2001a and 2001b) studied population genetic diversity of *C. alismatifolia* using isozyme data. Phunchaisri et al. (1998) studied several *Curcuma* species using RAPD data. However, there is no substantial research work available in Bangladesh or even in India, which can be based genetic improvements or any other conservation programmes on this species.

1.2. Significance of plant genetic diversity

It is well recognized that only by representing genetic variation, a given species is able to respond to the environmental force, change and survive in the long-term basis. Estimation of genetic diversity of plant populations has therefore been recognized as elementary topic not only to delineate *in situ* and *ex situ* conservation strategies (Holsinger and Gottlieb, 1991; Morden and Loeffler, 1999), but also to establish forms of rational and sustainable exploitation of genetic resources (Chalmers et al., 1992; Lacerda et al., 2001). The ability of a particular genotype to tolerate drought or inundation, grow in poor or rich soil, resistance against insects, pests or diseases, to offer higher protein yields or to produce a better-tasting food are traits passed on naturally by its genes. This genetic substance constitutes the raw material that plant breeders and biotechnologists utilize to produce new varieties or cultivars.

Without this diversity the ability of better adaptation to ever-changing conditions and needs will be lost (Menini, 1998). This mode of reproduction largely determines the transmission of genes in time and space; hence it is widely expected to be a major determinant of population genetic structure (Eckert et al., 2003). Most perennial plants combine sexual reproduction through seeds with clonal reproduction by means of vegetative propagation (Richards, 1986), and the production and recruitment of sexual versus clonal progeny may often vary within a species responding ecological and/or genetic factors that limit one or other reproductive mode and eventually affect the genetic structure of natural populations (Eckert, 2002). Clonal plants are generally characterised by the capability to generate genetically identical genet that can produce potential independent ramets. For the reason of this life history trait, low genetic diversity and gene flow between populations are expected in clonal plants (Eckert et al., 2003). However, such theoretical expectations sometimes do not take place in the natural populations (Auge et al., 2001; Paisooksantivatana et al., 2001a).

Plant breeders, the ultimate users of germplasm, generally agree that the required genetic diversity within the economically important plant species has never been greater. It is thought that crops are lacking of genetic variation compared to their wild relatives (Tanksley and McCouch, 1997). Germplasm is thus an aggregate genetic material of wild and domesticated species that can be used to reproduce directly through hybridization and selection or using biotechnology for enhancing the quality of target species. Hence, conserving genetic resources is a means of safeguarding the living materials that have been using at long-run to provide food, medicines, cloths, fuels, and also for industrial commodities.

1.3. Use of molecular markers in studying genetic diversity

Genetic markers have contributed to the studies of plant biodiversity and population genetics by providing techniques for detecting genetic variability among individuals, populations and species (Avise, 1994). In earlier days although, such studies were made on the basis of morphological traits, which have numerous realistic limitations because: 1) morphological variations are not detectable in many species, 2) studies utilizing morphological characters are generally limited to only one locus, 3) many characters have to be scored relatively late in the life cycle (Cruzan, 1998). In addition to that, many morphological characters are not discrete rather having a continuous and overlapping between species that hinder the precise plant biodiversity analysis. Since the use of morphological characters in studying genetic variability embraces a variety of impediments, the establishment of new molecular marker techniques

has given emphasis by the population geneticists as well as by the conservationists (Cruzan, 1998; Haig, 1998;Vekemans and Jacquemart, 1997).

Exploration of genetic variation in plant populations was greatly made possible by the development of protein based markers (i.e. allozymes) over three decades ago (Hamrick and Godt, 1990; Loveless and Hamrick, 1984). Since then a rapid advances in molecular technologies have opened a new era in population biology and species conservation (Haig, 1998). The new DNA-based techniques have enabled us to understand and, hence, better manage and use the genetic diversity of cultivated plants and their wild relatives. A number of techniques, DNA sequencing, MHC (major histocompatibility complex), minisatellite, microsatellite, RFLP (random fragment length polymorphisms) and some more recent PCR (polymerase chain reaction) based techniques such as RAPD (randomly amplified polymorphic DNA, AFLP (amplified fragment length polymorphisms), ISSR (intersimple sequence repeat polymorphism) provide more sophisticated analysis of population genetic structure and other events of their evolutionary biological processes (Albertson et al., 1999; Degen et al., 2001; Gerber et al., 2000; Hardy, 2003; Hill and Weir, 2004; Jacquemyn et al, 2004; Kjølner et al., 2004; Welsh and McClelland, 1990; Williams et al., 1990; Wolfe and Liston, 1998; Vos et al., 1995). These wide ranges of molecular technologies are being extensively used to resolve the problems in plant population genetics and conservation, since they allow investigators to obtain large amounts of data on variation within and among populations and species being studied (Holsinger et al., 2002). Current approaches to partitioning genetic diversity as assessed either by assuming that the inbreeding coefficient within populations is known (Lynch and Milligan, 1994; Zhivotovsky, 1999), or treating the multilocus phenotype as a haplotype and using a similarity index (Nei and Li, 1979), or Euclidean distance (Schneider et al., 2000) to describe distances among haplotypes using AMOVA (Excoffier et al., 1992; Isabel et al., 1999). Among different maker based techniques random amplified polymorphic DNA (RAPD) is one of the simplest, cost effective and powerful tool for the investigation of genetic variation. A number of recent publications have recognized that RAPD technique is a powerful tool to analyse population genetic consequences because RAPD usually displays a large number of polymorphic (di-allelic) loci that can be obtained relatively easily even for species for which no prior genetic information is available (Eckert et al., 2003; Fu et al., 2003; Hardy, 2003; Jordano and Godoy, 2000; Kjølner et al., 2004; Masumbuko et al., 2003; Torimaru et al., 2003). In fact, in comparison of RAPD markers with other popular marker types were found to produce similar and

comparable results for AFLP (Díaz et al., 2001; Kjølner et al., 2004; Nybom, 2004; Uptmoor et al., 2003), ISSR (Nybom, 2004; Uptmoor et al., 2003) and allozyme (Buso et al., 1998; Waycott, 1998). Indeed, RAPD markers were used in most of the 307 studies (published in 1993-2003) estimating intraspecific genetic diversity with nuclear DNA markers which were recently evaluated by Nybom (2004).

1.4. Approaches for genetic diversity conservation

The irreversible loss of cultivated and wild species embraces the loss of varieties, single genes, and combinations of characteristics during the past 100–150 years is of major concern to gene banks and plant professionals (Hammer et al., 2003). The basic elements of plant genetic resource conservation are considering the genes within the entire gene pools of a target species. For a successful conservation, these gene pools should as whole be considered and conserved. Genetic conservation is a process that enthusiastically maintains and manages the diversity of the gene pool with a view to definite or prospective utilisation. Maxted et al. (1997) have projected a model, which is an attempt to construct unequivocal and fundamental elements of genetic resource conservation, as well as to determine the interrelations among them. This model discriminates two primary complementary conservation strategies, *ex situ* and *in situ*, each of which includes a range of different techniques that can be implemented to achieve the aim of the conservation scheme. Indeed, no one alone can effectively conserve the gene pool, though; biodiversity security results only from the application of a range of *ex situ* and *in situ* techniques applied in a complementary manner. One technique performs as a backup to the others (Maxted et al., 1997 and 2002).

Furthermore, genetic polymorphism is the central issue of plant genetic conservation since it is the primary source of variation of the morphological and physiological appearance of plants. This leads to evolutionary changes within and among species subsequently to genetic diversity (Hammer et al., 2003). Therefore, it presents a basis to adapt actively to changing environmental conditions and finally the genetic constitute allows it to respond to the challenges of the future. For this reason a successful conservation programme solely depends on the strategies that consider the genetic polymorphism of plants and succeeding events in terms of sustainable environmental adaptation.

1.5. Application of biotechnology in conservation programmes

The tools of modern biotechnology are being increasingly applied for plant genetic diversity assessment and characterization. They have also undoubtedly a major role in assisting plant conservation programmes (Benson, 1999; Menini, 1998; Villalobos et al., 1991). However, biotechnology is advancing so rapidly that it may be sometimes difficult for potential conservation users to assess the value and role of new techniques as well as procedures within their own specific area. It is important to recognize that the effective integration of biotechnology in conservation programmes requires interdisciplinary co-operation of activities. In order to justify an integration of biotechnology into conservation programme, Benson (1999) projected an outline of the key steps that must be considered when embarking on conservation strategy, which has the potential for incorporating biotechnology. This indicates that the integration of biotechnology into conservation programmes should be determined on the basis of following factors such as evaluation of the conservation need, an appraisal of existing conservation methods, biotechnological options, appropriate methods and regular evaluation of different conservation approaches.

In order to conserve a target plant species, it is essential to consider the relationship between conservation and sustainable utilization of different techniques. Prior to consider a conservation programme it is a prerequisite to evaluate field status of the species at genomic level using molecular markers (Benson, 1999; Uyoh et al., 2003). The elucidation of population structures and gene distribution patterns within ecosystems provides information that can be used to support conservation programme (Callow et al., 1997; Uyoh et al., 2003). Tissue culture or *in vitro* technologies have had a major impact on the *ex situ* conservation of plant genetic resources. Crop plants that are vegetatively propagated present particular conservation problems as their seeds are not available for banking. In addition to that, germplasm maintained in the field gene banks can be at risk by pathogen attack and climatic damage. Owing to these drawbacks, *in vitro* regeneration consequently conservation of *in vitro* germplasm using active growing state or under slow growth provides cost effective, medium-term conservation options. However, maintenance of plant germplasm in the active or slow growth states provides only medium-term storage option, while the long-term conservation of *in vitro* derived plant germplasm is increasingly achieved using cryopreservation in liquid nitrogen. Cryopreservation is thus applied to plant germplasm that cannot be conserved using traditional seed banking techniques. Bension (1999) stated that there are four main areas of biotechnology which can directly assist plant conservation

programmes which are: 1) molecular markers technology, 2) molecular diagnostics of plant, 3) tissue culture or *in vitro* technologies and 4) cryopreservation.

1.6. *In vitro* techniques for genetic improvement and conservation

In vitro culture of higher plants is the culture of plants, seeds, embryos, organs, explants, tissues, cells and protoplasts on nutrient media under sterile condition. This type of culture has shown magnificent development since 1975, resulting in the production and regeneration of viable individuals of many plant species. In addition, since 1980 plant tissue culture has evolved into a major and indispensable element in many areas of fundamental science and applied biotechnology. Plant *in vitro* technology has been successfully used for the commercial production of pathogen-free plants and to conserve the germplasm of rare and endangered species (Fay, 1992; Mikulík, 1999).

Numerous important medicinal plant species including *Curcuma* species are thought to be declining at an alarming rate as consequences of rapid agricultural and urban development, deforestation and indiscriminate collection of genetic resources. Plant tissue culture technology would likely to be appropriate to conserve rare and endangered *Curucma* species in the tropical countries since they are sterile seedless species. A number of protocols for *in vitro* multiplication of *C. longa* have already been established by different workers (Balachandran et al., 1990; Dekkers, 1991; Nadgauda et al., 1978; Salvi et al., 2002; Shirgurkar et al., 2001; Sunitibala et al., 2001; Yasuda et al., 1988; Yusuf et al., 2001), however, further improvement is required to meet the future demand.

Recently *in vitro* formation of storage organs such as bulbs, corms, tubers and rhizomes came into focus because these kinds of propagules can be directly transferred to the field without any acclimatization and hardening procedures. In addition to that, these organs can easily be transported across the national borders, as they do not require any culture medium or any other special measures. Only a few reports are available and still under progress. Microrhizome induction in turmeric (Nayak, 2000; Shirgurkar et al., 2001; Sunitibala et al., 2001) and in ginger (Nirmalbabu et al., 1994; Sharma and Singh, 1995) is available within the family Zingiberaceae.

In vitro storage of germplasm was first suggested in the mid-1970s (Henshaw, 1975). Storage of organised structures of plants like shoot apices or meristems are more stable and also

propagate more rapidly rather than unorganised cells that may have the ability to regenerate full plant. It is due to that the meristematic areas do not have to differentiate after recovery from storage. Effective storage systems are often inexpensive and easy to maintain, and should reduce the overall work load in gene banks. Frequent monitoring of culture viability and for microbial contamination should not be necessary. The germplasm of vegetatively propagated crops is normally stored and shipped as tubers, corns, rhizomes, roots, or in the case of woody perennials as cuttings. Although some of these crops have seeds that could be stored, they are frequently highly heterozygous and thus do not breed true to type from seed. Perennials are usually maintained *ex situ* either in plantations or are stored during the winter and planted the following spring. For these and other crops whose seeds are short lived, there are a number of potential advantages in storing them as *in vitro* cultures. These include economies in space and labour as well as provided an appropriate conservation method with greater genetic stability. Disadvantages, however, include the need for special facilities and trained technicians and some experiences in the use of *in vitro* methods for germplasm conservation on a large scale.

The slow growth approach of *in vitro* storage involves applying retardant chemicals or reducing the culture temperature. Subculture intervals can be extended up to 1 or 2 years, thereby greatly reducing the time, labour, and materials required maintaining the cultures. Slower growth reduces the frequency of cell division and consequently the number of times a random mutation is multiplied in the culture. Such genetic changes that occur in tissue cultures are called somaclonal variations. Stress is an intrinsic factor in slow growth, and little is known about its effect on somaclonal variation. What began as a clonal culture may change into a population of cells consisting of the original genotype plus variant genotypes. Also, stress factors may act differently on such a population of genotypes, favouring some somaclonal variants. This could result in a changed population of cells and the failure to conserve the genetic integrity of the original clonal material. Undifferentiated callus cultures are more susceptible to somaclonal variation than organized tissue systems, such as shoot cultures. Only organized cultures are therefore recommended for slow-growth storage. Medium-term conservation techniques have been developed for a wide range of plant species but they are still used routinely for the genetic resources conservation of a limited number of species only such as Musa, potato or cassava (Engelmann, 1997).

1.7. Cryopreservation: a potential tool for long-term storage of germplasm

In the tropics, numerous vegetatively propagated crop plants traditionally play a vital role in the rural economy as potential sources of agricultural and horticultural commodities, industrial raw materials, and indigenous medicines. These important crop genetic resources are always under threat of extinction since they do lack sexual reproduction and eventually lack genetic diversity within their entire gene pool. Most of the germplasm of vegetatively propagated species is mainly conserved in field gene banks. Increasing numbers of accessions require intensive labour and cost only to maintain a small proportion of diversity in the field conditions. This method of conservation, however, also presents certain drawbacks, which limit its efficiency and threaten the security (Takagi et al., 1998). Owing to this, there is a mounting demand for stable long-term storage of the germplasm of vegetatively propagated crop plants. It is currently well recognised that, cryopreservation of plant germplasm is a preferable option that has obvious advantages over *in vitro* storage (Engelmann, 1997; Wilkinson, 2003). Long-term conservation of such vegetatively propagated plants is fundamental because *in vitro* technique can only be used for the medium term conservation of germplasm, which is not only time consuming and labour-intensive, but in addition may not ensure good genetic stability of *in vitro* micropropagated plantlets (Sarkar and Naik, 1998). Cryogenic storage has therefore been recognized as a key method for long term storage of the base collection of vegetatively propagated crop plants since these plants have been yet received a little attention (Engelmann, 1991 and 1997; Sarkar and Naik, 1998; Takagi et al., 1998).

Cryopreservation involves suspending growth by keeping cultures at an ultra-low temperature, typically that of liquid nitrogen (-196°C). It offers the prospect of storage for indefinite periods with minimal risk. Two new approaches to cryopreservation may lead to more widespread applications for genetic conservation. They focus on reducing cell damage from ice crystal formation. One approach is through vitrification of cellular water by a cryoprotectant mixture and the other involves encapsulation of specimens within an alginate gel that is then dehydrated. For vitrification the specimen is infused with a cryoprotectant mixture that promotes the conversion of much of the cellular water into a noncrystalline, vitreous solid when rapidly cooled (Sakai et al., 1990). Recent years, many workers described the vitrification procedure as a successful technique for long-term conservation of germplasm. A number of reports are also available on some vegetatively propagated plants (Halmagyi et al., 2004; Helliot et al., 2003; Leunufna and Keller, 2003; Sarkar and Naik, 1998; Takagi et

al., 1998; Thinh et al., 1998). To the best of knowledge, no work has been published on the cryopreservation of *Curcuma* germplasm. The present study is therefore undertaken to standardise an effective cryopreservation technique of vitrification procedure using *in vitro* grown axillary buds of *C. longa*. An achievement in *Curcuma* genus will likely to be immensely helpful to further establishment of cryopreservation procedure for the family of Zingiberaceae, which is crucial since most of the ginger species are vegetatively propagated plants.

1.8. Problem statement in the genus *Curcuma*

A number of areas of *Curcuma* biology where substantial research is required including inter- and intra-specific genetic variations, genetic structures of populations and species, cytogenetic studies, reproductive behaviours, *in vitro* regeneration and conservation issues. The following key problems are concerned in the genus *Curcuma*:

- Lack of the information on present status of the *Curcuma* biodiversity in Bangladesh as well as other regions where they grow naturally.
- Among the native species of the genus *Curcuma*, no information is available on genetic variations that persist within and among populations and species.
- Rate of genetic depletion of *Curcuma* species and thus the potential medium to long-term risks have not been yet investigated.
- Experimental results and data are not available on differentiation between populations and the correlation with geographical and genetic distance, which can be based *in situ* conservation.
- Inadequate information on genome size of different species and their ploidy levels and their origin.
- Chromosome numbers of the species are partially investigated, though there is no report available in Bangladesh.
- Taxonomic uncertainty in many cases.
- A wide range of morphological variations as well as occurrence of overlapping morphological traits.
- *In vitro* regeneration techniques are yet developing and further study is needed.
- Cryopreservation techniques suitable for long-term preservation have not been yet investigated in this genus.

1. 9. Aims and Objectives

In general, this study is aimed to explore the *Curcuma*'s genetic diversity in Bangladesh including genetic structures of the populations. Another important aim is to establish *in vitro* systems for genetic improvement, regeneration and long-term conservation of *Curcuma* germplam. The outcome of the research work would likely to be contributed genetic conservation and sustainable management of *Curcuma* diversity in Bangladesh. In extension to that the present work will likely to be helpful to crop improvement programme in this genus since classical breeding programme is not suitable since they reproduce vegetatively. The following areas are specifically considered in this study:

- Exploration of plant diversity of the genus *Curcuma* in Bangladesh and identification of species through consultation of herbarium materials and available literature.
- Estimation of genetic variation existing within and among the species, which belong to the genus *Curcuma*.
- Comparison of estimated genetic variations of different species to conclude population genetic structures of the species.
- Determination of the genetic loss and threats of extinction to the species that are found in nature with small and scattered populations.
- Documentation of geographical variation existing within and among the species.
- Recommendations of expansion program for the small and fragmented populations of *Curcuma* species based on population genetic information.
- Investigation on chromosome number and ploidy levels, as they are also important biodiversity characters.
- Estimation of genome size to confirm their ploidy levels and also to correlate other genetic parameters.
- Development and optimisation of plant tissue culture protocols for efficient multiplication of *C. longa* L. as a case study.
- Establishment of microrhizome induction protocol since they are suitable in some cases.
- Establishment of long-term preservation of *Curcuma* genetic diversity of Bangladesh.

2. MATERIALS AND METHODS

2.1. Plant materials and study area

2.1.1. Collected plant samples

Different *Curcuma* species were investigated in this study. A total ninety six accessions were collected from six different sampling areas in Bangladesh namely Srimangal, Chittagong, Sitakundu, Savar, Birganj and Kapasia. Initially plant accessions were grown in the garden of Bangladesh National Herbarium (DACB), Dhaka, Bangladesh. Details of the sampling areas including geographical descriptions are presented in Table 2.1. Eco-geographically, the locations are generally distinguished as three habitats such as hilly areas (Chittagong, Sitakundu and Srimangal), plain lands (Savar) and Pleistocene plateau lands (Birganj and Kapasia). Genetic diversity analyses of different species considered all ninety six accessions collected from all of the above mentioned sampling areas, while the study on population genetic structures of *C. zedoaria* (Chrism.) Rosc. included a total of 42 accessions collected from five populations of Chittagong, Sitakundu, Srimangal, Savar and Birganj. An image of *Curcuma* species is presented in Fig 2.1.

Table 2.1. Accessions of *Curcuma* collected from six sampling areas; regional, geographical and ecological characteristics of the study areas

Sampling areas	District	Latitude	Longitude	Ecological remarks
Srimangal	Sylhet	24º 55' N	91º 55' E	Mixed deciduous forest margins and fallow lands besides tea gardens
Chittagong	Chittagong	22º 21' N	91º 50' E	Slope of the hill, shrubby woodland or open fields
Sitakundu	Chittagong	22º 35' N	91º 42' E	Mixed evergreen forest margins on the slope of the hills
Savar	Dhaka	23º 46' N	90º 23' E	Alluvial plain land, agricultural areas or fallow lands
Birganj	Dinajpur	25º 63' N	88º 63' E	Terrace soils of Pleistocene plateau land of Barind tract, remnant Sal forest and agricultural area
Kapasia	Gazipur	23º 80' N	90º 65' E	Terrace soil of Pleistocene plateau land of Madhupur tract, Sal forest margins and agricultural land

Fig 2.1. A) Image of a leafy shoot *C. aeruginosa* Roxb. contains inflorescence, B) a large view of inflorecsens represents yellow flowers and pink coma bracts

2.1.2. Species distribution and samplings areas

Hilly areas comprising higher diversity while plain and plateau land habitats contain rather less diversity and abundant of *Curcuma* species. Distribution patterns and collected sampling areas are pointed out in Fig 2.2. Bangladesh is located in between $20^{0}34'$ and $26^{0}38'$ north latitude and between $88^{0}01'$ and $92^{0}41'$ east longitude with an area of 148,393 km^{2}. The country is mostly surrounded by India, except for a short south-eastern frontier of 283.36 km with Myanmar (Burma) and a highly indented southern coastline with offshore islands in the Bay of Bengal. The boundary with India on the west, north and east is about 4092.62 km long. The maximum length of this land, between the north-west and the south-east extreme points is about 760 km, and the widest width from east to west extreme points is about 467 km.

2.1.3. *Curcuma* species occurred in Bangladesh

A total sixteen species were identified from a total of ninety six individuals that were collected from six different areas of Bangladesh. The selection of sampling areas was chiefly based on hilly, plain and plateau lands. The hill tracts areas are more diverged containing higher number of species in compare to plain land and Pleistocene Plateau land. Among the

studied species, thirteen species are wild and the rest of three species known to be cultivated in Bangladesh. However, the most widely cultivated species is *C. longa* L. In addition to that, *C. angustifolia* Roxb. is occasionally cultivated in some regions of the country to produce starch while *C. zedoaria* (Chrism.) Rosc. cultivated for aurvedic medicines. The details of the species and the accession number are presented in the Table 2.2.

Fig 2.2. Distribution of *Curcuma* species in Bangladesh and sampling areas; solid dots – general distribution of the species, triangles- sampling areas

Table 2.2. Different *Curcuma* species and the accessions used in this study

Species	Scientific name	Accession No.	Distribution	Wild/ cultivated
Sp01	*C. elata* Roxb.	S03, S06, S08, S09, S10, S11	Srimangal and Sylhet	Wild
Sp02	*C. angustifolia* Roxb.	C32, S02, S04, S07, S17, S21, S22, S23, S24, S27, S32, S33, S36, S41, S42	Chittagong and Srimangal	Wild Rarely cultivated
Sp03	*C. zanthorrhiza* Roxb.	S01, S05, S13, S14, S25, S26	Srimangal	Wild
Sp04	*C. australasica* Hook f.	S35, S38	Srimangal	Wild
Sp05	*C. viridiflora* Roxb.	S20, S44, D01	Srimangal and Dinajpur	Wild
Sp06	*Curcuma sp.*	C03, C09	Chittagong	Wild
Sp07	*C. latifolia* Rosc.	C10, C17, C30, C31	Chittagong	Wild
Sp08	*C. rubescens* Roxb.	C08, R03	Chittagong and Dinajpur	Wild
Sp09	*C. zedoaria* (Chrism.) Rosc.	S15, S18, S28, S29, S30, S31, S34, S37, C01, C02, C11, C12, C13, C15, C19, C16, C20, C21, C22, C23, C24, C25, C27, C28, C29, C26, C33, C35, C36, C37, D02, D03, D04, D05, D06, D08, R01, R02, R04, R05, R06, R07	Chittagong, Dhaka, Srimangal, Dinajpur	Wild Rarely cultivated
Sp10	*C. petiolata* Roxb.	C14	Chittagong	Wild
Sp11	*C. aeruginosa* Roxb.	C04, C06, C07, G04, G06, G07	Chittagong, Gazipur	Wild
Sp12	*C. amarissima* Rosc.	G03, G05	Gazipur	Wild
Sp13	*C. caesia* Roxb.	C05	Chittagong	Wild
Sp14	*C. longa* Linn.	G01, G09	Gazipur	Cultivated
Sp15	*C. aromatica* Salisb.	C18	Chittagong	Cultivated (wild)
Sp16	*C. amada* Roxb.	G02	Gazipur	Cultivated (wild)

2.1.4. Establishment of the accessions in Germany

Planted clones of *Curcuma* species were shifted to Germany from the field gene bank of Bangladesh National Herbarium and planted into soil pots at the glasshouse of the Institute of Botany, University of Hannover, Germany to allow sprouting of buds. A single voucher specimen representative of the accessions was prepared and deposited at Bangladesh National Herbarium (DACB). After developing leafy shoots in summer, leaf samples were collected from each specimen from 3 weeks old leaves and immediately transferred to the liquid nitrogen (-196^{0}C) and finally stored at –72°C until use them for genomic DNA extraction. Plant accessions were kept in glasshouse to make available further leaf materials for flow cytometry investigation and also for developing rhizomes as well as axillary buds that were used for *in vitro* regeneration experiment.

2.2. Genetic Diversity estimation using RAPD markers

2.2.1. DNA extraction and purification

After developing leafy shoots in summer, leaf samples were collected from each specimen from 3 weeks old leaves and immediately transferred to the liquid nitrogen (-196^{0}C) and finally stored at –72°C until use. DNA was extracted from stored leaf materials according to a modified CTAB method (Doyle and Doyle, 1990) which included the following steps: 1-2 g of frozen (-72°C) leaf sample was grinded using liquid nitrogen and the leaf powder was immediately shifted to a 50 ml tube containing 10 ml CTAB buffer prepared by 100 mM Tris-HCl pH 8.0, 1.4 M Sodium chloride, 20 mM EDTA (ethylenediaminetetraacetic acid disodium salt), 2% CTAB (hexadecyltrimethyl-ammonium bromide), 2% PVP-40 and 0.2% β-mercaptoethanol, mixed and incubated for 1 h at 65°C. After centrifugation the solution for 20 min with 4,000 rpm (2,000 xg) at room temperature, supernatant was taken, treated with RNase for 30 min at 37°C. The same volume of chloroform:isomylalcohol (24:1) was added to the solution and centrifuged for 30 min with 4,000 rpm at room temperature. Supernatant was used again, mixed with the same volume of 2-propanol, incubated overnight at 4°C, and centrifuged for 30 min at 10,000 rpm (12,520 xg) at room temperature. The supernatant was discarded and the pellet was washed with 2 ml 70% ethanol, centrifuged again for 10 min with 10,000 rpm at room temperature and dried using vacuumed desiccators. The pellet was then resuspended with 100 µl double distilled water, incubated for 15 min at 50°C and centrifuged for 5 min with 10,000 rpm at room temperature. Finally, the DNA solution was transferred to a new 1.5 ml Eppendorf tube. Approximate DNA concentrations were determined by comparing the brightness of sample bands against uncut λ DNA standards of known concentration on 1.0% agarose gels stained with ethidium bromide. Yields obtained were between 1.5 and 3.5 µg $\mu l^{-1.}$ This method of determining DNA concentration was found to be sufficiently accurate for RAPDs, which were quite robust to variation in the added the amounts of DNA per reaction ranging from 30 - 50 ng.

2.2.2. RAPD reactions

DNA amplification was performed using an MJ Research 96-well PTC-200 Peltier Thermal Cycler with hot lid according to the following programme: one cycle of 94 °C for 3 min, 42 cycles of 94 °C for 30 s, 34 °C for 30 s, 72 °C for 2 min, and finally one more cycle for 72 °C for 8 min. PCR products were visualized by electrophoresing 15-20 µl aliquots on 1.5% agarose gels for 120 V in a 1xTAE buffer system. One liter 50 x TAE buffer was prepared by

using 242 g Tris base, 57.1 ml Acetic acid, 100ml 0.5 M EDTA and required volume of ddH2O; pH to 8.5. After completion of electrophoresis gels were stained with ethidium bromide solution for 20-25 and photographed with a BioRad GelDoc 1000 computer system.

The RAPD protocol was optimised for a selection of primers for concentrations of Mg^{2+} (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mM), DNA template (5, 10, 20, 30, 40, 50 and 60 ng per reaction), primer (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 µM), and Sigma Red TAQ DNA polymerase (0.5, 1, 1.5 and 2 units per reaction). Amplification was equally successful for $MgCl_2$ concentrations between 2.5 and 3 mM. The range of DNA concentrations tested had significant effect on the patterns of amplification wherein 30-50 ng produced better amplification, whereas for primer and polymerase enzyme concentration there was a limit below which no amplification occurred (0.1 µM and 1 unit, respectively) and above which the same fragments were consistently amplified, but varied rarely in intensity. Optimised conditions were chosen based on the clearest reproducible RAPD profiles; thus, each 25 µL reaction contained: 10x Red Taq reaction buffer (Sigma), 3 mM $MgCl_2$ (ThermoHybaid), 0.1 mM each of four dNTPs (ThermoHybaid), 0.1 µM primer (Operon Technologies), 1 unit of Red Taq DNA polymerase (Sigma), and 30-50 ng of genomic DNA.

A selection of 10-mer primers from the Operon Technologies RAPD primer set OPX and five additional primers were screened for amplification on five DNA samples. Out of 25 primers screened, 13 yielded consistent patterns of amplified products and all of these primers which successfully amplified DNA had a C + G content of ≥ 60% (Table 2.3). Duplicated PCR reactions were conducted to confirm the reproducibility of the RAPD products.

Table 2.3. RAPD primers used for *Curcuma* genetic diversity analysis

Primer	Sequences
OPX 01	5´-CTG GGC ACG A-3´
OPX 03	5´-TGG CGC AGT G-3´
OPX 04	5´-CCG CTA CCG A-3´
OPX 07	5´-GAG CGA GGC T-3´
OPX 08	5´-CAG GGG TGG A-3´
OPX 09	5´-GGT CTG GTT G-3´
OPX 11	5´-GGA GCC TCA G-3´
OPX 12	5´-TCG CCA GCC A-3´
OPX 14	5´-ACA GGT GCT G-3´
OPX 15	5´-CAG ACA AGC C-3´
OPX 19	5´-TGG CAA GGC A-3´
P 92	5´-CCT GGG CTT T-3´
P 24	5´-ACA GGG CTG A-3´

2.2.3. Phenetic analysis

RAPD products between 200 and 2000 bp were scored for presence (1) and absence (0) irrespective of band intensity since each PCR product of identical molecular weight was supposed to represent a single locus. Products in this range are considered to be the most reliable because extremes of size may not be consistently amplified (Bussell, 1999; Harris, 1995; Stewart and Porter, 1995). The data were analysed based on the Dice Coefficient (Dice, 1945) for binary data via SIMQUAL of the NTSYSpc ver. 2.1 Package (Rohlf, 2000). Landry and Lapointe (1996) compared several coefficients for use with RAPD markers and suggested to use of Dice (1945) and Jaccard (1908) coefficients with no less than 12 primers. Analysis utilized the SAHN clustering programme of the NTSYSpc package (Rohlf, 2000) to construct an UPGMA (Unweighted Pair Group Method of Arithmetic Mean) dendrogram. Similarity coefficients from the squared data matrix were used for Principal Coordinates Analysis (PCoA) via PCOORDA of NTSYSpc package. The pairwise Nei's (Nei, 1987) unbiased genetic distance matrix of the populations was used to construct a phylogenetic tree by using Neighbour joining (NJ) method of the programme MEGA2 (Kumar et al., 2001).

2.2.4. Diversity analyses of the species and populations

To estimate genetic variation within and among populations the RAPD phenotypes of each genet were used. One of the most commonly employed methods to estimate within population diversity is the expected heterozyogosity H_E (Nybom, 2004), which is equivalent to Nei's unbiased gene diversity H_s (Nei, 1978). To further avoid biased results, Lynch and Milligan (1994) suggest that the analyses should be restricted to bands with an observed frequency below 1-(3/N), where N is the sample size. Estimates of fixation indices based on dominant markers like RAPDs rely on the assumption of Hardy-Weinberg equilibrium ($F_{is} = 0$) in the populations examined (Jordano and Godoy, 2000). Estimated genetic diversity was calculated for each population (H_s) and overall species (H_t) for each locus as follows:

$$H = 1 - (p^2 + q^2)$$

Where, p is the frequency of the dominant allele and q is the frequency of the null allele. Allele frequencies were estimated from the number of null/null homozygotes present in the population (Kwon and Morden, 2002; Morden and Loeffler, 1999). The component of diversity within populations is H_s/H_t and the component between populations G_{ST} is (H_t-

H_s)/H_t (Nei, 1978). The overall G_{ST} value was calculated from the average per marker values of each primer. Despite limitations of RAPD data to assess population genetic statistics due to their dominant nature, Shannon's information measures were used that assume Hardy-Weinberg equilibrium (Bussell, 1999; Chalmers et al., 1992). Shannon's Index (Lewontin, 1972) for each RAPD locus was calculated for each population as:

$$H'_j = -\sum p_i log_2 p_i$$

where p_i is the frequency of the presence or absence of a RAPD in a population. The over all average diversity of the populations was calculated for each locus as

$$H'_{pop} = 1/N \sum H'_j$$

Where, N is the number of populations. The species diversity was calculated for each locus as

$$H'sp = -\sum p_s log_2 p_s$$

Genetic diversity was partitioned for each primers since most investigators have followed Chalmers et al. (1992) when using Shannon's Index to analyse RAPD diversity (Wolff et al., 1997; Bussell, 1999), This involves subtotalling H'_j, H'_{pop}, and H'_{sp}, and partitioning diversity for each primer to enable comparison of the levels of diversity detected by different primers, The overall G_{ST} is then calculated from the average per-primer values for H'_j, H'_{pop} and H'_{sp}. The precise criteria of these analyses are stated in the following box.

H'_{pop} : average genetic diversity within populations
H'_{sp} : average genetic diversity among populations
H'_{pop} / H'_{sp} : proportion of genetic diversity within populations
G_{ST} [(H'_{sp} - H'_{pop}) / H'_{sp}] : proportion of genetic diversity between populations

2.2.5. AMOVA analysis

The AMOVA procedure was employed using ARLEQUIN ver. 2.000 (Schneider et al., 2000) to estimate the variance components of RAPD phenotypes. AMOVA was performed in order to test the significance of the partition of genetic variance resulting from groupings of the

species based cultivated / wild, and dominant / rare characteristics. Further analysis associated to the regional, geographical and topographical nested structure of the populations of *C. zedoaria*, with partitioning the variation of individuals within and among populations as well as regions. Regions were defined on the basis of i) a strict regional arrangement of the populations of north eastern (Srimangal) / south eastern (Chittagong and Sitakundu) / central (Savar) / north western regions (Birganj); ii) eco-geographical distribution strictly based on two eco-geographical arrangement of hilly areas comprising diverse plant composition and less agricultural impacts (Srimangal, Chittagong and Sitakundu) / non-hilly areas mostly open grassland comprising high agricultural impacts (Savar and Birganj); and iii) a strict edaphic distribution patterns of hill soils (Srimangal, Chittagong and Sitakundu) / alluvial and flood plain soils (Savar) / terrace soils (Birganj). The AMOVA was based on the pairwise squared Euclidean distances between RAPD phenotypes that allowed us to calculate both variance components among groups, populations and within populations and the analogues of F_{ST}, so-called Φ_{ST}. The significance levels of variance components were calculated by 1,000 permutations in each analysis. The parameters of molecular variance analysis are stated in the following box.

Φ_{SC} : correlation of the molecular diversity of individuals within populations, relative to that of individuals of the groups

Φ_{CT} : correlation of the molecular diversity of individuals within a group of populations, relative to that of individuals in the whole species

Φ_{ST} : correlation of the molecular diversity of individuals within populations, relative to that of individuals in the whole species (Excoffier et al., 1992)

2.3. Cytology and flow cytometry

2.3.1. Chromosomal investigation in the genus *Curcuma*

In order to establish protocol for root tip squash two different techniques were followed. First one is standard Feulgen method involved a staining with fuchsic acid while second one followed a complex procedure of squashing including DAPI staining. This study was carried out in the Laboratory of Cytogenetics, IPK, Gatersleben.

2.3.1.1. Feulgen method

About 1 cm long root tips from adult plants were collected from the glass house and incubated for 24 hours in ice water to amplify metaphases and shorten chromosomes. Root tips were fixed in ethanol : acetic acid (3:1). After hydrolysis in 1N hydrochloric acid for 15 min the roots were stained in fuchsic acid according to the standard Feulgen method. Chromosome spreads were prepared in propion orcein. Because chromosomes could not be spreaded in one focus layer an Axiophot microscope (Zeiss, Jena, Germany) integrated into a Digital Optical 3D Microscope System (Schwertner GmbH, Jena, Germany) was used to take image stacks to produce 3D images for chromosome counting. The image stacks were also used for karyogram establishment via the Ikaros software (MetaSystems GmbH, Altlussheim, Germany).

2.3.1.2. DAPI staining method

Similar to the above, about 1 cm long root tips from adult plants were collected from the glass house and incubated for 24 hours in ice water to amplify metaphases and shorten chromosomes. Root tips were fixed in 3:1 ethanol/ acetic acid for at least 5 hours then washed 3 x 10 min in 10 mM citrate buffer (pH 4.8). Incubated for about six hours to overnight with 2% pectinase and cellulase to soften tissue then washed again 3x 10 min in 10 mM citrate buffer (pH 4.8). Root tips were then transferred to 45 % acetic acid and squashed meristems in 45 % acetic acid. Removed the coverslip after freezing on dry ice. Slides were dehydrated in ethanol series of 70, 90 and 96%, air dried and mounted with 1µg/ml DAPI in anifade (Vectashield). Images were taken using a Zeiss Axiophot fluorescence microscope integrated into a Digital Optical Microscope system (digitaloptics, Jena, Germany).

2.3.2. 2C DNA values and genome size estimation using flow cytometry

In this study leaf samples of *Curcuma* were used. For *C. zedoaria* leaf samples were used from different individuals from five populations. For preparation of suspensions of nuclei, approximately 30 mg tissue was chopped with a razor blade together with material from *Raphanus sativus* as reference plant with a 2C DNA value = 1.38 pg (Doležel et al., 1998) in a petridish in 1 ml ice-cold staining buffer according to Galbraith et al. (1983) and filtered through a 35-mm mesh (Falcon 12x75 mm tube with a 35-mm strainer cap). The Galbraith buffer was supplemented with 50 µg/ml propidium iodide (PI; Molecular Probes, Eugene, OR) and 50 µg/ml DNase-free RNase (Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany). The analysis was done with a FACStar PLUS flow cytometer (Becton

Dickinson, San Jose, CA) equipped with two argon lasers INNOVA 90-5 (Coherent, Palo Alto, CA) using the analysis program CellQuest. PI fluorescence was excited with 500 mW at 514 nm and measured in the FL1 channel using a 630-nm band pass filter. The formula used for converting florescence values to DNA content was: Nuclear DNA content = (mean position of unknown peak)/(mean position of known) x DNA content of known standard. Finally the genome size was calculated in bp following the equation of number of base pairs = mass in pg x 0.978 x 10^9 (Doležel et al., 2003).

2.3.2.2. Statistical procedure

Significant differences in genome size and genetic distance were calculated trhough Tukey test using SAS statistical software, Release 8 (SAS Institute Inc., Cary, NC). To test whether genome size variations were correlated with genetic distances of the populations, correlation coefficient was analysed using statistical programme SigmaPlot, ver. 8.0 (SPSS Inc., Chicago, Illinois).

2.4. *In vitro* regeneration and microrhizome induction

2.4.1. *In vitro* regeneration of *C. longa* using axillary buds

In vitro regeneration of *C. longa* was followed different key steps including successful achievement of contamination free axillary buds and optimisation of *in vitro* conditions for stable rate of shoot multiplication of established culture lines.

2.4.1.1. Source of materials and surface sterilization

Collected rhizomes of *C. longa* var. Surma of Bangladesh plsanted to produce fresh rhizomes containing immature sprouted axillary buds in summer in the glasshouse of the Institute of Botany, University of Hannover, Germany. Sprouted immature shoots (ca. 1 cm long axillary buds covered with several leaf sheaths) were collected from the glasshouse and used as the source of material. Buds were cleaned with running tap water and then soaked with detergent (Tween-20) for 5 min and again rinsed the explants thoroughly under running tap water for 2-3 minutes. Explants were then soaked with 70% ethanol for 30-40 sec before submerging the explants into the disinfectant solution of 0.1% $HgCl_2$ to which 2-3 drops of Tween-20 were added in 100 ml solution. Under sterile conditions, $HgCl_2$ solution was decanted and the explants were rinsed 5-6 times with sterile distilled water.

2.4.1.2. Initial culture and regenerations

Sterilized sprouts were dissected under aseptic condition to remove the outer few layers of leaf sheaths. Excised shoot buds were initially cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with 18 µM BA and 0.6 µM NAA, and 3% sucrose. The medium was gelled (solidified) by adding 0.8% agar (Duchefa, NL) after adjusting the pH to 5.8 and sterilized by autoclaving at 121^0C (1.06 Kg^{-1} m^{-2}) for 20 min. Sterile streptomycin sulphate (500 mg l^{-1}) was added to a portion of the autoclaved medium to investigate the effect of the antibiotic in decreasing bacterial contamination. About 25 ml from each of the media with and without antibiotic (M1 and M2 media respectively) were dispensed into the sterile plastic culture tubes 'De Wit' (Duchefa, NL) and initial explants were cultured on these media in order to get contamination free cultures since contamination of the explants taken from underground rhizome was a major problem (Balachandrarn et al., 1990; Salvi et al., 2002). After 4 weeks, contamination free explants were transferred to the bigger container containing M2 medium for another 6 weeks for shoot multiplication. Vigorous multiple regenerates were found around the initial cultures. These multiple shoots were excised and transferred to the M3 medium (M2 medium devoid of growth hormones) and cultured for another 4 weeks to avoid carry over effects of the growth regulators and subsequently used as the source of explants for all experiments.

2.4.1.3. Optimising *in vitro* growth conditions

The effects of different cytokinins were tested using the M2 medium differing only in the concentrations of 6, 12 and 18 µM BA, Kn, 2iP and TDZ. Similarly, auxins NAA, IAA and IBA in the concentrations of 0.1, 0.3 and 0.6 µM were analysed using M4 medium (M2 medium modified with 12 µM BA) to select the suitable auxin. In order to optimise the strength of MS basal medium, explants were cultured on M5 medium (M2 medium modified with 12 µM BA and 0.3 µM NAA) with different strengths as 1.00x, 0.75x, 0.50x, and 0.25x strength of MS salts. To test the effect of agar concentrations, explants were cultured on M6 medium (used 0.75x strength of MS salts) solidified with 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5% agar. The effect of sucrose concentration was also tested by growing the explants on M6 medium, and the effects of different concentrations of 1, 3, 5 and 7 % sucrose were studied.

2.4.1.4. Hardening and establishment of plants in soil

In vitro regenerated plants were washed in tap water to remove agar from roots. Old yellowish leaves were removed and the explants transferred to the pots and stored in a small chamber

maintaining a high percentage of humidity for 4 weeks, followed by the final transplantation to the glasshouse, where they were grown to maturity.

2.4.1.5. Data analysis

Results presented in tables and figures in this paper are pooled mean ± standard error (SE) of two repeated experiments with 15 replications. The statistical significant tests were done through Tukey test at 5% level of significance using SAS statistical software, Release 8 (SAS Institute Inc., Cary, NC).

2.4.2. Microrhizome induction in *C. longa*

2.4.2.1. Initial explants

Axillary buds of *C. longa* var. Surma were used for regeneration of multiple shoots following the above procedure of *in vitro* regeneration. After six weeks multiple shoots were excised and transferred to the M2 medium (M1 medium devoid of growth hormones) and cultured for another 4 weeks to avoid carry over effects of growth regulators and subsequently used for all subsequent experiments using liquid medium in the 100 ml flask.

2.4.2.2. Investigation of the effects of sucrose, BA, Kn, NAA and MS salts

An experiment was carried out to determine the effect of the concentration of sucrose using M3 medium (M1 medium devoid of agar and 0.75x MS salts) with 1, 3, 5, 7, 9 and 11% sucrose concentrations. All treatments were investigated under both complete darkness and 16 h light (50 µM m^{-2} s^{-1}) condition. Sixteen combinations with different concentrations (3 – 18 µM) of BA and Kn alone or in presence of 0.3 µM NAA were investigated under fully dark condition using M4 medium (M3 medium modified with 9% sucrose). Effects of different concentration of 0.3, 0.6, 0.9 and 1.2 µM of NAA were also studied using M5 medium (M4 medium modified with 12 µM BA). In order to optimise the strength of MS basal medium, explants were cultured on M6 medium (M5 medium modified with 0.3 µM NAA) with different strengths as 1.00x, 0.75x, 0.50x, and 0.25x strength of MS salts.

2.4.2.3. Development of the plantlets and glasshouse evaluation

In vitro produced microrhizomes were isolated after 60 days. Harvested microrhizomes were directly transferred to the soil for plantlet development. These potted rhizomes were

maintained in the glasshouse at ambient temperature. After 4 months of culture, the plants developed from three different sizes of 0.5 – 1.0 cm (small), 1.1 – 2.0 cm (medium) and > 2.0 cm (large) microrhizomes were evaluated using various morphological characters.

2.4.2.4. Data analysis

Presented results in the tables and in the figures are the pooled means ± standard errors (SE) of two repeated experiments each with 15 replications with an exception of table 3 in which 10 replicates were used without any repetition. The statistical significant test was done by means of Tukey's test at 5% level of significance using SAS statistical software, Release 8 (SAS Institute Inc., Cary, NC).

2.5. Cryopreservation techniques for *C. longa* germplasm conservation

2.5.1. Establishment of initial explants

Axillary buds of *C. longa* var. Shruma were used for regeneration of multiple shoots following the above procedure of *in vitro* regeneration. After 6 weeks, contamination free explants were transferred to the bigger boxes containing the same medium (M1) for another 6 weeks for shoot multiplication. Multiple vigorous regenerates were found around the initial cultures. These multiples shoots were excised and further incubated in the same medium for 3 weeks to obtain required young axillary buds.

2.5.2. Preconditioning and preculture of the explants

Clamps containing 5 - 7 axillary buds were preconditioned for 6 different time periods of 1, 3, 5, 7, 10 and 15 days in the above mentioned multiplication medium varied with different concentrations of 0.10, 0.15, 0.20, 0.30, 0.40 and 0.50 M sucrose. Single young axillary buds were excised after preconditioning under a stereomicroscope for subsequent cryopreservation experiments. Prepared axillary buds were placed on filter paper wetted with MS medium containing 0.3M sucrose in a Petridish and incubated overnight under normal growth conditions before vitrification treatment. Survival rates of different sizes of axillary buds (< 3 mm, 3-4 mm and > 4 mm) were also investigated in order to select standard size of buds for cryopreservation.

2.5.3. Vitrification procedures

The vitrification procedures involved the following steps: (a) loading of pre-cultured buds with loading solutions containing MS medium components supplemented with 2 M glycerol and 0.4 M sucrose for 20 min with an exception in the case of Steponkus solution where loading solution contained MS medium components supplemented with 1.5 M ethylene glycol (b) dehydration of the loaded axillary buds by concentrated vitrification solutions of PVS (22% w/v glycerol, 15% w/v ethylene glycol, 15% w/v propylene glycol and 7% w/v DMSO) (Uragami et al., 1989) or PVS2 (30% w/v glycerol, 15% w/v ethylene glycol and 15% w/v DMSO) (Sakai et al., 1990) or Steponkus solution (40% w/v ethylene glycol, 15% w/v sorbitol and 6% w/v BSA) (Langis et al., 1990) for 20 min in room temperature (c) optimum PVS2 incubation period was also tested by using a series of time periods of 5, 10, 15, 20, 30 and 40 min in room temperature; (d) treated buds were then transferred to small droplets on an aluminium foil (0.7 cm x 2 cm x 0.03 mm and each foil contained six droplets of 2.5 µl) (e) pre-cooled cryovials were placed in a polystyrene box containing liquid nitrogen and each of aluminium foils bearing the vitrified axillary buds were plunged into a cryovial filled with LN and the lid of the cryovial loosely closed and immediately transferred to the LN storage container for at least one hour. For each experiment, 15 or 20 buds were used with at least three replicates.

2.5.4. Thawing and recovery

Cryovials were transferred from LN storage and opened under sterile conditions. Aluminium foils bearing frozen buds were rapidly submerged upside down in a Petri dish containing MS medium components supplemented with 1.2 M sucrose at room temperature. Each 5 min this unloading solution is refreshed for a total period of 10 min. Unloaded buds were picked up and placed on a sterile filter paper which was laid over the surface of a fresh semi-solid MS medium supplemented with 0.3 M sucrose in a Petri dish for overnight. Axillary buds were then transferred onto semi-solid MS medium supplemented with 0.1 M sucrose, 6 µM BA and 0.3 µM NAA and initially placed in dark for first two weeks at 25 ± 1^0C, then they were placed under 16 h light (white fluorescent light with 50 µM m^{-2} s^{-1} light intensity) / 8 h dark photoperiod. Survival of buds was checked 8- 10 weeks after control treatment or thawing of buds stored in LN. Buds those recovered green colour and growth up to that time were considered to have survived.

2.5.5. Data analysis and statistical procedure

In all experiments measurements for each condition were performed three times, using 15 - 20 buds for each replica. Each experiment was conducted twice. The percentage data were pooled over the two repeated experiments. The obtained percentage values were tested statistically using Tukey's test at 5% level of significance using SAS statistical software, Release 8 (SAS Institute Inc., Cary, NC).

3. GENETIC DIVERSITY ANALYSES USING RAPD MARKERS

3.1. Introduction

3.1.1. Randomly amplified polymorphic DNA (RAPD) as a genetic marker

Assessing the level and distribution of genetic variation within and among populations is a primary goal of population genetics and conservation biology. Last decade, significant progresses have been made on molecular marker based population genetic studies. Among different popular markers, Random Amplified Polymorhic DNA (RAPD) is one of the most useful markers (Welsh and McClelland, 1990; Williams et al., 1990) which may have its most significant impact in conservation biology since it provides robust data for this purpose. Ultimately the number of loci available for sampling is virtually unlimited which makes RAPD particularly valuable when isozyme profiles are completely or nearly monomorphic. However, there are two weaknesses in the standard use of the RAPD assay for describing the genetic structure of populations relative to isozyme and RFLP e.g. two alleles are only assumed in the case of dominant loci, and heterozygotes are not usually detectable with RAPD using conventional procedures. Therefore, the mean number of alleles per locus and observed heterozygosity are two standard measures in population genetics are not applicable to RAPD data. Consequently, useful statistics such as percentage of polymorphic loci and expected heterozygosity cannot be calculated without invoking additional assumptions. The inability to detect heterozygosity does not mean that estimates of genetic diversity cannot be calculated, but it does mean that the statistical power of such calculations will be reduced relative to codominant markers systems (Lamboy, 1994).

Despite these shortcomings, RAPD has been shown highly useful in studies of genetic variation within species. It is a robust method for testing hypothesis of clonal population structure, and can also be used to assess the distribution of genetic variation within and among populations as long as certain precautions are taken. Lynch and Milligan (1994) have provided the calculation necessary for relatively unbiased measures of population structure including gene diversity within and among populations, population subdivision, degree of inbreeding, and individual relatedness using RAPD approach, as thus RAPD markers are well established and well recognised as important genetic tools. The RAPD procedure neither requires previous genomic knowledge nor radioactivity, rather requires only small amount of

DNA (Caetano-Anolles et al., 1991; Hadrys et al., 1992; Huff et al., 1993). Furthermore, in comparison to codominant markers (isoyme, RFLP), RAPD can be developed quite easily even for species for which no prior genetic information is available and at a relatively low cost (Mueller and Wolfenbarger, 1999).

RAPD bands are produced by PCR, using a single random primer that amplifies segments of DNA flanked by two primer-binding regions that theoretically are exactly complementary to the primer. The primer binding sites must be close enough that amplification proceeds over the entire DNA segment spanning them. Because of base pair mismatch, though a single base change in the genomic DNA can prevent amplification (Williams et al., 1990). Most often, polymorphisms between different DNA samples occur when a segment that is amplified in a sample, whose primer-binding site is exactly complementary to the primer, and not amplified in another sample, whose primer-binding site is not an exact complement to the primer (Williams et al., 1990; Klein-Langhorst et al., 1991). Polymorphisms may also result from deletions within a priming site, insertions that separate priming sites by too great a distance to support amplification, or insertions that change the size of the segment without affecting amplification (Williams et al., 1990). Segments that can be amplified in once genetic background but not in another (Heun and Helentjaris, 1993), polymerase slippage during replication, non template-directed addition of nucleotides by Taq polymerage, or the amplification of *in vitro* recombinants (Riedy et al., 1992) also result production of polymorphisms. Even changes in PCR parameters, such as primer/template ratios, annealing temperature and Mg^{2+} concentration can also produce polymorphisms (Ellsworth et al., 1993). Impurity of DNA containing RNA, polysaccharides and phenolics etc. can also cause polymorhisms (Carlson et al., 1991; Ellsworth et al., 1993). Whatever their source, artifactual RAPD bands belong to one of two types: false positive (bands that appear in a lane but should not) or false negative (bands that do not appear in a lane but should), and this designation is arbitrary to a certain degree (Lamboy, 1994).

There are three similarity measures that are considered for coefficient analysis such as the simple matching coefficient (Williams et al., 1990), Jaccard's coefficient (Jaccard, 1908; Vierling and Nguyen, 1992) and Nei and Li's (i.e. Dice) coefficient (Chalmers et al., 1992; Nei and Li, 1979). Among these coefficient measures Nei and Li's coefficient preferably recommended as it displays less percent of bias than other two measures (Lamboy, 1994).

3.1.2. RAPD PCR Products and data analysis

Studies on estimating genetic diversity have addressed the dominance problem in one of two ways. Hardy-Weinberg equilibrium has been assumed, or band phenotype has been scored as presence/absence characters. The assumption of the Hardy-Weinberg equilibrium allows one to calculate allele frequencies, because the frequency of the null-null genotype for each locus is known (band absent). Under Hardy-Weinberg, the frequency of the null allele is the square root of this frequency of the band allele is therefore one minus the null allele frequency. Once the allele frequency is calculated, it is possible to estimate the expected heterozygosity (H_e) and other descriptors dependent on allele frequency. Lynch and Milligan (1994) have provided the calculation necessary for relatively unbiased measures of population structure using this approach including gene diversity within and among populations, population subdivisions, degree of inbreeding, and individual relatedness. However, they emphasized that for the measures to be valid the marker alleles for most dominant loci should be in low frequency (e.g. <0.94 for N=50). Furthermore, two to ten times as many individuals (ideally at least 100) must be sampled per locus for dominant markers (Fritsch and Rieseberg, 1996).

Several methods of analysis have been employed for estimating genetic diversity based on band on band phenotype such as Shannon´s information measure (Lewontin, 1972), Nei´s gene diversity statistics (Nei, 1973) and Φ statistics from AMOVA (Exocoffier and Smouse, 1994). The advantage of using these methods is that the Hardy-Weinberg assumption is not needed. Out of these three measures, AMOVA shows the most promise, since measures of diversity can be tested for significance (Excoffier et al., 1992). This method has been applied in many recent studies on natural plant populations (Fritsch and Reiseberg, 1996). The main drawback with RAPD procedure is their dominant nature, which does not provide any genotypic information and thus hinders several population genetic investigations which require heterozygosity data and eventually RAPD have found to be little useful in estimation of breeding systems (see Fritsch and Reiseberg, 1992; Gaiotto et al., 1997). They are also incompatible to estimate the population genetic parameters such as F-statistics and G_{ST} unless assumptions are made regarding levels of selfing and null homozygote frequencies and their relationship to heterozygosity (Clark and Lanigan, 1993; Lynch and Milligan, 1994). Another method for partitioning variation, AMOVA (Exocoffier and Smouse, 1994) also requires the same assumptions. Thus, RAPD have been used for population genetic assessment and quantification in the cases where the results have been interpreted either in light of prior knowledge of breeding systems or genotypic data were available (Bussell, 1999).

However, an alternative approach for quantifying and partitioning of genetic diversity that neither requires any assumption of breeding nor estimation of heterozygosity is Shannon´s Index (Bussell, 1999). Dawson et al. (1993) reported that Shannon´s Index was relatively insensitive to skewing effects caused by the inability to detect heterozygous loci. Shannon´s Index is preferably applicable for partitioning genetic diversity of plants using data generated by different markers, such as allozymes (Brown and Weir, 1983) rDNA (King and Schaal, 1989; Gustafsson and Gustafsson, 1994), AFLPs (Travis et al., 1996), and RAPDs (Chalmers et al., 1992; Kwon and Morden, 2002; Martin et al., 1997; Wolff et al., 1997).

3.1.3. Purposes of this study

In this study, an investigation was made on genetic diversity and relations among different *Curcuma* species that occurred in Bangladesh using RAPD technique. The main goal of this study was to investigate questions concerning genetic variation of the species. Major questions were: a) do *Curcuma* species contain low genetic variation within species as they are clonally reproduced plant, b) do they possess significant genetic variation between species c) do wild/cultivated and rare/dominant species comprise significant genetic differentiation. In addition to these primary goals, the relation among species was also investigated to make available some basic information on the genetic diversity of the genus *Curcuma* in Bangladesh.

Furthermore, the population genetic structures of extremely differentially adapted populations of *C. zedoaria* in Bangladesh were investigated using the RAPD technique. The main goal of this study was to investigate questions concerning the population genetic variation of the species to support further conservation programmes. Major questions were: a) does *C. zedoaria* contain low genetic variation within the populations as it is a clonally reproduced plant, b) does it possess a significant genetic variation among the populations of different ecogeographical habitats and c) do the non hilly anthropogenic populations have less genetic diversity than that of natural hilly populations. Extending to that, the relations among individuals as well as populations were investigated to make available some basic information on the genetic diversity of *C. zedoaria* populations from Bangladesh.

3.2. Results

3.2.1. Genetic variation among different *Curcuma* species in Bangladesh

3.2.1.1. The RAPD profile of different *Curcuma* species

In this study a total sixteen species including 96 individual accessions were used. The details of the species and the accession number are presented in the Table 3.1. Among the studied species, thirteen species were wild and the rest of three species cultivated. However, the most widely cultivated species in Bangladesh is *C. longa*. Geographically, the hill tracts area are more diverged containing higher number of species in comparison to plain land and Pleistocene Plateau land.

Among the tested primers, a total of 13 primers showed reliable banding patterns with high reproducibility and clear band resolution were used for further analyses. These 13 primers produced a total of 217 distinct amplification products ranging from 300 to 2000 bp. Some representative agarose gels are presented in the Fig 3.1. The number of scored bands per primer ranged from 11 (P24) to 24 (OPX 14), with a mean number of 16.70 per primer (Table 3.2). These two primers are also representatives of the lowest (9) and the highest (22) number of polymorphic products respectively. Among these amplified products, a total of 191 were polymorphic. The average number of polymorphic markers across the primers was 87.65%, ranging from 75% produced by the primer OPX 19 to 100% obtained by OPX 04.

3.2.1.2. Genetic distance among the species

A distance matrix of different species was obtained by using the POPGEN ver. 1.32 (Yeh et al., 1999) which is presented in Table 3.3. Among different *Curcuma* species, the lowest distance (0.040) was observed in between species *C. angustifolia* (Sp02) and *C. zedoaria* (Sp09) and the highest distance (0.520) between *C. petiolata* (Sp10) and *C. amada* (Sp16). Using this matrix data a Neighbour Joining tree was constructed via MEGA 2.1 (Kumar et al., 2001), which presented in Fig 3.2. The cultivated species *C. longa* (Sp14), *C. aromatica* (Sp15), *C. amada* (Sp16) are separated from the wild species, however *C. amada* is the distinct species in the tree, while *C. longa* and *C. aromatica* are more close to each other among these three species. Two more distinct clusters were also found in the tree. The first cluster contained the species an unidentified *Curcuma* sp. (Sp06) and *C. caesia* (Sp13) whereas the second cluster are more divergent comprising the rest of species of *C. elata*

(Sp01), *C. angustifolia* (Sp02), *C. zanthorrhiza* (Sp03), *C. australasica* (Sp04), *C. viridiflora* (Sp05), *C. latifolia* (Sp07), *C. rubescens* (Sp08), *C. zedoaria* (Sp09), *C. petiolata* (Sp10), *C. aeruginosa* (Sp11) and *C. amarissima* (Sp12). Within the last large cluster *C. petiolata* is distinct while *C. elata*, *C. angustifolia*, *C. zanthorrhiza, C. latifolia* and *C. zedoaria* grouped together in a sub cluster. One more nearest sub cluster of this contained the species *C. australasica* and *C. viridiflora*.

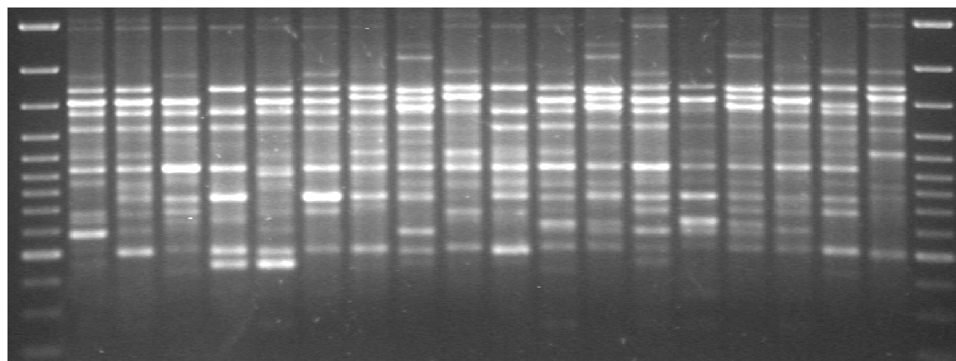
3.2.1.3. Genetic variation within species

Estimation of genetic diversity within each population was obtained by following Shannon's Information Index (Lewontin, 1972). The results for each primer and the mean values (*H'*) averaged across markers are presented in Table 3.4. The RAPD primers varied in their power to detect diversity within populations. Averaged over all markers, *C. zedoaria* (Sp09) displayed the highest genetic diversity (0.335 ± 0.117) and the unidentified *Curcuma sp.* (Sp06) the lowest (0.018 ± 0.028) diversity.

Table 3.1. Different *Curcuma* species and the accessions used in this study

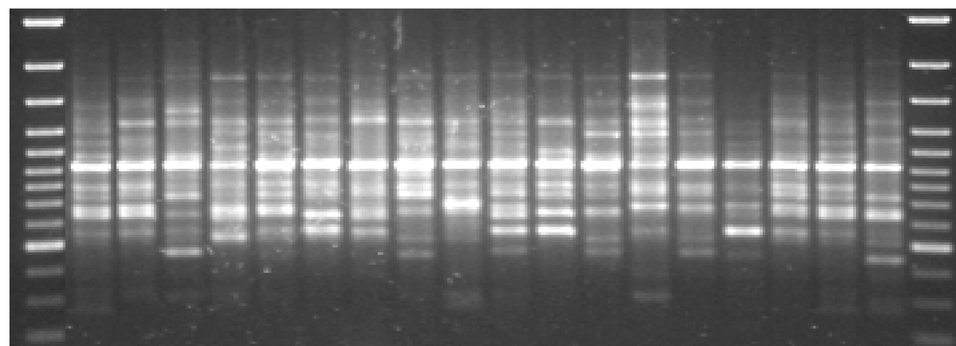
Species	Scientific name	Accession No.	Distribution	Remarks
Sp01	*C. elata* Roxb.	S03, S06, S08, S09, S10, S11	Srimangal Sylhet	Wild
Sp02	*C. angustifolia* Roxb.	C32, S02, S04, S07, S17, S21, S22, S23, S24, S27, S32, S33, S36, S41, S42	Chittagong Srimangal	Wild
Sp03	*C. zanthorrhiza* Roxb.	S01, S05, S13, S14, S25, S26	Srimangal	Wild
Sp04	*C. australasica* Hook f.	S35, S38	Srimangal	Wild
Sp05	*C. viridiflora* Roxb.	S20, S44, D01	Srimangal Dinajpur	Wild
Sp06	*Curcuma sp.*	C03, C09	Chittagong	Wild
Sp07	*C. latifolia* Rosc.	C10, C17, C30, C31	Chittagong	Wild
Sp08	*C. rubescens* Roxb.	C08, R03	Chittagong Dinajpur	Wild
Sp09	*C. zedoaria* (Chrism.) Rosc.	S15, S18, S28, S29, S30, S31, S34, S37, C01, C02, C11, C12, C13, C15, C19, C16, C20, C21, C22, C23, C24, C25, C27, C28, C29, C26, C33, C35, C36, C37, D02, D03, D04, D05, D06, D08, R01, R02, R04, R05, R06, R07	Chittagong, Dhaka, Srimangal, Dinajpur	Wild
Sp10	*C. petiolata* Roxb.	C14	Chittagong	Wild
Sp11	*C. aeruginosa* Roxb.	C04, C06, C07, G04, G06, G07	Chittagong, Gazipur	Wild
Sp12	*C. amarissima* Rosc.	G03, G05	Gazipur	Wild
Sp13	*C. caesia* Roxb.	C05	Chittagong	Wild
Sp14	*C. longa* Linn.	G01, G09	Gazipur	Cultivated
Sp15	*C. aromatica* Salisb.	C18	Chittagong	Cultivated
Sp16	*C. amada* Roxb.	G02	Gazipur	Cultivated

A



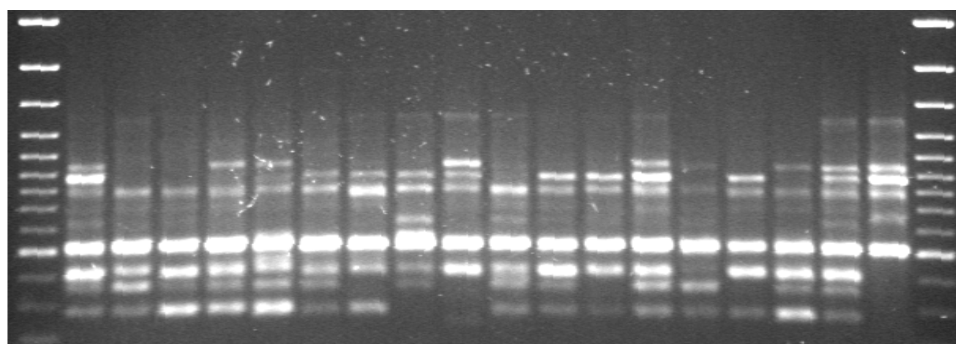
M C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12 C13 C14 C15 C16 C17 **C18 M**

B



M C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12 C13 C15 C14 C16 C17 **C18 M**

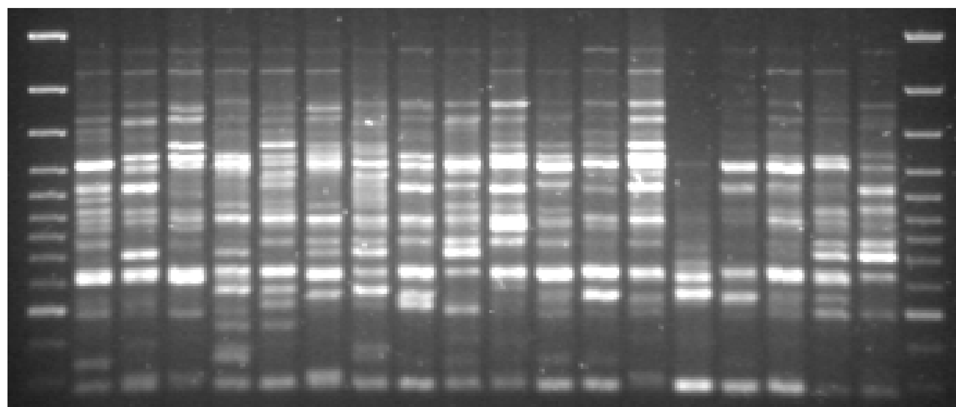
C



M C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12 C13 C14 C15 C16 C17 **C18 M**

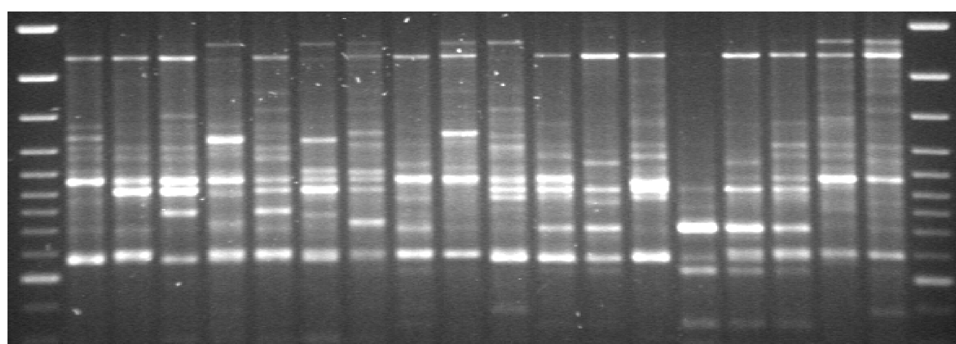
Fig 3.1. Results of gel electrophoresis of PCR products obtained by using primers A) OPX 01, B) OPX 08 and C) OPX 11; Accessions contained *C. zedoaria* (C1, C2, C11, C12, C15, C16), *Curcuma sp*. (C03, C09), *C. aeruginosa* (C04, C06, C07), *C. ceasia* (C05), *C. rubescens* (C08), *C. latifolia* (C10, C17), and *C. aromatica* (C18); M-100 bp ladder plus (Figure continued in next page)

D



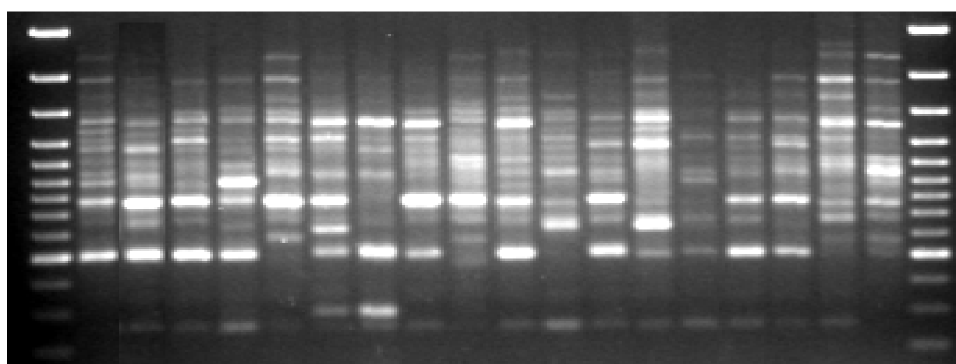
M C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12 C13 C14 C15 C16 C17 **C18 M**

E



M C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12 C13 C14 C15 C16 C17 **C18 M**

F



M C01 C02 C03 C04 C05 C06 C07 C08 C09 C10 C11 C12 C13 C14 C15 C16 C17 **C18 M**

Fig 3.1. Results of gel electrophoresis of PCR products obtained by using primers D) OPX 12, E) OPX 14 and F) OPX 15; Accessions contained *C. zedoaria* (C1, C2, C11, C12, C15, C16), C. sp. (C03, C09), *C. aeruginosa* (C04, C06, C07), *C. ceasia* (C05), *C. rubescens* (C08), *C. latifolia* (C10, C17), and *C. aromatica* (C18); M-100 bp ladder plus M-100 bp ladder plus

Table 3.2. RAPD primers used in the study; number of amplified products, number of polymorphic bands and percentage of polymorphism scored from agarose gels

Primers	Sequence	No of bands	Polymorphic bands	Polymorphism (%)
OPX 01	5´-CTG GGC ACG A-3´	16	15	93.75
OPX 03	5´-TGG CGC AGT G-3´	15	14	93.33
OPX 04	5´-CCG CTA CCG A-3´	16	16	100
OPX 07	5´-GAG CGA GGC T-3´	17	15	88.23
OPX 08	5´-CAG GGG TGG A-3´	20	18	90
OPX 09	5´-GGT CTG GTT G-3´	13	10	76.92
OPX 11	5´-GGA GCC TCA G-3´	12	10	83.33
OPX 12	5´-TCG CCA GCC A-3´	23	19	82.61
OPX 14	5´-ACA GGT GCT G-3´	24	22	91.66
OPX 15	5´-CAG ACA AGC C-3´	21	19	90.48
OPX 19	5´-TGG CAA GGC A-3´	16	12	75
P 92	5´-CCT GGG CTT T-3´	13	12	92.31
P 24	5´-ACA GGG CTG A-3´	11	9	81.82
Total		217	191	
Mean		16.70	14.70	87.65

Table 3.3. Nei´s (1972) genetic distance matrix of sixteen *Curcuma* Species of Bangladesh calculated by using POPGEN ver.1.32 (Yeh et al., 1999)

Species	Sp01	Sp02	Sp03	Sp04	Sp05	Sp06	Sp07	Sp08	Sp09	Sp10	Sp11	Sp12	Sp13	Sp14	Sp15
Sp02	0.056														
Sp03	0.062	0.044													
Sp04	0.123	0.083	0.121												
Sp05	0.121	0.086	0.118	0.098											
Sp06	0.333	0.278	0.316	0.380	0.372										
Sp07	0.099	0.073	0.095	0.151	0.146	0.274									
Sp08	0.219	0.154	0.180	0.175	0.176	0.360	0.198								
Sp09	0.092	0.040	0.067	0.083	0.068	0.277	0.092	0.124							
Sp10	0.207	0.173	0.174	0.196	0.206	0.412	0.196	0.209	0.136						
Sp11	0.168	0.137	0.153	0.226	0.221	0.305	0.149	0.257	0.115	0.268					
Sp12	0.179	0.155	0.178	0.216	0.189	0.388	0.159	0.228	0.137	0.266	0.213				
Sp13	0.277	0.252	0.284	0.344	0.363	0.363	0.224	0.296	0.266	0.311	0.308	0.303			
Sp14	0.334	0.282	0.334	0.360	0.374	0.482	0.312	0.364	0.294	0.431	0.342	0.389	0.416		
Sp15	0.373	0.343	0.391	0.425	0.406	0.427	0.329	0.413	0.349	0.431	0.370	0.381	0.403	0.278	
Sp16	0.382	0.336	0.388	0.427	0.434	0.447	0.356	0.465	0.352	0.520	0.405	0.391	0.490	0.306	0.475

3.2.1.4. Partitioning of genetic diversity based on Shannon's index

Average values for intra-specific diversity (H'_{pop}) and diversity values in the whole samples (H'_{sp}) were used to calculate the level of genetic diversity within and between species. Results obtained for the RAPD analyses of each primer are shown in Table 3.5. Values for H'_{pop} / H'_{sp} were found to vary for different primers ranging from 31% (OPX01) to 61% (P92) of total diversity was found within species. However, all the primers detected more variability

between rather than within species. The mean overall markers value for the within species variation was 0.453 ± 0.074, while that between species G_{ST} (H'_{sp} - H'_{pop} / H'_{sp}) value was 0.547 ± 0.074.

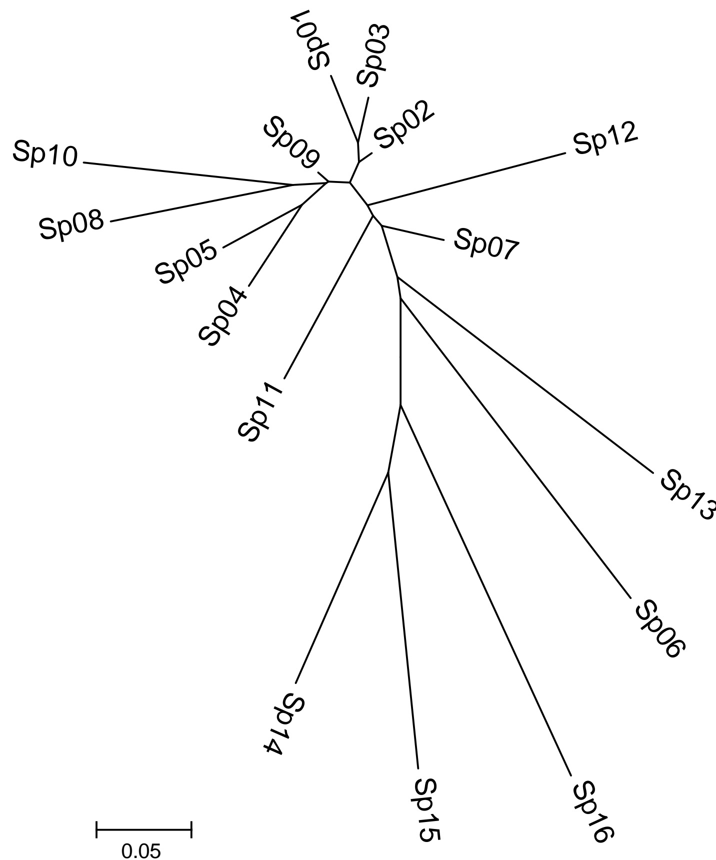


Fig 3.2. The Neighbour Joining tree constructed via MEGA 2.1 (Kumar et al., 2001) using the data of lower diagonal distance matrix of sixteen species calculated by POPGEN 1.32 (Yeh et al., 1999): *C. elata* (Sp01), *C. angustifolia* (Sp02), *C. xanthorrhiza* (Sp03), *C. australasica* (Sp04), *C. viridiflora* (Sp05), *Curcuma sp.*(Sp06), *C. latifolia* (Sp07), *C. rubescens* (Sp08), *C. zedoaria* (Sp09), *C. petiolata* (Sp10), *C. aeruginosa* (Sp11), *C. amarissima* (Sp12), *C. caesia* (Sp13), *C. longa* (Sp14), *C. aromatica* (Sp15), *C. amada* (Sp16)

3.2.1.5. Principal Coordinate Analysis (PCoA)

The Principal Coordinate Analysis (PCoA) was utilised through NTSYS pc ver. 2.0 (Rohlf, 2000) to discriminate all individuals of sixteen species (Fig 3.3). The individuals of cultivated species *C. longa,* and *C. aromatica* are found to be distinct in the coordinate plot, while *C. amada* reasonably distinct from them with a close association with Sp06 (unidentified species) and *C. aeruginosa*. The individuals *C. australasica*, *C. viridiflora*, *C. latifolia* and *C.*

caesia are also to some extent distinct in the plot, while *C. elata*, *C. angustifolia*, *C. zanthorrhiza*, *C. rubescens*, *C. zedoaria*, *C. petiolata* and *C. amarissima* showed a mixed distribution pattern from where they could not be discriminated.

Table 3.4. Shannon's Information Index (Lewontin, 1972) of different species of *Curcuma* calculated using POPGEN version 1.32 (Yeh et al., 1999); SD(±): standard deviations

Primers	H′ (Shannon's index)											
	Sp01	Sp02	Sp03	Sp04	Sp05	Sp06	Sp07	Sp08	Sp09	Sp11	Sp12	Sp14
OPX 01	0.122	0.287	0.128	0.076	0.060	0.000	0.038	0.000	0.357	0.207	0.076	0.038
OPX 03	0.259	0.330	0.250	0.202	0.277	0.000	0.361	0.186	0.391	0.312	0.040	0.000
OPX 04	0.394	0.503	0.294	0.265	0.260	0.038	0.443	0.216	0.549	0.414	0.151	0.076
OPX 07	0.177	0.265	0.338	0.142	0.096	0.000	0.158	0.202	0.393	0.286	0.050	0.285
OPX 08	0.288	0.391	0.298	0.151	0.163	0.091	0.264	0.134	0.414	0.141	0.091	0.121
OPX 09	0.046	0.107	0.052	0.000	0.073	0.000	0.107	0.110	0.060	0.197	0.093	0.093
OPX 11	0.121	0.255	0.157	0.101	0.096	0.050	0.209	0.060	0.302	0.170	0.050	0.000
OPX 12	0.201	0.211	0.155	0.026	0.130	0.000	0.232	0.173	0.304	0.198	0.026	0.026
OPX 14	0.180	0.321	0.296	0.176	0.190	0.025	0.224	0.082	0.316	0.169	0.176	0.050
OPX 15	0.203	0.220	0.189	0.144	0.175	0.029	0.194	0.064	0.345	0.228	0.029	0.000
OPX 19	0.259	0.182	0.085	0.038	0.162	0.000	0.112	0.000	0.197	0.229	0.000	0.113
P 92	0.272	0.385	0.442	0.326	0.231	0.000	0.390	0.055	0.420	0.435	0.140	0.279
P 24	0.214	0.224	0.310	0.055	0.149	0.000	0.127	0.134	0.304	0.284	0.055	0.000
Mean	0.210	0.283	0.230	0.131	0.159	0.018	0.220	0.109	0.335	0.252	0.075	0.083
SD(±)	0.088	0.104	0.113	0.096	0.069	0.028	0.120	0.073	0.117	0.092	0.053	0.098

Note: Sp10, Sp13 and Sp16 were not used due to lack of required individuals.

Table 3.5. Partitioning of the genetic diversity (Shannon's index) within and between of *Curcuma* species; SD(±): standard deviations

Primers	H'_{pop}	H'_{sp}	H'_{pop} / H'_{sp}	$G_{ST}(H'_{sp} - H'_{pop}) / H'_{sp}$
OPX 01	0.116	0.368	0.315	0.685
OPX 03	0.217	0.446	0.487	0.513
OPX 04	0.300	0.564	0.532	0.468
OPX 07	0.199	0.419	0.475	0.525
OPX 08	0.212	0.462	0.459	0.541
OPX 09	0.078	0.172	0.453	0.547
OPX 11	0.131	0.360	0.364	0.636
OPX 12	0.140	0.344	0.407	0.593
OPX 14	0.184	0.425	0.435	0.567
OPX 15	0.152	0.373	0.408	0.592
OPX 19	0.115	0.234	0.491	0.509
P 92	0.281	0.460	0.611	0.389
P 24	0.155	0.345	0.450	0.551
Mean	0.175	0.382	0.453	0.547
SD(±)	0.065	0.101	0.074	0.074

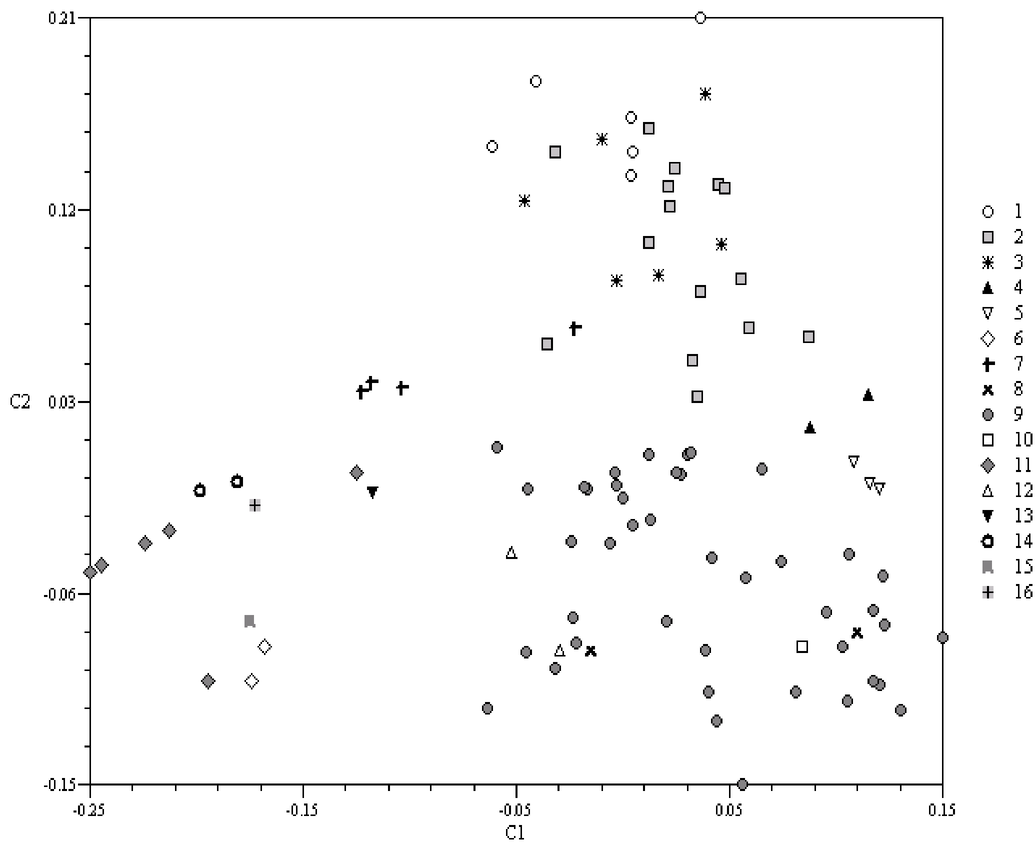


Fig 3.3. Principal coordinate analysis (PCoA) using RAPD data of 96 individuals of *Curcuma* accessions collected from Bangladesh; 1. *C. elata* (Sp01), 2. *C. angustifolia* (Sp02), 3. *C. zanthorrhiza* (Sp03), 4. *C. australasica* (Sp04), 5. *C. viridiflora* (Sp05), 6. *Curcuma sp.* (Sp06), 7. *C. latifolia* (Sp07), 8. *C. rubescens* (Sp08), 9. *C. zedoaria* (Sp09), 10. *C. petiolata* (Sp10), 11. *C. aeruginosa* (Sp11), 12. *C. amarissima* (Sp12), 13. *C. caesia* (Sp13), 14. *C. longa* (Sp14), 15. *C. aromatica* (Sp15), 16. *C. amada* (Sp16)

3.2.1.6. AMOVA analysis

AMOVA was performed in order to test the significance of the partition of genetic variance resulting from groupings of the species based on cultivated/wild and dominant/rare characteristics (Table 3.6). Different arrangements of species into groups demonstrated significant values ($P<0.001$). It was observed that the wild and cultivated species are significantly partitioned (Φ_{CT} value 0.265, $P<0.003$) while rare and dominant species partitioning was not significantly different (Φ_{CT} value 0.020, $P<0.001$). It is also evident that in all cases the highest level of genetic variability was attributed within species, i.e. differences among individual plants within species 58.90 and 79.55 % of the total variation for cultivated/wild and dominant/rare groupings respectively. In case of wild/cultivated species grouping remarkable genetic differences (26.48 %) was attributed to among groups, in contrast, rare/dominant plant grouping did not show any significant partition (2.05 %) among

groups. In both scenarios of wild/cultivated and rare/dominant, the Φ_{ST} values were considerably high (0.411 $P<0.001$, and 0.205, $P<0.001$ respectively) which indicated a great genetic differentiation among the species according to the interpretation of Wright (1978).

Table 3.6. Analyses of molecular variance (AMOVA) for wild and cultivated species of *Curcuma* species using ARLEQUIN ver. 2.000 (Schneider et al., 2000)

Source of variation	d.f.	Sum of squares	Variance	% of total variation	Φ Statistics	*P*
Wild/ cultivated species						
Among groups	1	113.69	10.13	26.48	Φ_{CT} 0.265	<0.003
Among species within groups	14	713.97	5.59	14.62	Φ_{SC} 0.199	<0.001
Within species	80	1803.04	22.54	58.90	Φ_{ST} 0.411	<0.001
Rare/dominant species						
Among groups	1	51.04	0.58	2.05	Φ_{CT} 0.020	<0.035
Among species within groups	11	589.18	5.24	18.40	Φ_{SC} 0.188	<0.001
Within species	79	1788.54	22.64	79.55	Φ_{ST} 0.205	<0.001

3.2.2. Genetic variation of *C. zedoaria* (Christm.) Rosc.

3.2.2.1. Genetic diversity of *C. zedoaria* populations

Five populations with a total of 42 individual accessions were used to analyse population genetic structures of *C. zedoaria*. The details of the collection sites and exact geographical locations are presented in Table 3.7 and Fig 3.4. Among these, populations of hill tract areas are much bigger and more diverse and widely distributed than that of Pleistocene plateau and plain land populations. Hill tracts populations comprise higher number of individuals and estimates have been made up to 50,000 - 80,000 for the population Chittagong, the highest number of individuals in a single population. Sitakundu and Srimangal populations are two comparable populations contained about 10,000-20,000 individuals, whereas populations Savar (plain land) and Birganj (plateau land) cover only about 2,000-3,000 individuals.

3.2.2.2. The RAPD profile of *C. zedoaria* populations

Among the tested primers a total of 13 primers showed consistent banding patterns with high reproducibility and clear band resolution that were used further to pursue detail analysis. These 13 primers produced a total of 189 distinct amplification products ranging from 300 to 2000 bp. The number of scored bands per primer ranged from 7 for OPX 09 to 21 for OPX 14, with a mean number of 14.5 per primer (Table 3.8). These two primers are also

representatives of the lowest (4) and the highest (18) number of polymorphic products respectively. Among these amplified products, a total of 151 were polymorphic. The average number of polymorphic markers across the primers was 78.2%, ranging between 57.5% produced by the primer OPX 09 and 100% obtained by OPX 14. No polymorphic product was fixed exclusively in a single population.

Table 3.7. Accessions of *C. zedoaria* (Chrism.) Rosc. collected from five different populations; regional and geographical locations of the sampling areas

Populations	No	Accession code	District	Latitude	Longitude
Srimangal	8	S15, S18, S28, S29, S30, S31, S34, S37	Sylhet	24º 55’ N	91º 55’ E
Chittagong	14	C01, C02, C11, C12, C13, C15, C16 C19, C20, C21, C22, C23, C24, C25	Chittagong	22º 21' N	91º 50' E
Sitakundu	8	C26, C27, C28, C29, C33, C35, C36, C37	Chittagong	22º 35’ N	91º 42’ E
Savar	6	D02, D03, D04, D05, D06, D08	Dhaka	23º 46’ N	90º 23’ E
Birganj	6	R01, R02, R04, R05, R06, R07	Dinajpur	25º 63' N	88º 63' E

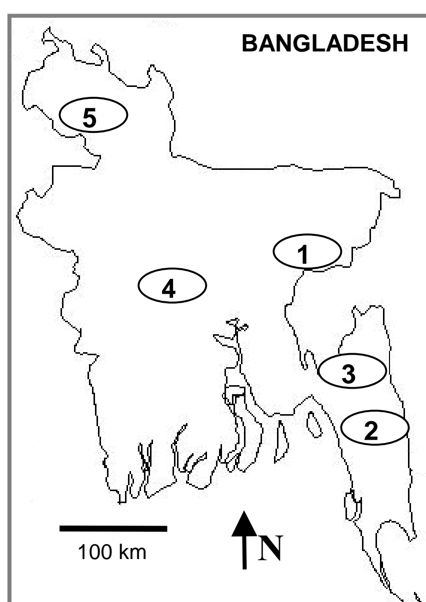


Fig 3.4. Locations of the studied areas in Bangladesh; 1. Srimangal, 2. Chittagong, 3. Sitakundu, 4. Savar, and 5. Birganj populations. Among these populations Srimangal, Chittagong and Sitakundu encompass the hilly areas while Savar and Birganj populations are from plain land and plateau areas respectively

Table 3.8. RAPD primers used in the survey of *C. zedoaria*; number of amplified products and percentage of polymorphisms scored from agarose gels

Primer	Sequence	No of bands	Polymorphism (%)
OPX 01	5´-CTG GGC ACG A-3´	15	80.0
OPX 03	5´-TGG CGC AGT G-3´	14	92.9
OPX 04	5´-CCG CTA CCG A-3´	16	100.0
OPX 07	5´-GAG CGA GGC T-3´	15	80.0
OPX 08	5´-CAG GGG TGG A-3´	16	87.5
OPX 09	5´-GGT CTG GTT G-3´	7	57.1
OPX 11	5´-GGA GCC TCA G-3´	10	60.0
OPX 12	5´-TCG CCA GCC A-3´	20	65.0
OPX 14	5´-ACA GGT GCT G-3´	21	85.7
OPX 15	5´-CAG ACA AGC C-3´	20	80.0
OPX 19	5´-TGG CAA GGC A-3´	13	69.2
P92	5´-CCT GGG CTT T-3´	13	92.3
P24	5´-ACA GGG CTG A-3´	9	66.7
Total		189	
Mean		14.5	78.2

The dendrogram based on the RAPD data of all 42 individuals presented in Fig 3.5 which showed three major clusters, where the first and second clusters represent the Chittagong and Sitakundu populations including a single individual of Srimangal population (S37). The third cluster represents individuals from all populations distributed in four sub-clusters. However, among these sub-clusters, Savar and Birganj populations grouped together in a single cluster, though they were also restricted in two different branches.

3.2.2.3. Genetic variation within populations

Estimation of genetic diversity within each population was obtained by following Shannon's Information Index (Lewontin, 1972). The results for each primer and the mean values (H') averaged across markers which are presented in Table 3.9. The RAPD primers varied in their power to detect diversity within populations. Some primers, such as OPX 04 and OPX 08, revealed high diversity level in most of the populations, whereas OPX 01, OPX 09 detected very low variation across the populations. Averaged over all markers, the Chittagong population displayed the highest genetic variation (0.349 ± 0.128) and the Birganj population the lowest (0.149 ± 1.04) diversity. It is also revealed that populations of hilly areas Srimangal, Chittagong and Sitakundu are somewhat natural populations showing very similar level of genetic diversity, whereas the more anthropogenic populations Savar and Birganj showed less genetic diversity. The mean diversity value across the 5 populations was 0.264 ± 0.083.

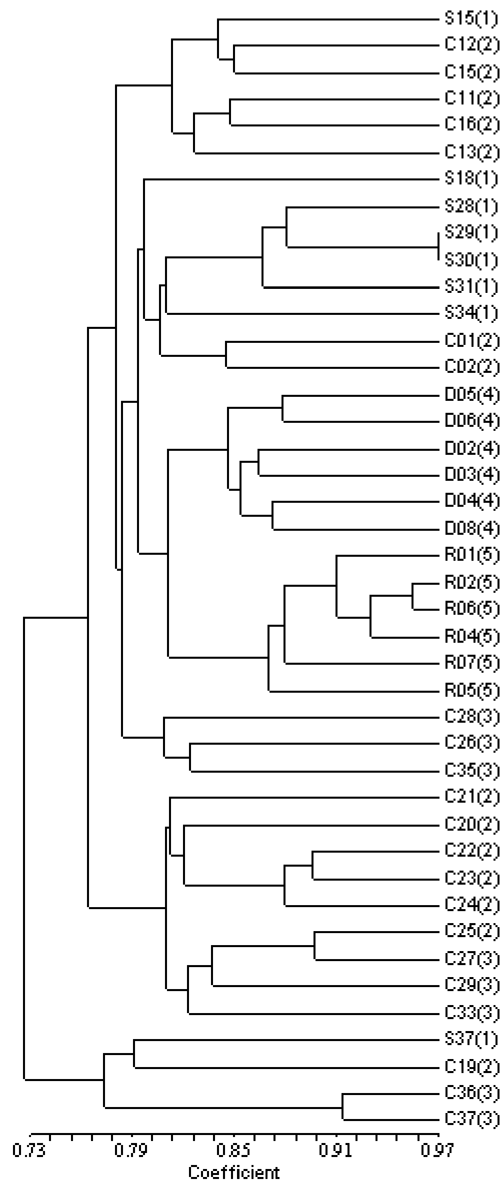


Fig 3.5. Dendrogram showing the relation of 42 individuals of *Curcuma zedoaria* based on Dice (1945) coefficient of similarity matrix performed by NTSYSpc software; 1 – 5 in parenthesis indicate populations of Srimangal (1), Chittagong (2), Sitakundu (3), Savar (4) and Birganj (5)

3.2.2.4. Partitioning of the diversity based on Shannon's index

Average values for intrapopulational diversity (H'_{pop}) and diversity values in the whole samples (H'_{sp}) were used to calculate the level of genetic diversity within and between populations. Results obtained for the RAPD analyses of each primer are shown in Table 3.10. Intrapopulational value (H'_{pop}) across the primers was 0.264 (±0.089) while whole samples (H'_{sp}) value was 0.370 (±0.109). Values for H'_{pop} / H'_{sp} were found to vary for different primers ranging from 53% (OPX 01) to 87% (OPX 09) of total diversity found within

populations. However, all the primers detected more variability within rather than between populations. The mean overall markers value for the within population variation was 0.717 ± 0.090, while that between populations G_{ST} (H'_{sp} - H'_{pop} / H'_{sp}) was 0.283 ± 0.089.

3.2.2.5. Partitioning of genetic diversity based on Nei's genetic diversity analysis

According to Nei's genetic diversity measure, the average values for within population diversity (H_s) and the diversity values in the whole samples (H_t) were employed to compute the intra- and interpopulational diversity. Table 3.11 presents the results that obtained from Nei´s unbiased genetic diversity measures. Across over the primers the mean within population value was found 0.177 (±0.063), whereas the value for whole samples was 0.240 (±0.077). It was observed that within population as well as among populations diversity values obtained from each primer varied remarkably. The values for intrapopulational genetic diversity (H_s/ H_t) were also found to vary in different primers ranging from 57% (OPX01) to 92% (OPX09), which are comparable to the Shannon's information measures. Averaged overall markers value for within population diversity was 74.1% while overall variation between populations G_{ST} (H_t - H_s / H_t) was 25% ranging from 7% to 42%. These results are also very similar to the Shannon's information index. All the primers and also over across ther primers intrapopulatinal variability was found rather than interpopulational variability. These results are comparable to Shannon´s Index.

Table 3.9. Shannon's Information Index (Lewontin, 1972) for different population of *C. zedoaria* calculated using POPGEN version 1.32 (Yeh et al., 1999); SD(±) – standard deviations

Primers	Diversity values (*H´*) of each population				
	Srimangal	Chittagong	Sitakundu	Savar	Birganj
OPX 01	0.193	0.364	0.208	0.080	0.052
OPX 03	0.309	0.419	0.267	0.263	0.064
OPX 04	0.485	0.560	0.568	0.212	0.283
OPX 07	0.354	0.448	0.411	0.283	0.217
OPX 08	0.404	0.434	0.409	0.410	0.351
OPX 09	0.056	0.060	0.211	0.129	0.042
OPX 11	0.346	0.285	0.124	0.151	0.256
OPX 12	0.231	0.337	0.332	0.102	0.109
OPX 14	0.296	0.297	0.328	0.135	0.120
OPX 15	0.285	0.364	0.210	0.189	0.164
OPX 19	0.244	0.242	0.239	0.250	0.052
P 92	0.311	0.483	0.448	0.172	0.193
P 24	0.366	0.250	0.378	0.325	0.033
Mean	0.299	0.349	0.318	0.208	0.149
SD(±)	0.106	0.128	0.123	0.095	0.104

Table 3.10. Partitioning of the genetic diversity (Shannon's index) within and between population of *C. zedoaria* for 13 random primers

Primers	H'_{pop}	H'_{sp}	H'_{pop} / H'_{sp}	G_{ST} $[(H'_{sp} - H'_{pop}) / H'_{sp}]$
OPX 01	0.179	0.337	0.531	0.469
OPX 03	0.264	0.387	0.682	0.318
OPX 04	0.422	0.548	0.771	0.230
OPX 07	0.343	0.442	0.776	0.224
OPX 08	0.402	0.509	0.790	0.210
OPX 09	0.100	0.115	0.870	0.131
OPX 11	0.232	0.346	0.671	0.330
OPX 12	0.222	0.354	0.627	0.373
OPX 14	0.235	0.356	0.660	0.330
OPX 15	0.242	0.357	0.678	0.322
OPX 19	0.205	0.252	0.814	0.187
P92	0.321	0.429	0.748	0.252
P24	0.270	0.384	0.703	0.297
Mean	0.264	0.370	0.717	0.283
SD (±)	0.089	0.109	0.090	0.089

Table 3.11. Partitioning of the genetic diversity based on Nei's unbiased analysis (Nei, 1987) of diversity within and between populations of *C. zedoaria*

Primers	H_s	H_t	H_s / H_t	G_{ST} $(H_s - H_t / H_t)$
OPX01	0.113	0.196	0.576	0.423
OPX03	0.174	0.231	0.753	0.247
OPX04	0.281	0.362	0.776	0.224
OPX07	0.235	0.286	0.822	0.178
OPX08	0.274	0.353	0.776	0.224
OPX09	0.059	0.064	0.922	0.078
OPX11	0.157	0.237	0.662	0.338
OPX12	0.150	0.236	0.636	0.364
OPX14	0.156	0.230	0.678	0.322
OPX15	0.164	0.230	0.713	0.287
OPX19	0.128	0.160	0.800	0.123
P92-1	0.208	0.277	0.751	0.249
P24-1	0.198	0.260	0.762	0.238
Mean	0.177	0.240	0.741	0.253
SD (±)	0.063	0.077	0.089	0.096

3.2.2.6. Pairwise migration (N_m) values, and genetic - geographic distances

It can be summarised that the effective number of migrants (Nm) between the five populations of *C. zedoaria* calculated from pairwise Φ_{ST} (analogue of Wright's F_{ST}) and relative geographic distances of the studied population (Table 3.12). The presented results indicate a positive correlation between genetic and geographic distances as well as the sample size. The lowest genetic distance was found between populations Chittagong and Sitakundu that are

also the geographically closest. Overall pairwise genetic distance markedly varied (2 – 33%) between the populations. The Birganj population was found genetically more distant to other populations, which supported the geographical distribution pattern of the populations. The Neighbour Joining (NJ) tree (Fig 3.6) was constructed using the genetic distance matrix through MEGA 2.1 (Kumar et al., 2001), where the populations showed two distinct clusters. The first cluster includes all hilly populations (Srimangal, Chittagong and Sitakundu), while the non-hilly populations of plain land (Savar) and plateau land (Birganj) are reasonably closely associated. However, the plateau land population is rather distinct in the tree.

Table 3.12. The effective number of migrants (*N*m) between the five populations of *C. zedoaria* (above diagonal) calculated from pairwise Φ_{ST} (analogue of F_{ST}) values (below diagonal); values within parenthesis are the geographical distances between populations

Populations	Srimangal	Chittagong	Sitakundu	Savar	Birganj
Srimangal	-	5.129	4.043	2.335	1.212
Chittagong	0.089 (220)	-	20.889	2.444	1.345
Sitakundu	0.110 (150)	0.023 (80)	-	2.651	1.236
Savar	0.176 (180)	0.170 (350)	0.159 (280)	-	1.007
Birganj	0.292 (450)	0.271 (600)	0.288 (520)	0.332 (320)	-

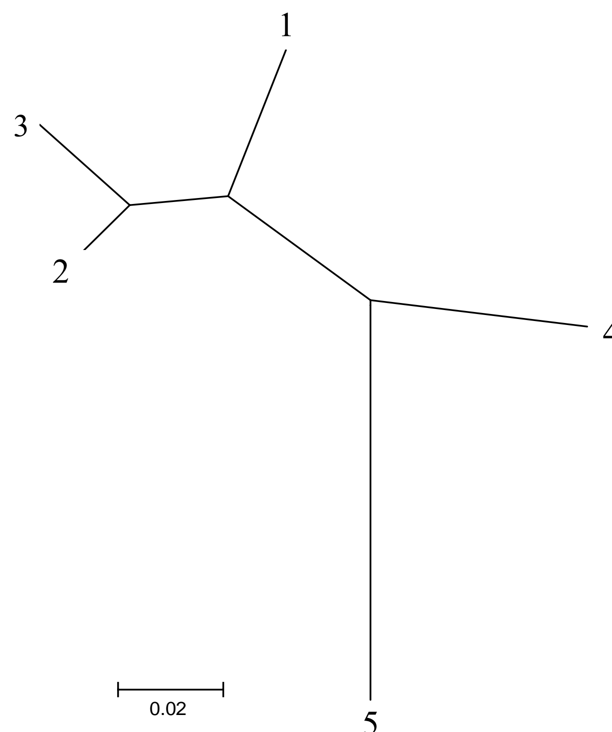


Fig 3.6. The Neighbour Joining (NJ) tree constructed via MEGA 2.1 (Kumar *et al.* 2001) using the data of lower diagonal distance matrix calculated by POPGEN 1.32 (Yeh et al., 1999); 1. Srimangal, 2. Chittagong, 3. Sitakundu, 4. Savar and 5. Birganj populations

3.2.2.7. Principal Coordinate Analysis (PCoA)

The Principal Coordinate Analysis (PCoA) was used to discriminate all individuals of the five different populations (Fig 3.7). The populations of hilly areas (Srimangal, Chittagong and Sitakundu) produced a mix distribution pattern from where they could not be discriminated, whereas the populations of plain land (Savar) and plateau land (Birganj) were very distinctively discriminated in the 2-D ordination plot. PCoA results imply that there are three distinct groups of populations: hilly, plain land and plateau land. The three hilly populations showed a very similar pattern of genetic diversity and comprised a similar pattern of ecological adaptations along with rigorous genetic interactions. In contrast, plain land and plateau land populations are more distinct without having any remarkable interactions amongst them.

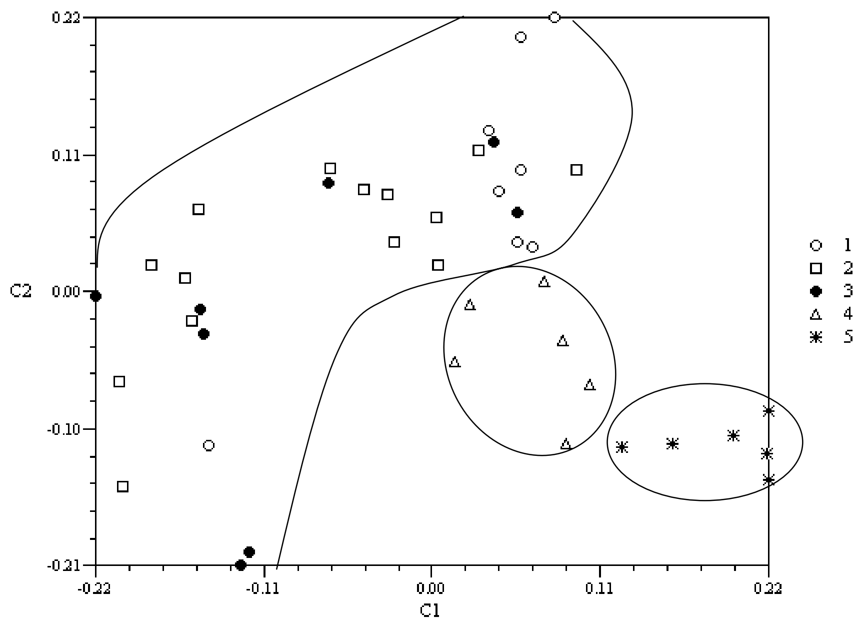


Fig 3.7. Principal coordinate analysis (PCoA) using RAPD data of 42 individuals of *Curcuma zedoaria* from five populations; 1-Srimangal, 2- Chittagong, 3- Sitakundu, grouped together where 4- Savar and 5- Birganj placed in separate groups

3.2.2.8. AMOVA analysis

AMOVA was performed in order to test the significance of the partition of genetic variance resulting from groupings of populations based on regional, eco-geographical or edaphic characteristics (Table 3.13). The results revealed different arrangements of populations into groups that demonstrated significant values ($P<0.001$). It was observed that the populations are moderately partitioned into regional (Φ_{CT} value 0.153, $P<0.001$) and edaphic (Φ_{CT} value

0.142, *P*<0.001) groups, while they were not very strict in eco-geographical attribute (Φ_{CT} value 0.093, *P*<0.001). It is also evident that in all cases the highest level of genetic variability was attributed within populations, i.e. differences among individual plants within populations (78.33 - 81.22 % of the total variation). But in case of regional and edaphic partioning an appreciable amount still separates groups (15.27 and 14.17 % respectively), and the differences among groups were small (3.51 and 7.49). In case of eco-geographical partition the diversity observed among groups was only 9.3 % of the total variation and was lower than the variation among populations within groups (11.05 %). In all cases of regional, eco-geographical and edaphic scenarios, the Φ_{ST} values (0.188 – 0.217, P<0.001) indicated a great genetic differentiation among populations according to the interpretation of Wright (1978).

Table 3.13. Analyses of molecular variance (AMOVA) for *C. zedoaria* populations under three alternative groupings using ARLEQUIN ver, 2000 (Excoffier et al., 2000)

Source of variation	d.f.	Sum of squares	Variance	% of total variation	Φ Statistics	*P*
Regional partition (Ch,Si/Sr/Dh/Bi)						
Among groups	3	193.0	3.993	15.27	Φ_{CT} 0.153	<0.001
Among populations within groups	1	306	0.917	3.51	Φ_{SC} 0.042	<0.003
Within populations	37	785.8	21.238	81.22	Φ_{ST} 0.188	<0.001
Eco-eographical partition (Ch,Si,Sr/Dh,Bi)						
Among groups	1	85.6	2.488	9.33	Φ_{CT} 0.093	<0.001
Among populations within groups	3	138.0	2.947	11.05	Φ_{SC} 0.122	<0.001
Within populations	37	785.81	21.238	79.63	Φ_{ST} 0.204	<0.001
Edaphic partition (Ch,Si,Sr/Dh/Bi)						
Among groups	2	142.1	3.843	14.17	Φ_{CT} 0.142	<0.001
Among populations within groups	2	81.5	2.031	7.49	Φ_{SC} 0.087	<0.001
Within populations	37	785.8	21.238	78.33	Φ_{ST} 0.217	<0.001

3.3. Discussions

3.3.1. Genetic variation among *Curcuma* species

In this study, the genetic diversity and relationships of different *Curcuma* species were investigated using RAPD technique. A total of sixteen species and a total of 96 individuals were used to investigate the genetic relationship and distance. Thirteen random primers were used in this study, which differed to a large extent in their ability to reveal diversity between species. This inferred the need of using a large number of RAPD primers in order to avoid bias in the inference of genetic parameters. Landry and Lapointe (1996) compared several coefficients for RAPDs data and suggested using of Dice (Dice, 1945) and Jaccard (Jaccard,

1908) coefficients along with no less than 12 primers. Calculation of the present study was followed Dice coefficient (Dice, 1945) to develop pairwise distance matrix of *Curcuma* species. The Neighbour Joining tree of sixteen species inferred that the cultivated species are genetically distinct from wild species.

Fig 3.8 illustrates the genetic as well as morphological relationships of sixteen species found in Bangladesh. Among the studied species, *C. longa, C. amada* and *C. aromatica* are three closely related species; however, the species *C. amada* is furthest among them. This result strongly supports the morphological characters of the species. For example these three species contain high amount of curcumin in the underground rhizomes with orange yellow colour. In addition, none of them contain any purple strip or any band in the leafy stem as well as in the leaf. Among these three species, *C. longa* and *C. aromatica* comprise larger size of rhizome with deep orange yellow colour while *C. amada* contains slightly light yellow colour with the flavour of young green mango. These morphological traits support RAPD data. However, the leaf morphological characters do not agree with the present results since the leaf of *C. aromatica* is comparatively larger and wide, hairy and abaxially pubescent whereas, *C. longa* and *C. amada* comprise very similar leaves which are narrowly lanceolate and abaxially glabrous. These findings also have disagreement with the result of Cao et al. (2001) where they found distinct clustering in between *C. longa* and *C. aromatica*.

Among the clusters of wild species *C. caesia* and one unidentified *Curcuma* species are separated from other wild species. *C. caesia* comprises unique characters of leaf containing very prominent dark and wide purple strip along the mid rib which can be a distinguishing character of this species. One closely associated species group in the dendogram contained *C. elata*, *C. angustifolia*, *C. zanthorrhiza* and *C. zedoaria*. This clustering supports their morphology since they have in general very large clumps of leafy stiffs and large rhizomes. Leafy stiff of these species higher that 1m and leafy stem and leaf more or less green without exceptions of few *C. zedoaria* clones which are sometimes contained very thin and narrow strip along the mid rib. These observations are in agreement with the results of Apavatjrut et al. (1999) as they described a close association of the above species except *C. angustifolia* that was not included in their study. The above species including *C. aeruginosa* were reported to be triploid with $2n = 3x = 63$ (Apavatjrut et al., 1996). In addition, *C. australasica* and *C. viridiflora* are the two newly reported species in Bangladesh, which are also closely associated with the above species. These two species are also morphologically very similar

except a little difference in leaf and rhizome characters. *C. viridiflora* leaf is linear-lanceolate where *C. australasica* leaf widely ovate lanceolate and comparatively bigger in size. In addition to that *C. viridiflora* contains a bit bigger rhizome comprising light yellow colour whereas *C. australasica* contains smaller rhizomes having a grey white colour. The close association of *C. aeruginosa* and *C. amarissima* supports their unique blue colour of rhizomes, however the rhizomes of *C. aeruginosa* are comparatively smaller and containing blue circle in the central portion only while the *C. amarissima* rhizomes are throughout dark blue and larger in size. In contrast, leaf morphology is also somewhat different in these two genetically related species. *C. aeruginosa* contained narrowly lanceolate or linear leaf and possesses a thin strip along midrib while *C. amarissima* contains broadly lanceolate or ovate shaped leaf that are throughout green. Another distinct species in the dendrogram was *C. latifolia*, which was also morphologically diverse comprising a very wide and large ovate shaped leaf containing light purple band along the mid rib. *C. rubescens* and *C. petiolata* are also closely associated in the tree but there morphological character does not support there close association since *C. rubescens* comprises very dark leafy stem and mid rib whereas *C. petiolata* having a green colour throughout. In addition, the leaf of *C. rubescens* are ovate lanceolate while the latter possesses a long lanceolate leaf with a pale white leaf margin.

From this study some critical comments on their evolutionary history cannot be made since their sequence were not interpreted. Recently however, preliminary molecular research on the genus has been carried out in The Royal Botanic Garden Edinburgh, UK. This revealed that the percentage of sequence divergence in the nuclear DNA internal transcribed spacer 2 (ITS2) among species of subgenus *Curcuma* was very low. It might indicate that the species have recently diverged.

3.3.2. Population genetic diversity of *C. zedoaria*

Population genetic analysis of *C. zedoaria* was followed Dice coefficient (Dice, 1945) to develop pairwise distance matrix as well as a dendrogram for all individuals of five populations of *C. zedoaria*. The produced dendrogram demonstrated that individuals of Srimangal, Chittagong and Sitakundu populations are close to each other while Savar and Birganj populations are genetically rather distinct (Fig 3.9). The possible explanation of this picture is that the first group possesses similar ecological attributes and that geographically they are closer compared to the second group.

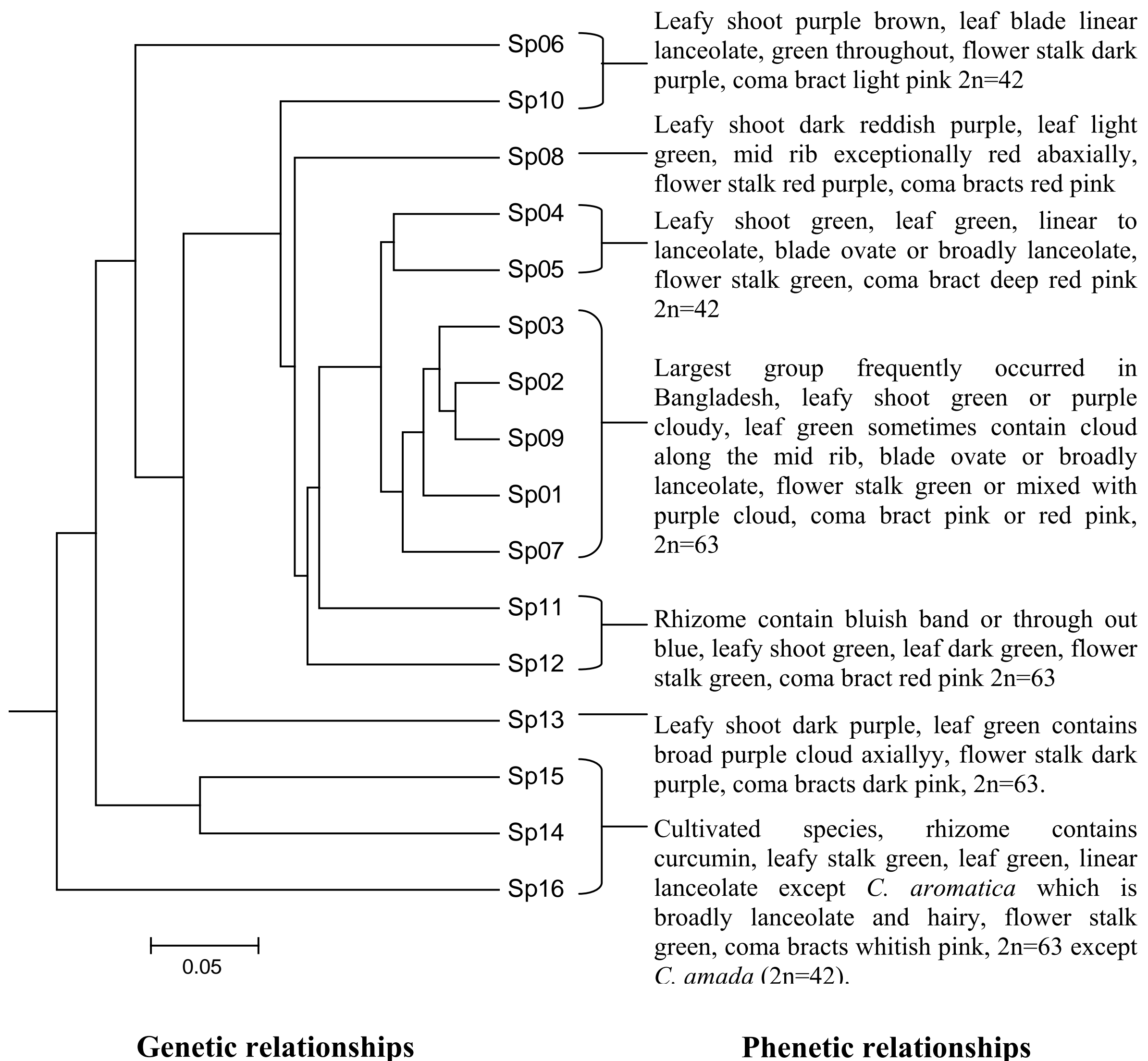


Fig 3.8. Rrelationships of sixteen *Curcuma* species based on genetic data and phenological characters: *C. elata* (Sp01), *C. angustifolia* (Sp02), *C. xanthorrhiza* (Sp03), *C. australasica* (Sp04), *C. viridiflora* (Sp05), *Curcuma sp.*(Sp06), *C. latifolia* (Sp07), *C. rubescens* (Sp08), *C. zedoaria* (Sp09), *C. petiolata* (Sp10), *C. aeruginosa* (Sp11), *C. amarissima* (Sp12), *C. caesia* (Sp13), *C. longa* (Sp14), *C. aromatica* (Sp15), *C. amada* (Sp16)

One of the main reasons that insisted to use the Shannon's index and AMOVA for partitioning genetic diversity of *C. zedoaria* populations is that these procedures do not require the Hardy-Weinberg assumption (Fritsch and Reiseberg, 1996). Shannon's information measures of each population indicated that the populations of hilly areas (Srimangal, Chittagong and Sitakundu) that comprise a higher number of individuals are attributed to comparably higher level of genetic diversity than those of plain land (Savar) and plateau land (Birganj) populations comprising a lower number of individuals. The high level

of genetic diversity in hilly areas located in the eastern part of the country supports the hypothesis of the close relation and influence of its Indo-Malayan centre of origin comprising greater diversity as described by many authors (Apavatjrut et al., 1999; Maciel and Criley, 2003; Purseglove, 1974; Sirirugsa, 1999). Moreover, it is remarkable that these hilly populations are reasonably undisturbed and possess wider ecological adaptations such as open and shady places of forest margins covering altitudes near sea level up to 500 m above the mean sea level, whereas, plain land and plateau land populations comprising ecologically rather homogeneous condition experienced with high disturbance due to intensive agricultural practices. These results also agree with the findings of Paisooksantivatana et al. (2001a) in *C. alismatifolia* as they found greater genetic variation in highland populations. It is also revealed that populations of hilly areas Srimangal (n = 8), Chittagong (n = 14) and Sitakundu (n = 8) are the natural populations showing very similar level of genetic diversity despite their difference in sample size. Such independence of Shannon's index from the sample size was also observed in other species by Cardoso et al. (1998) and Lacerda et al. (2001).

Considering the genetic structures of *C. zedoaria* populations, one can describe that RAPD markers revealed a significant genetic subdivision of zedoary populations in Bangladesh. It is also recognised that this subdivision is highly correlated with the ecogeographical parameters of the regions. According to Wright's interpretation of F_{ST} values (Wright, 1978), it can be delineated that the *C. zedoaria* populations evaluated in this study are moderately differentiated from each other and genetic divergent varied from populations. A high level of genetic variation within populations (78.33 – 81.22 %) was found in *C. zedoaria* which agrees the findings of Hamrick and Loveless (1989) as they showed tropical plants tend to present high levels of genetic diversity, most of it within populations. There is no information available on population genetic diversity of *C. zedoaria*, which does not allow comparing the present results. However, Paisooksantivatana et al. (2001a and 2001b) studied on the genetic diversity of *C. alismatifolia* Gagnep. in Thailand using allozyme polymorphism and revealed a comparatively higher level of genetic diversity in *C. alismatifolia*. It might be due to the result of a wide range of habitat conditions that encompasses by the populations of *C. alismatifolia* in Thailand. One more probable account supporting this statement is that their sampling areas were positioned within its centre of diversity, which might play a vital role for high level of genetic variations.

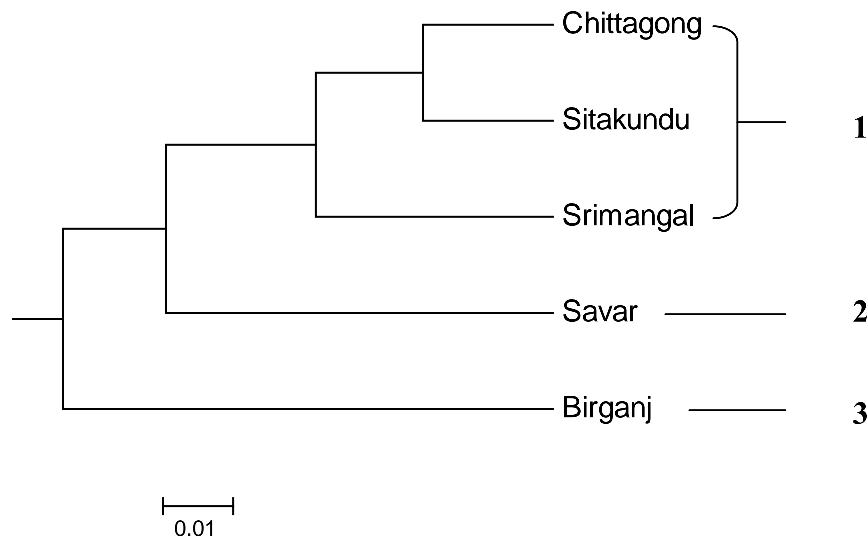


Fig 3.9. Relationships of *C. zedoaria* populations based on genetic data and ecogeographical parameters 1) Hilly populations mixed with mixed evergreen forest or shrubby woodlands contain higher diversity, 2) Plain land population characterised by alluvial soils encompasses high anthropogenic disturbance, 3) Plateau land composed of terrace soils population is a fragmented portion of remnant Sal forest elements experience with high agricultural impacts

The Neighbour Joining (NJ) dendrogram of the populations of *C. zedoaria* was developed based on pairwise genetic distance which showed a close genetic relation among hilly natural populations, in addition, intrapopulational genetic diversity patterns are also very similar within these populations. In contrast, the other two populations (Savar and Birganj) are rather distinct from hilly populations and comparatively more anthropogenic, which demonstrated a similar pattern of genetic variation. It is also inferred that the plain land population (Savar) that is located in the central part of the country builds a link between hilly and distinct plateau land populations. Paisooksantivatana et al. (2001a and 2001b) also reported in *C. alismatifolia* high level of genetic distance between high lands and low lands populations and a high level of genetic identity among hilly populations that agreed with these results. The most plausible explanation for the features plateau land population is that this is thought to be colonized and established as a natural population in the past when the area was covered by preliminarily '*Sal'* forest (*Shorea rubusta*) that was gradually fragmented due to habitat loss through occupying areas by modern agricultural crops and ultimately reduced the number of individuals from the natural populations in their evolutionary histories. Collection rate of zedoary plant from these populations by the local inhabitants are much higher than the hilly populations that might be another important reason of genetic loss of these populations.

4. CYTOLOGY AND FLOW CYTOMETRY

4.1. Introduction

4.1.1. Chromosome research and polyploidy in *Curcuma*

Chromosome numbers and karyomorphological data can be used in studying taxonomic relationships and evolutionary patterns within the groups (Joseph et al., 1999). The somatic chromosome numbers of *Curcuma* species have been reported as 2n = 20, 24, 28, 32, 34, 36, 42, 56, 62, 63 and 84 (Apavatjrut et al., 1996 and 1999; Beltran and Kiew, 1984; Chakravorti, 1948; Chattarjee et al., 1989; Chen et al., 1984; Darlington and Wylie, 1955; Das et al., 1999; Eksomtramage et al., 1996; Raghavan and Bhattacharya, 1943; Ramachandran, 1969; Roy et al., 1999; Sharma and Bhattacharya, 1959; Sirirugsa, 1999; Weerapakdee and Krasaechai, 1997; Venkatasubban, 1946). The variation in chromosome numbers within *Curcuma* species is evidence of existing both polyploidy and aneuploidy (Eksomtramage et al., 2002).

Chromosomes sizes of this genus were very small ranging from 0.5-2.0 micrometers (Apavatjrut et al., 1996; Joseph et al., 1999). Among these wide range of chromosome numbers the basic chromosome number n = 21 is very frequent within the genus *Curcuma* with 2n = 2x = 42, 2n = 3x = 63, 2n = 4x = 84 were described by different authors. However, there are still disagreements among some reports on chromosome numbers of some species that have to be reconfirmed. In addition to that there is no report available on chromosome research of *Curcuma* species of Bangladesh. It has also to be mentioned that the genetic improvement of this important crop through conventional breeding is handicapped due to incompatibility and high pollen sterility resulting in no seed set (Joseph et al., 1999).

Detailed karyomorphological studies on *Curcuma* species occurred in Bangladesh were not tried so far. It is might be the reason of very small size of chromosomes. However, this is a prerequisite for *Curcuma* taxonomy as well as for executing genetic improvement programmes in Bangladesh. In this study metaphase chromosome numbers of some species were counted to confirm their genetic relations. This would likely to be helpful for further studies on genetic diversity and evolution as well as crop improvement programme.

4.1.2. 2C DNA amounts and genome size of *Curcuma*

The plant scientists are currently emphasizing nuclear 2C DNA values or genome size values of plant species since they are very important biodiversity characters with fundamental biological significance utilities (Bennett and Leitch, 1995; Bennett et al., 2000). To determine C-values of plant species are highlighted and currently being increasing the percentage species with known C-values (Obermayer et al., 2002). Analysis of genome size can support further studies on plant population genetics and conservation programmes. In addition, genome size of the different plant populations might be used to interpret the composition of the communities and other aspects of geobotanical studies (Lysák et al., 2000). Inter- and intraspecific variation in nuclear DNA content among flowering plants has been well documented in the literature (Bennett and Leitch, 1995; Bennett and Smith, 1976 and 1991; Cavallini and Natali, 1991; Price, 1988). Though, a great deal on determinations of genome size has been made over the last decades, only a negligible percentage of the known angiosperm species are represented (Rayburn et al., 1997). A number of studies have been reported significant intra-specific variation in genome size and currently the original view of species-specific constancy is questioned (Bennett and Leitch, 1995). Intra-specific variability in genome size in correlation with the eco-geography has also been documented by many authors in several plant taxa, including *Poa annua* (Grime, 1983), *Milium effusum* (Bennett and Bennett, 1992), *Dactylis glomerata* (Reeves et al., 1998) and *Sesleria albicans* (Lysák et al., 2000).

Detailed genome size analysis and estimation of nuclear 2C DNA amounts of *Curcuma* was not comprehensively tried so far. Only the genome size of *C. zanthorrhiza* is available in the Kew database reported by Bharathan et al. (1994). Genome sizes of few other species of *C. amada*, *C. caesia* and *C. longa* have been reported by Das et al. (1999). There is also no information available about the genome size at population level of any species. The lack of this genome size information in the literature motivated to undertake this study. The main goal of the present study was to assess the extent of inter and intra-specific genome size variation in the genus *Curcuma*, since the inadequate information of genome size can not be based the further studies related to the genetic diversity and evolutionary patterns and relations in the genus *Curcuma.* A considerable amount of research investigation is still required to facilitate further taxonomic research as well as crop genetic improvement and conservation programmes. In this study, 2C DNA values and genome size of sixteen *Curcuma* species as well as different populations of *C. zedoaria* were measured.

4.2. Results

4.2.1. Chromosomal investigation

Two staining methods of Feulgen and DAPI were used to find out better squashing technique for *Curcuma* root tips. It was observed that the Feulgen technique was not very suitable for *Curcuma* root tips since it does not allow well spreading of the root tips tissue on slides. In contrast, DAPI staining was followed some additional steps that might help to spreading the tissues properly. For example digestion of tissues with mixture of cellulase and pectinase enzymes allowed to make the root tips softer and this consequently facilitate well spreading the root tips tissues.

In general characteristic notable in the genus *Curcuma* is that all the species encompass very small sized chromosome. The length varied approximately from 0.5 - 2µm. Fig 4.1 and Fig 4.2 illustrated the metaphase stage of chromosomes obtained from the young root tips of different *Curcuma* species. It was observed that the species possess different 2n chromosome numbers including 40 in an identified *Curcuma* species, 42 in *C. rubescens* (diploid accessions), *Curcuma viridiflora* and *C. amada,* 63 in *C. longa, C. zanthorrhiza, C. elata, C. caesia, C. amarissima, C. rubescens* (triploid accessions), *C. zedoaria* and *C. latifolia* and 84 (*C. aeruginosa* –tetraploid accession). Chromosome counts of different speices that occurred in Bangladesh and previous references are presented in Table 4.1. Chromosome numbers of some other species that have not been recorded in Bangladesh so far are listed in Table 4.2, which inferred that the genus *Curcuma* comprise rather wide range of chromosome numbers in other geographical regions especially in the regions of its centre of origin.

4.2.2. 2C DNA amounts and genome size of different *Curcuma* species

Nuclear genome size was analysed using different species and individuals of different species of *Curcuma*. *Raphanus sativus* was used as an internal reference standard, which was also found to be suitable by other authors (Doležel et al., 1998; Schmuths et al., 2004). In this study a wide range of 2C DNA values and genome size in *Curcuma* species were observed. Table 4.3 represents the 2C DNA value and the genome size of different *Curcuma* species. The 2C DNA values were ranged from 2.10 pg ± 0.018 – 5.30 pg ± 0.025. The highest DNA amount was obtained from the accession *C. aeruginosa* collected from Chittagong area while the lowest was found in *C. australasica*. Correspondingly, the highest genome size was calculated from *C. aeruginosa* (5185.36 Mbp) and the lowest value similarly from *C.*

australasica (2074.34 Mbp). From these results it is believed that the species are comprising the ploidy levels of diplod, triploid, tetraploid and pentapolid. All measurements resulted in histograms with two peaks representing *R. sativas* nuclei and the nuclei of *Curcuma* species. Flow cytometry histogram patterns of four expected ploidy levels are presented in Fig 4.3.

4.2.3. 2C DNA amounts and genome size of *C. zedoaria* populations

In the present study, 2C DNA amounts and genome size was also analysed in five populations of *C. zedoaria* including in total 42 accessions. *Raphanus sativus* was also used as an internal reference standard. All measurements resulted in histograms with two peaks representing *R. sativas* nuclei (peak A) and the nuclei of *C. zedoaria* (peak B). A representing histogram is presented in Fig 4.4. The close proximity of the peaks of both standard reference and *C. zedoaria* accession guaranteed an accurate analysis as well as minimized the risk of errors due to non-linearity (Lysák et al., 2000).

Table 4.1. Chromosome numbers of different *Curcuma* species investigated including previous references

Name of the species	Chromo. (2n)	Previous References
C. elata Roxb.	63	Apavatjrut et al., 1996
C. angustifolia Roxb.	42	Chakravorti, 1948; Sharma and Bhattacharya, 1959
C. zanthorrhiza Roxb.	63	Apavatjrut et al., 1996; Sirirugsa, 1999
C. zedoaria (Chrism.) Rosc.	63	Apavatjrut et al., 1996; Ramachandran, 1969
	64*	Chakravorti, 1948; Ramachandran, 1969
	66*	Chattarjee et al., 1989
C. petiolata Roxb.	64*	Venkatasubban, 1946
	42	Apavatjrut et al., 1996
C. aeruginosa Roxb.	63	Apavatjrut et al., 1996; Roy et al., 1999
	84	
C. latifolia Rosc.	63	--
C. viridiflora Roxb.	42	--
C. caesia Roxb.	22*	Das et al., 1999
	63	Roy et al., 1999
C. longa L.	62*	Raghavan and Bhattacharya, 1943
	62*, 63, 64*	Chakravorti, 1948
C. aromatica Rosc.	42*	Raghavan and Bhattacharyya, 1943; Sirirugsa, 1999
	63	Chen et al., 1984; Sirirugsa, 1999
C. amada Roxb.	40*	Das et al., 1999
	42	Chakravorti, 1948; Ramachandran, 1969

*Chromosome numbers that have not been recorded in this study

Table 4.2. Chromosome number of some non-native *Curcuma* species reported by previous workers

Name of the species	Chromosome no. (2n)	References
Curcuma aff. *oligantha* Trimen	42	Eksomtramage et al., 2002
C. rhabdota Sirir. & Newm.	24	Eksomtramage et al., 2002
C. parviflora Wall.	32	Eksomtramage et al., 2002
	28, 34, 36	Apavatjrut et al., 1996
C. roscoeana Wall.	42	Apavatjrut et al., 1996
C. attenuata Wall.	84	Apavatjrut et al., 1996
C. alismatifolia Gagnep.	32	Apavatjrut et al., 1996
C. thorelii Gagnep	14, 17, 28	Apavatjrut et al., 1996
C. decipiens Dalz.	42	Ramachandran, 1969
C. neilgherrensis Wt.	42	Ramachandran, 1969
C. comosa Roxb.	42	Roy et al., 1999
C. haritha Mangly & Sabu	42	Roy et al., 1999
C. malabarica Vel., Ama. & Mur.	42	Roy et al., 1999
C. raktacanta Mangly & Sabu	63	Roy et al., 1999
C. yunnanensis Liu & Chen	63	Sirirugsa, 1999
C. wenyujin Chen & Ling	63	Sirirugsa, 1999
C. phaeocaulis Val.	63	Sirirugsa, 1999
C. kawangsiensis Lee & Liang	84	Sirirugsa, 1999
C. aurantiaca van Zijp.	42	Bettran and Kiew, 1984

The analysed data of all accessions revealed that *C. zedoaria* comprises considerable amounts of variation in 2C DNA amounts that ranged from 3.145 ± 0.010 – 3.373 ± 0.011 pg and did not showed any relative increase or dicrease in the nuclear DNA amounts (Table 4.4). Statistical analysis showed significant differences between individual populations ($P<0.001$). 2C nuclear DNA content of individuals from entire samples ranged from 3.10 - 3.44 pg, however, mean of the populations varied from 3.145 ± 0.010 - 3.373 ± 0.011 pg. Among the five populations, the highest DNA amount was obtained from the population Chittagong (3.373 pg) and the lowest in Birganj population (3.145 pg). Correspondingly, the highest genome size was calculated from Chittagong population was 3298.79 Mbp and the lowest value of Birganj population was 3075.81 Mbp, while the average value of entire population was 3188.28 Mbp. According to the Tukey's grouping there is no significant variation in between populations Chittagong and Sitakundu. Similarly, Birganj and Savar populations showed similar amount of 2C DNA. However, the 2C DNA amounts of Birganj population significantly varied from Srimangal, Chittagnong and Sitakundu populations.

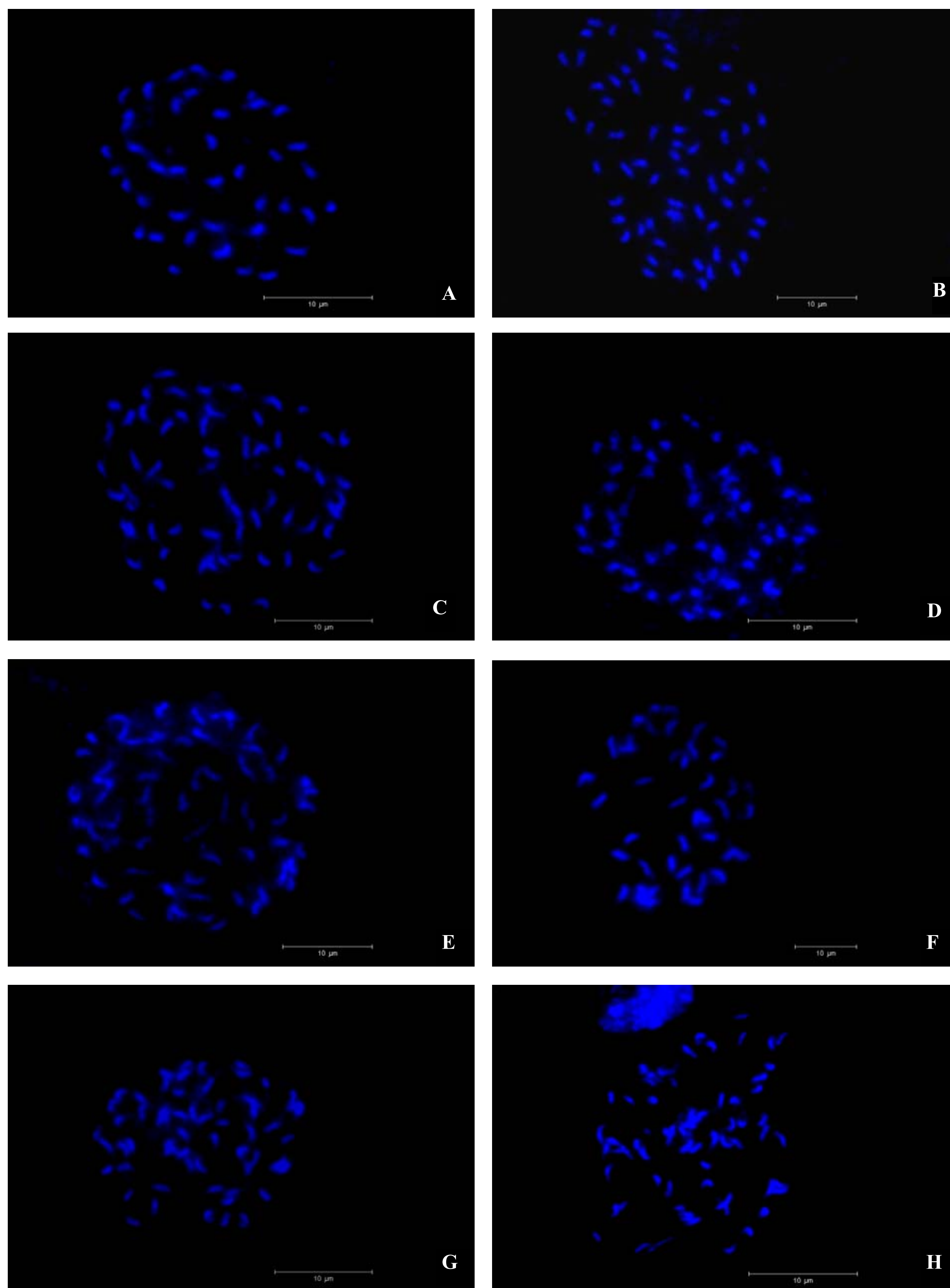


Fig 4.1. Somatic metaphase stage of chromosomes obtained from the young root tips of different *Curcuma* species; A- *Curcuma* sp. - 2n= 2x=40, B- *C. zedoaria* - 2n = 3x = 63, C- *C. zanthorrhiza* - 2n = 3x = 63, D- *C. amarissima* - 2n= 3x =63, E- *C. rubescens* - 2n = 3x = 63, F- *C. rubescens* - 2n = 2x= 42, G- *C. longa* - 2n=3x=63, H- *C. caesia* - 2n=3x=63; bars = 10µm.

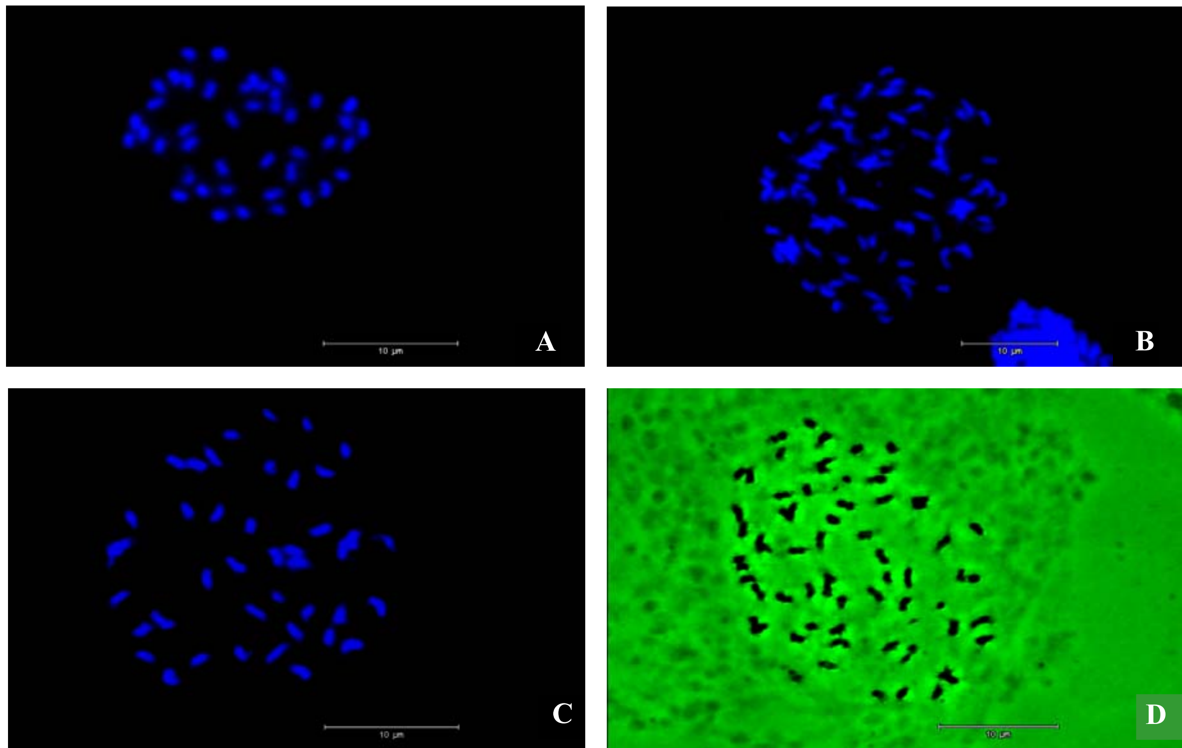
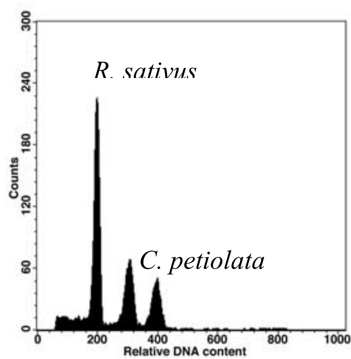
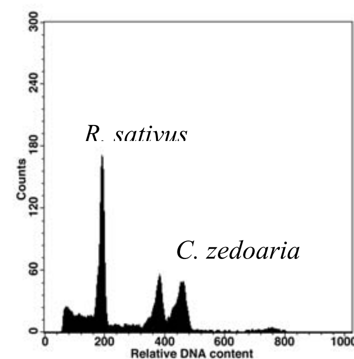


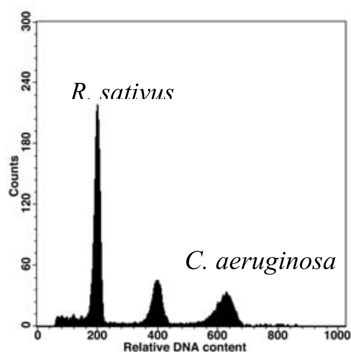
Fig 4.2. Somatic metaphase stage of chromosomes obtained from the young root tips of different *Curcuma* species; A- *C. viridiflora* 2n=2x= 42, B- *C. aeruginosa* 2n=4x= 84, C- *C. amada* 2n= 42 and D- *C. lalifolia*2n = 63; bars = 10µm.



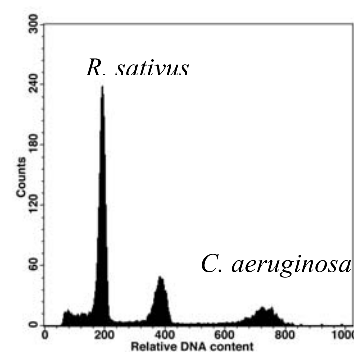
a) C14: *C. petiolata* (2n =2x = 42)



b) D04: *C. zedoaria* (2n = 3x =63)



c) C07: *C. aeruginosa* (2n = 4x = 84)



d) G06: *C. aeruginosa* (2n = 5x = ?)

Fig 4.3. Flowcytometry histograms patterns of leaf nuclei from different species/accessions of *Curcuma* measured by using *R. sativus* as an internal standard; a-d expected diploid, triploid, tetraploid and pentaploid respectively

Table 4.3. 2C DNA amounts and genome size of several *Curcuma* species/accessions collected from Bangladesh

Species/accessions	2C DNA amount (pg)	Standard deviation	95% confid. interval	Genome size (Mbp)	Tukey's groupings
C. aeruginosa Roxb.	5.302	0.025	0.040	5185.36	a
C. aeruginosa Roxb.	4.469	0.024	0.038	4370.68	b
C. latifolia Rosc.	3.435	0.007	0.011	3359.43	c
C. caesia Roxb.	3.333	0.011	0.017	3259.67	d
C. zedoaria (Chris.)Rosc.	3.321	0.008	0.013	3247.94	d
C. amarissima Rosc.	3.289	0.030	0.047	3216.64	de
C. zanthorrhiza Roxb.	3.285	0.014	0.022	3212.73	de
C. longa L.	3.275	0.009	0.014	3202.95	de
C. aeruginosa Roxb.	3.230	0.025	0.040	3158.94	ef
C. aromatica Salisb.	3.184	0.011	0.017	3113.95	f
C. elata Roxb.	3.181	0.092	0.146	3111.02	f
C. rubescens Roxb.	2.204	0.011	0.017	2155.51	g
C. sp.	2.195	0.033	0.053	2146.71	gh
C. australasica Hook. f.	2.181	0.009	0.014	2133.02	ghi
C. viridiflora Roxb.	2.173	0.008	0.013	2125.19	ghi
C. viridiflora Roxb.	2.164	0.011	0.018	2116.39	ghi
C. australasica Hook. f.	2.153	0.007	0.011	2105.63	ghi
C. petiolata Roxb.	2.143	0.016	0.025	2095.85	ghi
C. angustifolia Roxb.	2.143	0.011	0.018	2095.85	ghi
C. angustifolia Roxb.	2.141	0.016	0.026	2093.90	ghi
C. amada Roxb.	2.132	0.009	0.014	2085.10	hi
C. angustifolia Roxb.	2.121	0.018	0.029	2074.34	i

Tukey's groupings were made by using SAS statistical software at 5% level of significance
Number of base pairs = mass in pg x 0.978 x 10^9 (Doležel et al., 2003)

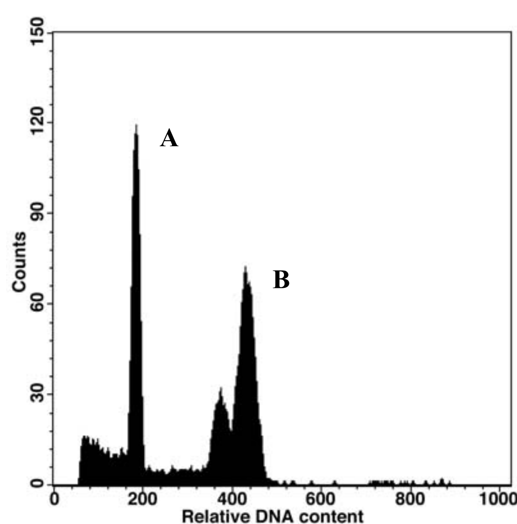


Fig 4.4. Flowcytometry histogram of leaf nuclei from the accession of *C. zedoaria* corresponds to the peak B while peak A is the internal standard *Raphanus sativus* (2C = 1.38 pg)

Table 4.4. 2C DNA amounts and genome size of different populations of *C. zedoaria* (Chrism.) Rosc. collected from five different locations

Populations	No of measures	2C DNA value (pg)	SE(±)	Min. – Max. (differences)	Genome size (Mpp)	Tukey's grouping
Srimangal	12	3.247	0.018	3.15 - 3.38 (0.23)	3175.57	b
Chittagong	16	3.373	0.011	3.25 - 3.44 (0.19)	3298.79	a
Sitakundu	10	3.339	0.021	3.19 - 3.42 (0.23)	3265.54	a
Savar	12	3.198	0.019	3.12 - 3.32 (0.20)	3127.64	bc
Birganj	8	3.145	0.010	3.10 - 3.32 (0.06)	3075.81	c
Mean		3.260	0.042		3188.67	

Tukey's groupings were made by using SAS statistical software at 5% level of significance
Number of base pairs = mass in pg x 0.978 x 10^9 (Doležel et al., 2003)

4.3. Discussions

4.3.1. Chromosomal investigation in *Curcuma*

A wide range of chromosome numbers in the genus *Curcuma* was reported by previous authors (Apavatjrut et al., 1996 and 1999; Beltran and Kiew, 1984; Chakravorti, 1948; Chattarjee et al., 1989; Chen et al., 1984; Darlington and Wylie, 1955; Das et al., 1999; Eksomtramage et al., 1996 and 2002; Raghavan and Bhattacharya, 1943; Ramachandran, 1969; Roy et al., 1999; Sharma and Bhattacharya, 1959; Sirirugsa, 1999; Weerapakdee and Krasaechai, 1997; Venkatasubban, 1946). In general the chromosomes of *Curcuma* species are very small ranging from 0.5 – 2.0µm along with a large number of chromosomes make difficult for exact counting of chromosomes. In this study about ten species were investigated where 2n = was found to be 40, 42, 63, and 84. The basic number n= 21 found to be more frequent in different species with three different ploidy levels of diploid, triploid and tetraploid. However, the variation in chromosome numbers of *Curcuma* genus indicates both polyploidy and aneuploidy (Eksomtramage et al., 2002).

In this study the chromosome numbers of *C. latifolia* and *C. viridiflora* were determined for the first time. The species *C. zedoaria, C. zanthorrhiza, C. elata, C. aeruginosa, C. caesia, C. longa, C. aromatica*, *C. amarissima* and *C. latifolia* were found to be triploid. This result supports the investigation of Apavatjrut et al. (1996). They studied diploid and haploid chromosome numbers from Thai *Curcuma* species and reported a wide range of 2n chromosome numbers in *Curcuma* species which include 2n = 42 (*C. roscoeana* Wall., *C. petiolata* Wall.), 2n = 63 (*C. zedoaria* Rosc., *C. zanthorrhiza* Roxb., *C. elata* Roxb. and *C.*

aeruginosa Roxb.), 2n = 84 (*C. attenuata* Wall.), 2n = 32 (*C. alismatifolia* Gagnep.) 2n = 24 (*C. thorelii Gagnep*), 2n = 28, 34, 36 (*C. parviflora* Wall.). However some other studies reported higher number of chromosomes in *C. zedoaria* which are 2n = 64 (Chakravorti, 1948; Ramachandran, 1969) and 66 (Chattarjee et al., 1989). Several reports also indicate a variation of chromosome number in *C. longa* such as 2n = 62 (Raghavan and Bhattacharya, 1943; Chakravorti, 1948) and 64 (Chakravorti, 1948). Some other species such as *C. amada, C. angustifolia* and *C. petiolata* were reported as 2n = 42 by previous workers (Apavatjrut et al., 1996; Chakravorti, 1948; Das et al., 1999; Ramachandran, 1969; Sharma and Bhattacharya, 1959) which agreed with the present results. In this study, some species were found to be diploid e.g. *C. amada, C. viridiflora*, an accession of *C. rubescens* and an unidentified species. Previous report was also support the ploidy levels of *C. amada* (Chakravorti, 1948; Das et al., 1999; Ramachandran, 1969), however no report is available on *C. viridiflora.*

4.3.2. 2C DNA amounts and genome size estimation

In this study the flow cytometry technique was utilised for analysis of interspecific and interpopulational genome size variations. To estimate interspecific genome size analysis different *Curcuma* species were considered while in the case of interpopulational genome size estimation a single species of *C. zedoaria* was considered since this species is one of the important wild species in Bangladesh as well as this species having reasonable number of individuals of five different populations. During this experiment it was found that the method was reliable and sensitive in detecting small differences in DNA content. A number of recent studies have also reported that flow cytometry can be employed successfully for determination of genome size and ploidy levels of plant species (Bennett and Bennett, 1992; Bennett and Leitch, 1995; Bennett and Smith, 1991; Bennett et al., 2003; Bureš et al., 2004; Lysák et al., 2000; Obermayer et al., 2002; Rayburn et al., 1997; Tuna et al., 2001).

4.3.2.1. 2C DNA amounts and genome size of different *Curcuma* species

It was revealed from the flow cytometry results that the members of the genus occurred in Bangladesh comprise a wide range of genome size which indicated different ploidy levels of diploid, triploid, tetraploid and pentaploid. The accessions of *C. rubescens* showed diploid and triploid ploidy levels whereas *C. aeruginosa* found to be more diverse containing triploid, tetraploid and pentaploid individuals within the species. While, *C. zedoaria* includes a large number of accessions in this study which were restricted to the one single ploidy level of

triploid. From this result it can be concluded that the genus *Curcuma* comprises a wide range of ploidy levels. Some species contained individuals with more than one ploidy level while others were found to be restricted to a single ploidy level. Detailed genome size analysis and estimation of nuclear DNA amounts of *Curcuma* species has not comprehensively been tried so far. Only the genome size (2C DNA value) of *C. zanthorrhiza* (2.60 pg) is available in the Kew database reported by Bharathan et al. (1994). 4C DNA (pg) content of few other species of *C. amada* (4.234 pg ± 0.092 SE) *C. caesia* (3.120 pg ± 0.048 SE) *C. longa* var. Suroma (5.263 pg ± 0.0123 SE) and *C. longa* var. TC-4 (5.100 pg ± 0.031 SE) have been reported by Das et al. (1999).

4.3.2.2. 2C DNA amounts and genome size of *C. zedoaria* populations

A considerable amount of variation was found in the genome size within and among *C. zedoaria* populations. Genome size variations among the individuals within the populations varied from 0.06 – 0.23 pg, while the differences in genome size among the populations was 0.23 pg. The largest difference between populations was 6.99% while the difference among whole samples was 9.82%. In addition, the largest difference within population relative to population mean was found in Srimangal which was 7.08%. Some cases the genome size varition in *C. zedoaria* was found to be significant. These significant differences among the accessions might be due to the aneuploidy of the species as reported in *Curcuma* species (Beltran and Kiew, 1984; Eksomtramage et al., 2002). Palomino et al. (2003) reported 2.5% variation in 2C DNA of the diploid varieties of *Agave tequilana*, however, no significant differences were detected among all diploid varieties. Vilhar et al. (2002) determined 2C value of *Dactylis glomerata* L. and they reported 2.1% variation among five different populations. Lysák et al., (2000) reported 1.6% intraspecific variation in *Sesleria albicans*, which was almost identical range of variation of 1.8% in the same species, previously reported by (Lysák and Doležel, 1998) where only a few populations were analysed. This result inferred that that the genome size variation in *C. zedoaria* based on the populations of Bangladesh is likely to be the alike irrespective of the broad geographical distances among the tropical Asian populations. Unfortunately, this data could not be compared since there is no report available on *C. zedoaria* and the present result can be considered as a first report in this species. However, for further confirmation of the genome size of *C. zedoaria*, a detail study is required covering the populations of broad geographical areas of south- and south-east Asian populations.

5. *IN VITRO* REGENERATION AND MICRORHIZOME INDUCTION

5.1. Introduction

The neIntroduction cessity of the development of *Curcuma in vitro* regeneration and multiplication systems is precisely in the earlier chapter. In this study *C. longa* was used since it is the most important species within the genus. The rate of rhizome multiplication in this species is very low (6-10 times) with the yield ranging between 15 and 25 tons/hectare (Balachandran et al., 1990), while a huge number of seed rhizomes about 2000-2500 kg that correspond to 10-20% of total yields are required for cultivation of one hectare of land (Shirgurkar et al., 2001). Maintenance of such huge amount of germplasm annually (every year) is expensive and labour intensive. Besides, many diseases and pests, particularly soft rot of turmeric caused by *Pythium myriotylum and P. gramnicolum* as well as bacterial wilt caused by *Pseudomonas solanacearum* take a heavy toll on the germplasm which is the major constraint in the production of turmeric (Balachandran et al., 1990; Nayak, 2000; Salvi et al., 2001 and 2002; Shirgurkar et al., 2001). Furthermore, turmeric cannot be improved by conventional breeding methods because flowering is rare and seed set does not occur presumably due to the triploidy of the plant. The alternatives are (1) the selection of desirable genotypes for *in vitro* multiplication and their use as planting stocks, and (2) the genetic transformation of useful genes. The establishment of an effective *in vitro* multiplication technique is essential to achieve both these objectives. It is also important to make available disease free planting material especially for newly developed line(s) or mutants that exist only in small quantities. A number of protocols for *in vitro* multiplication of *C. longa* have already been established by different workers (Balachandran et al., 1990; Dekkers, 1991; Nadgauda et al., 1978; Salvi et al., 2002; Shirgurkar et al., 2001; Sunitibala et al., 2001; Yasuda et al., 1988; Yusuf et al., 2001), however, further improvement is required to meet the future demand especially the elite variety of Surma of Bangladesh, since there is no report available on this variety. In this study, most of the recommended systems of *Curcuma* species were compared to each other and a simple cost effective high frequency regeneration system for *C. longa* L. has been developed.

In addtion, a number of reports are available on *in vitro* formation of storage organs such as bulbs, corms, tubers and rhizomes for different plants (Alizadeh et al., 1998; Abbott and Belcher, 1986; Garner and Blake, 1989; Grewal, 1996; Gopal et al., 1998; Hoque et al., 1996;

Pence and Soukup, 1993; Slabbert and Niederwieser, 1999; Vreugdenhil et al., 1998), but only a few reports are available that dealt with microrhizome induction in the member of Zingiberaceae. Microrhizome induction in ginger (Nirmalbabu et al., 1994; Sharma and Singh, 1995) and in turmeric (Nayak, 2000; Shirgurkar et al., 2001; Sunitibala et al., 2001) is available within this family. Till now there is no work has been done on the variety of Surma of Bangladesh that demands considerable research efforts on microrhizome induction. These protocols, however, require improvement to obtain bigger and efficient microrhizomes since the survival of small microrhizomes is very low and small rhizomes normally produce unhealthy stunted plants as reported by Shirgurkar et al. (2001). In addition, there is a disagreement between the reports of Nayak (2000) and Shirgurkar et al. (2001), while the earlier report described BA as a promoting growth regulator and the later mentioned BA as an inhibitory growth regulator. The present study therefore was also directed to investigate the effects of the *in vitro* growth conditions such as concentration of sucrose, strength of MS medium and light illumination on microrhizome induction in *C. longa* L. The effects of cytokinins BA and Kn as well as the auxin NAA were tested as they were described as effective growth regulators for the genus *Curcuma* (Balachandran et al., 1990; Dekkers, 1991; Nadgauda et al., 1978; Salvi et al., 2002; Shirgurkar et al., 2001; Sunitibala et al., 2001; Yasuda et al., 1988). Finally, an improved *in vitro* protocol would be recommended that could be used to induce microrhizome in *C. longa* providing planting material for the farmers as well as being suitable for germplasm storage and conservation.

5.2. Results

5.2.1. *In vitro* shoot multiplication of *C. longa* L. using axillary buds

5.2.1.1. Surface sterilization and establishment of contamination free initial culture

Freshly sprouted axillary buds (ca. 1 cm long) were found to be ideal as initial explants. Earlier workers (Balachandran et al., 1990; Salvi et al., 2002) reported that contamination of underground rhizome is very high and establishment of contamination free cultures was difficult, therefore, in this study, Tween 20 was incorporated with 0.1% $HgCl_2$ as a wetting agent to reduce surface tension and also to allow better surface contact. More than 70% of the explants remained contamination free till next subculture. Incorporation of antibiotic did not show any improvement in the result during this study. Fig 5.1 presented different steps of axillary bud multiplication in *Curcuma*.

5.2.1.2. Optimum growth conditions for high frequency regeneration

The concentrations of different cytokinins significantly influenced the number and length of shoots and roots (Table 5.1). BA was found to be suitable for shoot multiplication of *C. longa* and 12µM BA produced the highest average number of shoots (6.73 ± 0.48) with enough number of roots (5.13 ± 0.31). In many cases a single explant produced about 12 shoots (Fig 5.1d). From the values of shoot and root numbers and length it is inferred that BA is efficient enough to produce sufficient numbers of shoots within shorter period of time than another ever reported cytokinin used for *Curcuma* shoot proliferation. Kn (12µM) has produced the second highest number of shoots (5.20 ± 0.42) and the highest number of roots (5.27 ± 0.34). TDZ and 2iP did not produce any sufficiently enhanced number of shoot. 2iP significantly increased root number and length while TDZ produced unhealthy, stunted shoots and very few roots in the presence of 0.3µM NAA.

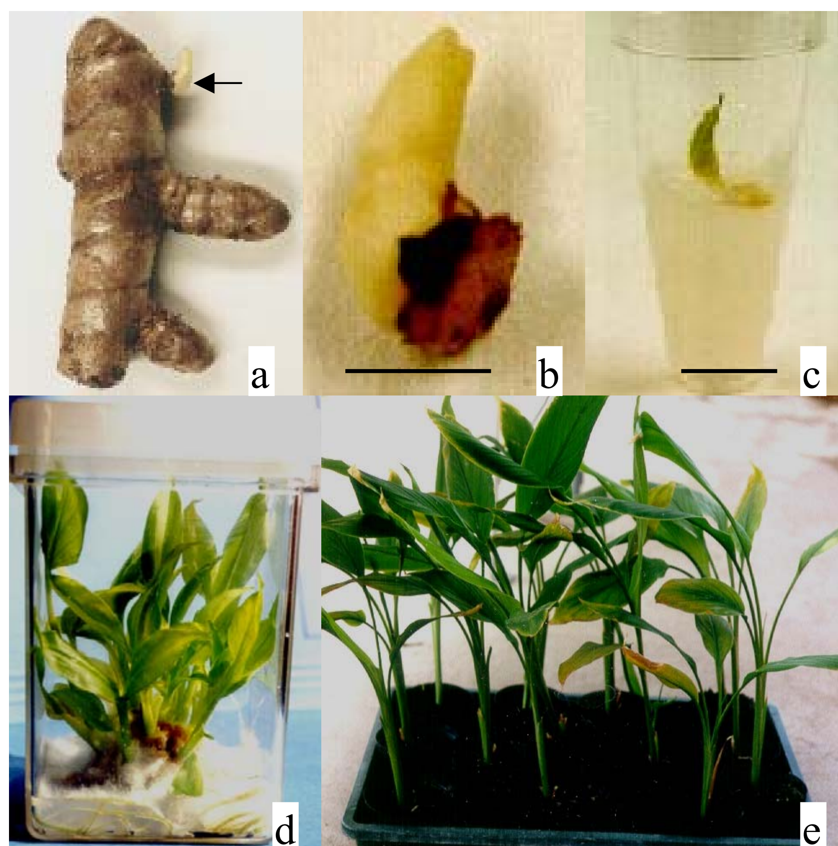


Fig 5.1. Developmental stages of *in vitro* shoot regeneration in *C. longa* a. sprouted rhizome, arrow indicates the immature bud , b. initial explants excised from rhizome , c. explants cultured for 4 weeks on MS medium, d. multiple shoots after 6 weeks , e. successful transplantation in the soil. bars = 0.5 cm

Different auxins (NAA, IBA and IAA) also had significant effects on the number and length of shoots and roots and NAA (0.3 µM) has found to be optimal for *C. longa* (Table 5.2). IAA (0.6 µM) was found to be very suitable for increasing root number and length but it decreased the shoot number remarkably, while IBA could be used as second alternative as it did not reduce shoots and roots dramatically. An increased amount of NAA (0.6 µM) produced very thick and healthy roots with an enormous number of visible root hairs but it reduced the number of shoots. Among the various strengths of MS salts, 1.00 and 0.75x strength of MS medium were found to be suitable for shoot's multiplication, however, the highest number of shoots was obtained by using 0.75x strength of MS salts (Fig 5.2). Lower strengths of the media produced lower number and shorter shoots and roots as well as leaf yellowing.

Table 5.1. Effects of different concentrations of the cytokinins BA, Kn, 2iP and TDZ on *in vitro* regeneration in *C. longa* (data recorded after 4 weeks and all experiments conducted using 0.75x MS medium, 3% sucrose, 0.8% agar and 0.3 µM NAA)

Cytokinins	No of shoot (±SE)	Length of shoot (cm) (±SE)	No of root (±SE)	Length of root (cm) (±SE)
6µM BA	3.00 (±0.24)^{de}	2.09 (±0.10)^{ef}	3.67 (±0.32)^{bc}	4.13 (±0.18)^{bc}
12µM BA	6.73 (±0.48)^{a}	3.37 (±0.21)^{abc}	5.13 (±0.31)^{a}	5.01 (±0.22)^{ab}
18µM BA	4.80 (±0.38)^{bc}	3.47 (±0.16)^{abc}	4.13 (±0.31)^{ab}	4.35 (±0.28)^{abc}
6µM Kn	3.27 (±0.27)^{de}	2.77 (±0.14)^{cde}	4.67 (±0.27)^{ab}	3.74 (±0.24)^{c}
12µM Kn	5.20 (±0.42)^{b}	3.50 (±0.17)^{abc}	5.27 (±0.34)^{a}	3.74 (±0.24)^{c}
18µM Kn	4.27 (±0.25)^{bcd}	3.79 (±0.14)^{ab}	4.73 (±0.33)^{ab}	5.12 (±0.22)^{ab}
6µM 2iP	4.13 (±0.32)^{bcd}	3.59 (±0.19)^{ab}	4.27 (±0.37)^{ab}	4.21 (±0.17)^{bc}
12µM 2iP	3.60 (±0.31)^{cd}	3.91 (±0.21)^{a}	3.47 (±0.27)^{bc}	3.93 (±0.19)^{c}
18µM 2iP	3.00 (±0.22)^{de}	3.07 (±0.16)^{bcd}	5.47 (±0.26)^{a}	5.34 (±0.24)^{a}
6µM TDZ	2.87 (±0.22)^{de}	2.46 (±0.12)^{def}	2.40 (±0.19)^{cd}	2.39 (±0.15)^{d}
12µM TDZ	1.87 (±0.26)^{ef}	1.67 (±0.19)^{fg}	1.67 (±0.25)^{d}	1.61 (±0.19)^{de}
18µM TDZ	1.40 (±0.31)^{f}	1.29 (±0.22)^{g}	1.40 (±0.27)^{d}	1.18 (±0.20)^{e}

Table represents pooled means from fifteen replicates in each of 2 repetitions. Means followed by the same letter(s) are not different significantly at the probability level 5% according to Tukey test.

Different concentrations of sucrose were investigated, as the sucrose is an efficient and frequently used source of carbohydrate for *in vitro* regeneration. It was observed that the sucrose concentration influenced shoot multiplication significantly and 3% sucrose was found to be optimum for *C. longa* with respect to the number and length of shoots and roots. There was a linear decrease in the number of shoots in both lower and higher concentrations of sucrose, however, a higher concentration increased the root number (Fig 5.3). Out of six

different agar concentrations, maximum shoots were obtained in 0.8% agar, and a linear decrease in shoot and root productions were found in both higher (1.0 and 1.5%) and lower (0.6, 0.4 and 0.2%) concentrations of agar (Fig 5.4).

5.2.1.3. Hardening and transfer to the field

About 100% plants survived and were successfully established in the soil while they were transferred to the pots after 4 weeks of acclimatization in the growth chamber of the glasshouse maintaining high humidity (fig 5.1e). In the present study, field level evaluation could not be made as turmeric is a tropical crop for which an appropriate natural condition is not available in the glasshouse.

Table 5.2. Effects of different concentrations of the auxins NAA, IAA and IBA on *in vitro* regeneration in *C. longa* (data recorded after 4 weeks and all experiments conducted using 0.75x MS medium, 3% sucrose, 0.8% agar and 6 µM BA)

Auxins	No of shoot (±SE)	Length of shoot (cm) (±SE)	No of root (±SE)	Length of root (cm) (±SE)
0.1µM NAA	5.73 (±SE0.37)^{ab}	3.25 (±SE0.15)^{abc}	4.60(±SE0.31)^{bcd}	3.97(±SE0.22)^{bc}
0.3µM NAA	6.73 (±SE0.47)^{a}	3.55 (±SE0.14)^{a}	5.87(±SE0.39)^{ab}	4.69(±SE0.15)^{ab}
0.6µM NAA	4.67 (±SE0.40)^{bcd}	3.39 (±SE0.17)^{ab}	6.27(±SE0.38)^{a}	4.45(±SE0.20)^{abc}
0.1µM IBA	4.87 (±SE0.38)bc	2.98 (±SE0.16)^{abcd}	3.47(±SE0.27)^{cd}	3.57(±SE0.25)^{c}
0.3µM IBA	5.67 (±SE0.27)^{ab}	2.97 (±SE0.13)^{abcd}	4.47(±SE0.29)^{bcd}	4.35(±SE0.21)^{abc}
0.6µM IBA	4.27 (±SE0.32)^{bcd}	2.59 (±SE0.10)^{cd}	5.87(±SE0.42)^{ab}	5.07(±SE0.24)^{a}
0.1µM IAA	3.53 (±SE0.27)^{cd}	2.57 (±SE0.12)^{d}	3.40(±SE0.27)^{d}	3.92(±SE0.24)^{bc}
0.3µM IAA	4.47 (±SE0.29)^{bcd}	2.96 (±SE0.18)^{abcd}	5.00(±SE0.39)^{abc}	4.47(±SE0.25)^{abc}
0.6µM IAA	3.27 (±SE0.23)^{d}	2.87 (±SE0.16)^{bcd}	6.20(±SE0.37)^{a}	5.13(±SE0.22)^{a}

Table represents pooled means from fifteen replicates in each of 2 repetitions. Means followed by the same letter(s) are not different significantly at the probability level 5% according to Tukey test.

5.2.2. Microrhizome induction

5.2.2.1. Efficient technique of microrhizome induction

In the case of microrhizome induction experiment, contamination free explants were cultured for four weeks and multiplied by using this established protocol that contained MS basal medium (pH 5.8), 3% sucrose, 0.8% agar, and a low concentration of BA (6 µM) and NAA (0.3µM). Multiplied shoots were then cultured on PGR free medium to avoid carry over effects. Fig 5.5 presented different developmental stages of *in vitro* microrhizome induction in *C. longa* and their successful recovery at field level without any acclimatisation.

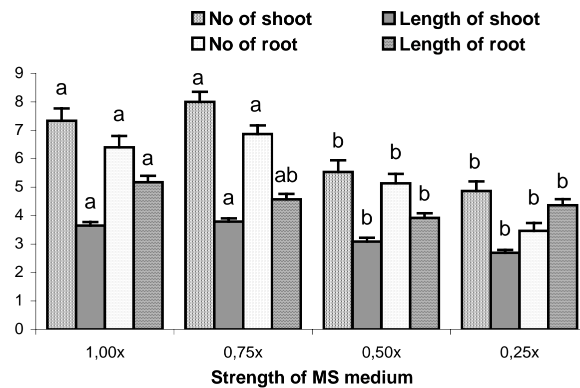


Fig 5.2. Effect of the strength of MS salts on *in vitro* shoot regeneration in *Curcuma longa* L. (experiments were conducted using 3% sucrose, 0.8% agar, 12µM BA and 0.3µM NAA; bars represent pooled means from fifteen replicates in each of 2 repetitions and means followed by the same letter(s) are not different significantly at the probability level 5% according to Tukey test.

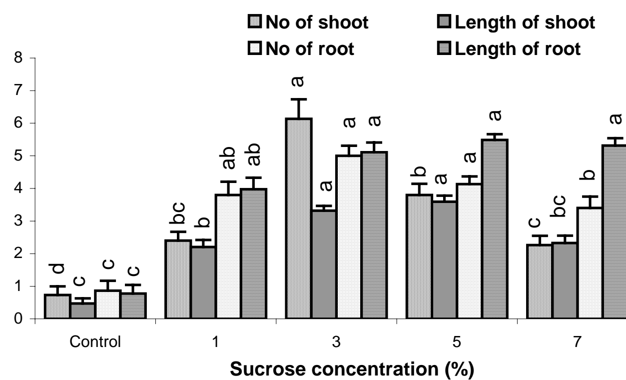


Fig 5.3. Effect of different concentrations of sucrose on *in vitro* shoot regeneration in *Curcuma longa* L. (experiments were conducted using 0.75x MS salts, 0.8% agar, 12µM BA and 0.3µM NAA); bars represent pooled means from fifteen replicates in each of 2 repetitions and means followed by the same letter(s) are not different significantly at the probability level 5% according to Tukey test.

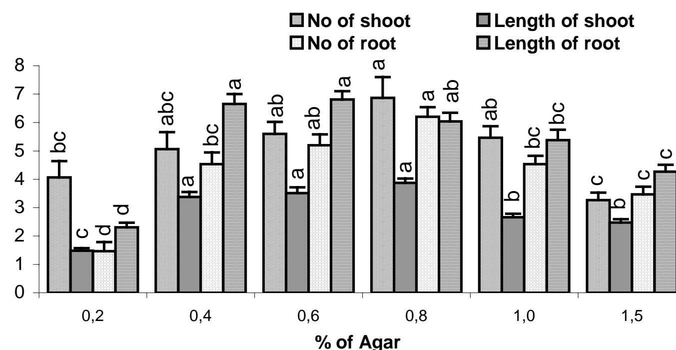


Fig 5.4. Effect of different concentrations of agar on *in vitro* shoot regeneration in *Curcuma longa* L. (experiments were conducted using 0.75x MS salts, 3% sucrose, 12µM BA and 0.3µM NAA), bars represent pooled means from fifteen replicates in each of 2 repetitions and means followed by the same letter(s) are not different significantly at the probability level 5% according to Tukey test.

5.2.2.2. Effects of sucrose

In order to develop an optimised standard protocol, different concentrations of sucrose were investigated under fully dark and 16 hrs light illumination conditions (white fluorescent light with 50 µM m^{-2} s^{-1} light intensity). It was observed that sucrose plays a significant role in terms of size and number of microrhizome in *C. longa.* 9% sucrose produced the highest number (8.3 ±0.32) and the biggest (0.88 g ± 0.03) of healthy microrhizomes in dark condition, while light treatment reduced the number (8.3 ± 0.35) and weight (0.82 g ± 0.03) slightly which were not found to be significant statistically (Fig 5.6). 7% sucrose reduced both number. However, both lower (0 - 5%) and higher (11%) concentrations of sucrose had significant inhibitory effects on microrhizome production.

Fig 5.5. Developmental stages of *in vitro* microrhzome induction in *C. longa* L. a) multiple shoots obtained from shoot multiplication medium after 4-6 weeks, b) explants cultured on liquid medium for microrhizome induction, c) after sixty days microrhizome is developed along with a clump of roots, d) collected microrhizomes, e) shoot development and establishment in the soil

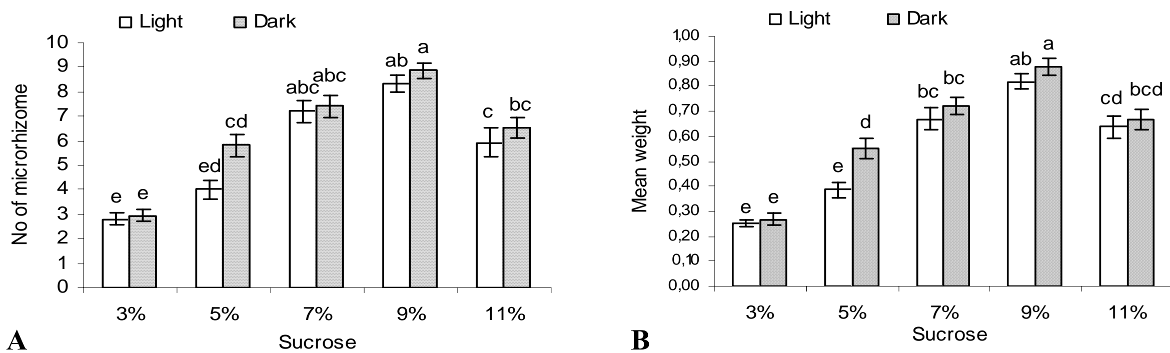


Fig 5.6. Effects of sucrose on *in vitro* microrhizome induction in *Curcuma longa* L.: experiments were conducted using 0.75 x MS medium – pH 5.8, 6 µM BA, 0.3 µM NAA under 16 h light and fully dark conditions. Results obtained from pooled means of two repeated experiments each of them included 15 replicates; bars are ± standard error; means with the same letters are not significantly different at 5% level according to Tukey's test of SAS programme; A) No of microrhizomes and B) mean weight of microrhizomes

5.2.2.3. Effects of BA and Kn

The presented results indicate that the plant growth regulators (PGR) BA, Kn and NAA had also significant effects on *in vitro* microrhizome induction (Table 5.3). Among the sixteen different combinations of PGR, 12 µM BA together with 0.3 µM NAA exhibited a better response than any other treatments in terms of mean number (8.1 ± 0.36) and the weight (0.67 g ± 0.03) of microrhizomes. In the present study, Kn alone or in presence of NAA did not show any promising result while it produced up to the number 4.5 ± 0.41 and weight 0.39g ± 0.02 at the concentrations of 12 µM BA and 0.3 µM NAA.

5.2.2.4. Effects of NAA

Effects of different concentrations of NAA were also investigated in a separate experiment and 0.3 µM NAA was found to be suitable to obtain the highest number (8.7 ± 0.36) of bigger microrhizomes (0.82 g ± 0.03), while both increasing and decreasing of NAA had significant decrease of the number and size of microrhizome (Fig 5.7).

5.2.2.5. Effects of MS salts

Fig 5.8 indicated the effects of MS salts on microrhizome induction in *C. longa*. Among the various strengths of MS salt, 0.75x strength of MS medium was found to be the most suitable in terms of number (8.3 ± 0.55) and weight (0.81 ± 0.04) of microrhizome, however, both half and full strength of MS salts decreased the number 6.5 ± 0.53 and 7.1 ± 0.54, and the size

0.65 ± 0.04 and 0.69 ± 0.04 respectively. Lower strength of the MS medium produced a lower number of smaller microrhizomes, while full strength produced a lower number but comparatively bigger size of rhizomes.

Table 5.3. Effects of BA, Kn alone or in presence of 0.3 µM NAA on *in vitro* microrhizome induction in *C. longa* (experiments were conducted using 0.75x MS medium–pH 5.8, 9% sucrose in the dark and data recorded after 60 days)

BA/Kn	NAA	Number (±SE)	Weight (g) (±SE)
Control	-	2.7 (0.21)de	0.31 (0.02)fg
3 µM BA	-	3.9 (0.32)bcde	0.32 (0.02)efg
6 µM BA	-	4.0 (0.29)bcde	0.36 (0.01)cdefg
12 µM BA	-	5.6 (0.35)b	0.48 (0.03)b
18 µM BA	-	5.0 (0.45)bc	0.45 (0.03)bcd
3 µM BA	0.3 µM	5.0 (0.34)bc	0.45 (0.02)bcd
6 µM BA	0.3 µM	5.3 (0.45)b	0.47(0.02)bc
12 µM BA	0.3 µM	8.1 (0.36)a	0.67 (0.03)a
18 µM BA	0.3 µM	4.0 (0.41) bcde	0.44 (0.03)bcde
3 µM Kn	-	3.1 (0.32)de	0.32 (0.02)efg
6 µM Kn	-	3.5 (0.31)cde	0.38 (0.02)bcdefg
12 µM Kn	-	3.9 (0.33)bcde	0.39 (0.02)bcdefg
18 µM Kn	-	2.9 (0.24)de	0.34 (0.02)defg
3 µM Kn	0.3 µM	4.4 (0.34)bcd	0.42 (0.02)bcdef
6 µM Kn	0.3 µM	4.1(0.48)bcde	0.35 (0.03)defg
12 µM Kn	0.3 µM	4.5 (0.41)bcd	0.39 (0.02)bcdefg
18 µM Kn	0.3 µM	2.5 (0.29)e	0.29 (0.02)g

Table represents pooled means from fifteen replicates in each of 2 repetitions.
Means followed by same letter(s) are not significantly different at 5% level according to Tukey's test of SAS programme.

5.2.2.6. Plantlets development and growth performance

Under *in vivo* conditions microrhizomes can successfully germinate shoots and grow upto maturity after transferring them directly to the soil (Fig 5.5e). Germination, survival rate, and morphological characters were varied among the different sizes of microrhizomes. The survival rate of the plantlets from small (0.5 –1.0 cm), medium (1.1-2.0 cm) and large (> 2.0 cm) of microrhizomes was 50, 70 and 90% respectively. Plants regenerated from bigger microrhizomes were also found more vigorous in the glasshouse in terms of their growth parameters of shoot, root and leaf (Table 5.4).

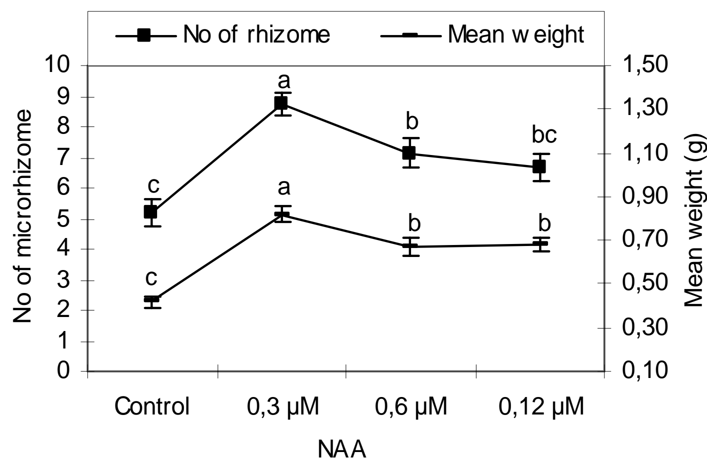


Fig 5.7. Effects of NAA on *in vitro* microrhizome induction in *Curcuma longa* L. (experiments were conducted using 0.75 x MS medium – pH 5.8, 12 µM BA and 9% sucrose under fully dark condition). Results obtained from pooled means of two repeated experiments each of them included 15 replicates; bars are ± standard error; means with the same letters are not significantly different at 5% levels according to Tukey's test of SAS programme

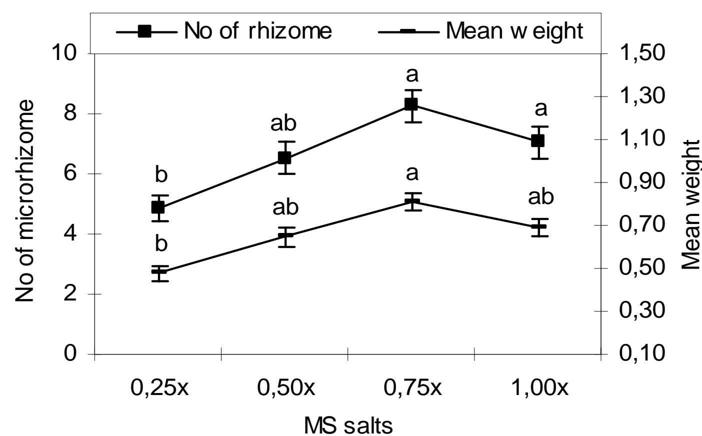


Fig 5.8. Effects of the strength of MS salts on *in vitro* microrhizome induction in *Curcuma longa* L. (experiments were conducted using 12 µM BA, 0.3 µM NAA and 9% sucrose in the dark). Results obtained from pooled means of two repeated experiments each of them included 15 replicates; bars are ± standard error; means with the same letters are not significantly different at 5% level according to Tukey's test of SAS programme.

Table 5.4. Morphological parameters were evaluated using plants regenerated from different size of microrhizomes of *C. longa* under *in vivo* condition (data recorded after 90 days cultivation in the glasshouse)

Parameters	0.5 – 1.0 cm (±SE)	1.1 – 2.0 cm (±SE)	> 2.1 cm (±SE)
Survival rate (%)	50	70	90
No of shoot	0.6 (0.22)b	1.1 (0.28)b	2.3 (0.33)a
Length of shoot (cm)	6.8 (2.32)b	9.3 (2.07)ab	14.6 (1.76)a
No of roots	3.9 (1.34)b	7.0 (1.63)ab	12.1 (1.46)a
Length of roots (cm)	5.4 (1.89)b	8.2 (1.94)ab	12.2 (1.50)a
Number of leaf	1.3 (0.47)b	2.7 (0.62)ab	3.3 (0.42)a
Leaf length (cm)	3.3 (1.12)b	5.6 (1.28)ab	10.7 (1.56)a
Leaf width (cm)	1.9 (0.66)a	3.6 (0.86)a	4.3 (0.58)a

Table represents pooled means from ten replicates. Means followed by same letters are not significantly different at 5% level according to Tukey's test of SAS programme.

5.2.3. Genetic stability of *in vitro* regenerated plantlets

To detect genetic stability of *in vitro* regenerated axillary buds of *C. longa* L. the RAPD procedure was used. Plant DNA was amplified from second, fourth and sixth subcultures using 14 different primers from OPX series of Operon technologies. Detail sequences and amplified products of primers are mentioned in Table 5.5. From this study it was observed that the *in vitro* regenerated *C. longa* plantlets showed considerable amount of genetic variation. Amplified PCR products varied from primers with a range of 8-18 bands. The highest number of bands (18) was scored from primer. The highest percentage of polymorphic bands (87.50%) was obtained from primer OPX 04 while the lowest (18.19%) equally from two primers OPX 08 and OPX 09. A total 171 bands were scored from PCR products, where 107 bands were monomorphic while 64 bands were found to be polymorphic. Mean number of amplified products per primers was 12.21 where 7.64 were monomorphic and 4.57 were polymorphic. Fig 5.9 presents the results of gel electrophoresis of PCR products of some representative agarose. Table 5.6 showed the degree of polymorphisms in different subcultures. It was observed that the second subculture produced 19 polymorphic bands i.e. 13.29% polymorphism while subcultures four and six produced higher number of polymorphic bands respectively 60 (37.27%) and 65 (38.69%). Mean polymorphic bands of different subcultures were 48 (29.75%). It was also observed that the control plant produced mostly similar amplification products to the second subculture.

Table 5.5. Different primers used for studying genetic stability of *in vitro* regenerated plant of *C. longa* L. using axillary buds.

Primers	Sequences	Total bands	Monomorphic bands (n)	Polymorphic bands (n)	Polymorphic band (%)
OPX 01	5'-CTGGGCACGA-3'	16	11	5	31.25
OPX 03	5'-TGGCGCAGTG-3'	13	8	5	38.46
OPX 04	5'-CCGCTACCGA-3'	8	1	7	87.50
OPX 05	5'-CCTTTCCCTC-3'	13	8	5	38.46
OPX 06	5'-ACGCCAGAGG-3'	9	6	3	33.33
OPX 07	5'-GAGCGAGGCT-3'	14	11	3	21.43
OPX 08	5'-CAGGGGTGGA-3'	11	9	2	18.19
OPX 09	5'-GGTCTGGTTG-3'	11	9	2	18.19
OPX 11	5'-GGAGCCTCAG-3'	12	8	4	33.33
OPX 12	5'-TCGCCAGCCA-3'	18	11	7	38.89
OPX 14	5'-ACAGGTGCTG-3'	13	7	6	46.15
OPX 15	5'-CAGACAAGCC-3'	11	5	6	54.54
OPX 17	5'-GACACGGACC-3'	14	7	7	50.00
OPX 19	5'-TGGCAAGGCA-3'	8	6	2	25.00
Total		171	107	64	
Mean		12.21	7.64	4.57	38.19

Table 5.6. Polymorphism of RAPD amplified PCR products in different subcultures

Subculture	No of polymorphic bands	No of monomorphic bands	Polymorphic bands (%)
2	19	124	13.29
4	60	101	37.27
6	65	103	38.69
Mean	48	109.33	29.75

5.3. Discussions

5.3.1. Establishment of contamination free culture

Establishment of contamination free cultures under *in vitro* conditions has already been described by other groups (Balachandran et al., 1990; Dekkers, 1991; Nadgauda et al., 1978; Salvi et al., 2002; Shirgurkar et al., 2001; Sunitibala et al., 2001; Yasuda et al., 1988). Many authors argued that contamination of underground rhizomes was very high and that the establishment of contamination free cultures was difficult. Salvi et al. (2002) used streptomycin sulphate (750mg/l) and they obtained 30% contamination free explants after 4 weeks. In this study the antibiotic streptomycene sulfate was also tested but did not improve the condition.

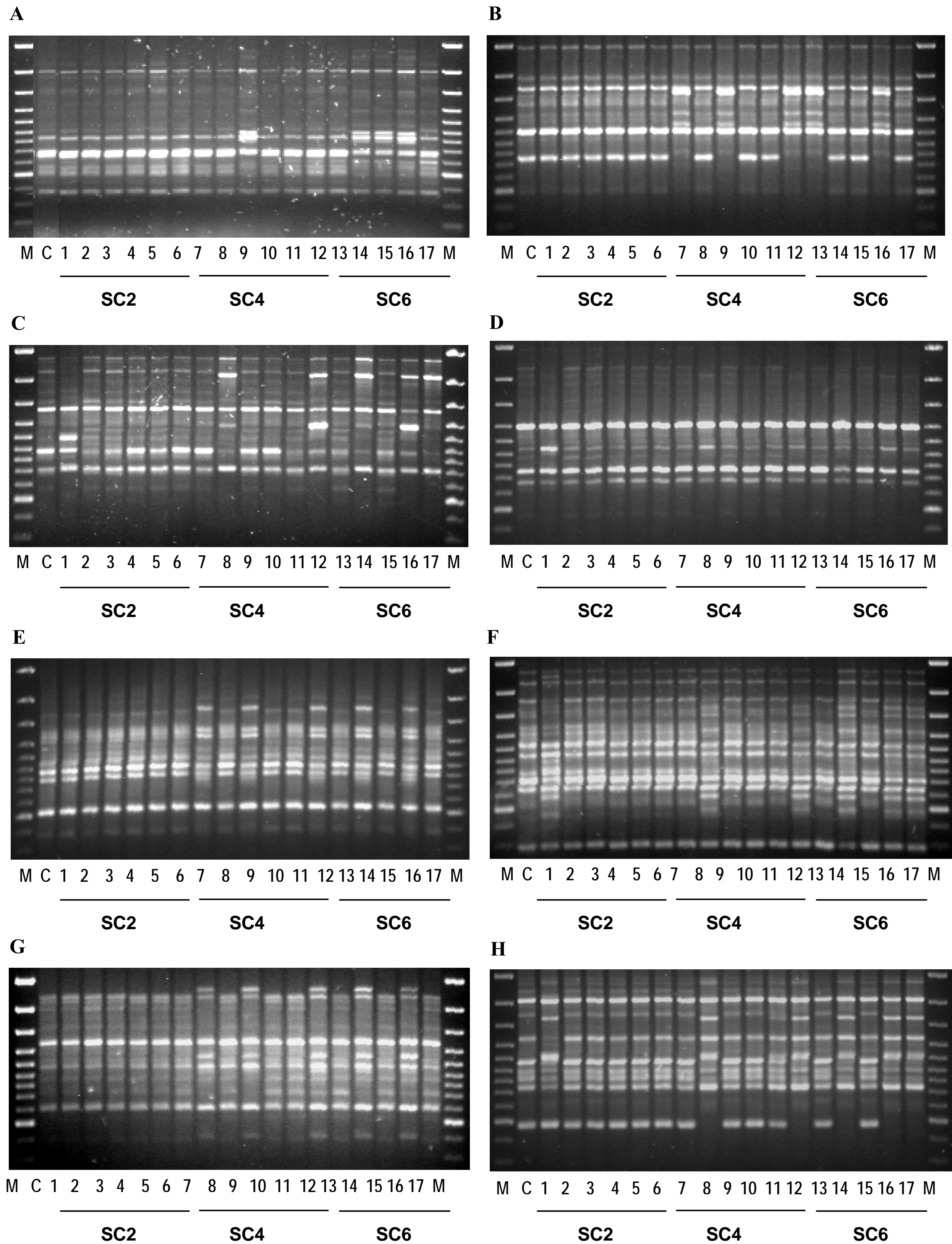


Fig 5.9. Results of gel electrophoresis of PCR products obtained by using A) OPX 01, B) OPX 03, C) OPX 05, D) OPX 09, E) OPX 11, F) OPX 12, G) OPX 14 and H) OPX 17; DNA was isolated from randomly collected *in vitro* cultured plant samples of 2nd (SC2; 1-6), 4th (SC4; 7-12) and 6th (SC6; 13-17) subcultures; M-100 bp ladder plus and C-control plant developed from rhizome

It seems that use of the wetting agent Tween-20 may help to get high percentage of contamination free explants as it allows better surface sterilization. A remarkable success was achieved by incorporating Tween 20 with 0.1% $HgCl_2$ as a wetting agent to reduce surface tension and also to allow better surface contact. Under these conditions more than 70% explants remained contamination free till next subculture. It seems that use of the wetting agent Tween-20 indeed helps to get high percentage of contamination free explants as it allows better surface sterilization.

5.3.2. *In vitro* regenerations of axillary buds

It is inferred from the obtained results that he concentrations of different cytokinins significantly influenced the number and length of shoots and roots. Among the cytokinins, 12µM BA was found to be suitable for shoot multiplication of *C. longa* and produced the highest average number of shoots along with sufficient number of roots. In many cases a single explant produced about 12 shoots. BA was also found suitable in shoot multiplications in the family *Zingiberaceae* (Balachandran, 1990; Dekkers, 1991; Shirin, et al., 2000; Rout et al., 1998; Sunitibala et al., 2001; Yusuf et al, 2001).

However, Salvi et al. (2002) reported some other expensive cytokinins rather than BA such as Kn-R, BA-R, 2iP and m-T. From the present study, it is inferred that BA is efficient enough to produce sufficient numbers of shoots within shorter period of time than other ever reported cytokinins used for *Curcuma* shoot proliferation. Kn produced the second highest number of shoots and the highest number of roots. Borthakur and Bordoloi (1992) and Nadgauda et al. (1978) have also reported Kn as an efficient cytokinin for plantlet formation and the development of *Curcuma* species. TDZ and 2iP did not enhance the production and multiplication rates in *C. longa*. Salvi et al. (2000) also found that TDZ was not as suitable as BA while they used segments of immature inflorescence for regeneration of turmeric. However, TDZ might be helpful for increasing shoots by induction of the explants for a shorter period of time (2-3 days) as it is a non-purine cytokinin-like compound. It has been shown to exhibit stronger effects than conventional cytokinins.

Different auxins had also significant effects on the number and length of shoots and roots. NAA was found to be optimal for *C. longa,* while IAA was found suitable for increasing root number and length but it decreased the shoot number remarkably. However, IBA could be used as second alternative as it did not reduce shoots and roots dramatically. The presented

results also support other works on the members of the Family *Zingiberaceae* (Balachandran et al., 1990; Dekkers, 1991; Nayak, 2000; Salvi et al., 2002 and 2000; Shirgurkar et al., 2001; Shirin et al., 2000). It seems that *Curcuma* and other ginger species may have a sufficient amount of internal auxin-activity and they do not need a higher amount of auxin for root development. However, it can be stated from the results of the present study that a low concentration of NAA (0.3µM) helps to get a higher number of healthy shoots and roots.

Among the various strengths of MS salts, 1.00 and 0.75x strength of MS medium were found to be suitable for shoot's multiplication. A similar trend was also reported by Shirin et al. (2000) in the production of plantlets of *Kaempferia galanga* (Zingiberaceae) and some other studies on *Trillium erectum* and *T. grandiflorum* (Pence and Soukup, 1993), and *Viola patrini* (Sato et al., 1995). However, comparable data were not available for *C. longa*, only the following information was available in the microrhizome production in turmeric (Shirgurkar et al., 2001), where half strength basal medium was found to be suitable, while Nayak (2000) used full strength MS basal medium for microrhizome induction in *C. aromatica* Salisb.

Different concentrations of sucrose were investigated, as the sucrose is an efficient and frequently used source of carbohydrate for *in vitro* regeneration. There was a linear decrease in the number of shoots in both lower and higher concentrations of sucrose, while a higher concentration increased the root number. However, Salvi et al. (2002) investigated the effect of various carbohydrate sources on *in vitro* shoot multiplication of *C. longa* and found that glucose was a more efficient carbohydrate source for shoot multiplication.

Out of six different agar concentrations, maximum shoots were obtained in 0.8% agar, and a linear decrease in shoot and root productions were found in both higher and lower concentrations of agar. However, lower concentrations of agar were found to be suitable for shoot multiplication of *C. longa* (Salvi et al., 2002). High percentage of survivability and establishment in the soil indicates the efficacy of the procedure. In this study, field level evaluation could not be made as turmeric is a tropical crop for which an appropriate natural condition is not available in the glasshouse, however Salvi et al. (2002) have already investigated and evaluated the field level efficiency of micropropagated turmeric plants and observed that micropropagated plants were superior to the conventionally propagated turmeric plants.

From the present study a simple protocol is recommended that can be used routinely for high frequency regeneration of *C. longa* in which 12 µM BA and 0.3 µM NAA were found to be optimum. It seems that the internal growth regulators (especially auxin) are very active in this plant so that they do not require higher amounts or any other PGR for *in vitro* regeneration. Moreover, the entire procedure could be completed without any callus induction, as it requires very low amount of PGR, which has an advantage since callusing may produce more variability among the regenerated plants. This regeneration system could also be used as a source of genetically identical germplasm for long long-term *in vitro* preservation.

5.3.3. *In vitro* microrhizome induction in *C. longa* L.

In order to develop an optimised standard protocol for microrhizome induction in *C. longa* L., four weeks old contamination free cultures were used and further multiplied for subsequent experiments. Different concentrations of sucrose were investigated under fully dark and 16 hrs light illumination conditions (white fluorescent light with 50 µM m^{-2} s^{-1} light intensity). It was observed that sucrose plays a significant role in terms of size and number of microrhizome in *C. longa.* Nine percent sucrose produced the highest number of healthy microrhizomes in dark condition, while light treatment reduced the number and size slightly, which were not found to be significant statistically. Seven percent sucrose reduced both numbers. However, both lower and higher concentrations of sucrose had significant inhibitory effects on microrhizome production. The presented results support some previous reports (Nayak, 2000; Shirgurkar et al., 2001; Sunitibala et al., 2001) since they described a comparable range of 6-9% sucrose for microrhizome induction in *Curcuma*. They also stated that a lower concentration of sucrose decreased the size and number of rhizomes or even prevented completely the induction of any microrhizome. Shirgurkar et al. (2001) obtained the highest number of microrhizome (5.6 ± 0.8 - 7.0 ± 1.1) at 6% sucrose while at 8% sucrose they found a reduction of the number (5.6 ± 0.5 – 5.8 ±1.1) but a slightly increase in their size. Nayak (2000) observed that at an obligatory concentration of sucrose (3%) of plants could not develop any microrhizome even by increasing the concentration of BA from 1-7 mg L^{-1} or by increasing the duration of photoperiod. Sharma and Singh (1995) reported that a concentration of 7.5% was required for *in vitro* rhizome induction in *Zingiber officinale* Rosc. In many other reports it is also stated that a high concentration of sucrose promotes the *in vitro* formation of storage organs such as bulbs, corms and tubers etc. remarkably (Alizadeh et al., 1998; Abbott and Belcher, 1986; Arora et al., 1996; Dantu and Bhojwani, 1987; Garner and Blake, 1989; Grewal, 1996; Gopal et. al., 1998; Hoque et al., 1996; Kim et al., 2003;

Slabbert and Niederwieser, 1999; Vreugdenhil et al., 1998). The enhanced rate of *in vitro* organ formation with increasing concentration of sucrose may be attributed to the presence of high carbon energy in sucrose since storage organs mostly stored carbohydrates (Nayak, 2000).

The presented results indicate that the plant growth regulators (PGR) BA, Kn and NAA had also significant effects on *in vitro* microrhizome induction. Among the sixteen different combinations of PGR, 12 µM BA together with 0.3 µM NAA exhibited a better response than any other treatments in terms of mean number and the weight of microrhizomes. Nayak (2000) reported that 5 µg L^{-1} enhanced microrhizome production in *C. aromatica* Salisb. while Sharma and Singh (1995) found 8 µg L^{-1} optimum in ginger. However, Shirgurkar et al. (2001) stated that BA had an inhibitory effect on *in vitro* microrhizome production in turmeric, though at the lowest level of 4.4 µM it did not have much adverse effect on the average number, weight and yield of microrhizome, but at the maximum concentration of 35.2 µM BA completely inhibited microrhizome production. In the present study, Kn alone or in presence of NAA did not show any promising result while it produced up to the number 4.5 ± 0.41 and size 0.39 ± 0.02 at the concentrations of 12 µM BA and 0.3 µM NAA. However, Sunitibala et al. (2001) reported that Kn (1mg L^{-1}) is suitable for *in vitro* rhizome induction in *C. longa* L. Grewal (1996) also stated Kn as an efficient cytokinin for enhancing microtuber induction of somatic embryos of *Bunium persicum*.

Effects of different concentrations of NAA were also investigated in a separate experiment and 0.3 µM NAA was found to be suitable to obtain the highest number of bigger microrhizomes, while both increasing and decreasing of NAA had significant decrease of the number and size of microrhizome. Sunitibala et al. (2001) also observed that NAA (0.1 mg/l) is suitable for induction of rhizomes. Peak and Murthy (2002) reported that NAA is the most effective auxin for the induction of bulblets *in vitro* from scale sections of *Fritillaria thunbergii*. Kim et al. (2003) also reported that 0.1 mg l^{-1} NAA along with 11% sucrose and 10 µM JA showed highest multiplication of bublets in garlic.

Effects of MS salts in microrhizome induction in *C. longa* were also investigated. Among the various strengths of MS salt, 0.75x strength of MS medium was found to be the most suitable in terms of number and size of microrhizome, however, both half and full strength of MS salts decreased both the number and size of microrhizomes. Lower strength of the MS medium had

produced a lower number of smaller microrhizomes, while full strength produced a lower number but comparatively bigger size of rhizomes. Shirgurkar et al. (2001) reported that half strength basal medium suitable for microrhizome production in turmeric while they obtained an average number (5.8 ± 0.7) with the biggest size (0.55 ± 0.06), while Nayak (2000) and Sunitibala et al. (2001) used full strength MS basal medium for microrhizome induction in *Curcuma*. Sharma and Singh (1995) also found full strength MS basal medium along with 7.5% sucrose and 35.2 µM BA optimal for the production of *in vitro* microrhizome in ginger.

Under *in vivo* conditions microrhizomes can successfully germinate shoots after transferring them into soil. Germination, survival rate, and morphological characters were varied among the different sizes of microrhizomes. Plants regenerated from bigger microrhizomes were found to be more vigorous in the glasshouse in terms of their growth parameters of shoot, root and leaf. Similarly, Shirgurkar et al. (2001) reported that bigger microrhizomes were more efficient and vigorous in the field and grew faster. However, they obtained a survival rate of 10.4%, 54.7% and 73.9% respectively from smaller (0.1 .0.4 g), medium (0.41 - 0.8 g) and large (>0.8 g) microrhizome. In this study much higher survival rate was achieved than previous studies. Average weight of the produced microrhizome was also higher which inferred that increasing the bigger microrhizome is a key factor in achieving commercial success in microrhizome induction in *C. longa* and the presented protocol is one step forwards to that of commercial need. Sharma and Singh (1995) reported that microrhizomes can be stored under moist conditions at room temperature and that more than 80% of the sprouted microrhizomes developed shoots and roots two months after they had been successfully transferred to the field.

In vitro shoot multiplication system for *C. longa* has already been studied by many authors previously that can be used in large-scale micropropagation. However, microrhizome induction in *Curcuma* is still under progress and this protocol can be used to produce a higher amount of large microrhizomes since this protocol provides with better results than other ever reported protocols. Produced *in vitro* microrhizomes would be a suitable source of disease free seed rhizomes that could be stored and transported easily which are advantageous over plantlets production. In addition to that, *in vitro* microrhizomes can eliminate the necessity of acclimatization in the field. The presented protocol is certainly a step forward towards an improved commercial propagation system for *C. longa* var. Surma in Bangladesh.

5.3.4. Genetic instability of *in vitro* regenerated plantlets

The detection of genetic variation using DNA markers among morphologically indistinguishable micropropagated plants emphasises the need for testing *in vitro* propagated plants at the molecular level. In this study genetic variability was detected from *in vitro* regenerated axillary buds of *C. longa* L. var 'Surma' using RAPD procedure. It was observed that second subculture produced 13.29% polymorphism while subcultures four and six produced higher number of polymorphic bands respectively 37.27% and 38.69%. Various factors are well recognised to induce somaclonal variations such as the *in vitro* process, duration, auxin and cytokinin concentrations, their ratio, and other nutritional conditions and *in vitro* stress, are all known to induce somaclonal variation (Devarumath et al., 2002). In addition to that, somaclonal variation may arise as a result of minor point mutations and rearrangements in nuclear or organellar DNA, the activation of transposable elements, polyploidy, aneuploidy, and epigenetic changes (Rahman and Rajora, 2001). Devarumath et al. (2002) detected somaclonal variation in *Camellia sinensis* (clone U26) by RAPD technique where 4.28% fragments were found polymorphic. RAPD markers have also been used to detect somaclonal variation among micropropagated plants of *Coffea arabica* (Rani et al., 2000), *Populus deltoides* clones (Rani et al., 1995 and 2001), banana (Damasco et al., 1996; Gimenez et al., 2001) and oil palm (Rival et al., 1998). Damasco et al. (1996) stated that, reliable detection of dwarf plants was achieved using RAPD marker that providing the only available means of *in vitro* detection of dwarfs. Other micropropagation-induced RAPD polymorphisms were detected but not associated with the dwarf trait. A somaclonal variant of banana CIEN BTA-03 resistant to Yellow Sigatoka disease was obtained by induction of adventitious shoots from excised sucker shoot tips of Williams cultivar (AAA) (subgroup Cavendish), grown in MS medium containing 15 mg BA (Gimenez et al., 2001).

Salvi et al. (2001) detected 16.5% polymorphism in *C. longa* plants that regenerated from leaf base callus using RAPD procedure. However, RAPD polymorphism was not detected in *C. longa* cv 'elite' by Salvi et al. (2002) using same primers while plantlets were regenerated from axillary buds. Hence it can be pointed out that the genetic constitution in each clone/variety which determined the stability/variability of the micropropagated plants under the given cultural conditions ((Devarumath et al., 2002). It would appear that *C. longa* var 'Surma' is inherently unstable under *in vitro* conditions and/or more prone to *in vitro*-induced stress leading to somaclonal variation. Similar results have been obtained in micropropagated plants of *Camellia sinensis* (Devarumath et al., 2002), and poplars (Rani et al., 1995 and

2001). A species/genotype-specific response to genetic stability/instability in tissue culture conditions is not uncommon and has been reported in many other plant taxa as well ((Devarumath et al., 2002; Mohmand and Nabors, 1990; Rani and Raina, 2000). Whether the genetic changes observed in the present study influence traits of interest or not, but the matter is that, this Bangladeshi turmeric variety Surma has likely found to be genetically unstable under *in vitro* conditions. The results of the present study in congruent with earlier reports ((Devarumath et al., 2002; Rani et al., 1995, 2000 and 2001; Damasco et al., 1996; Rani and Raina, 1998 and 2000; Rival et al., 1998; Gimenez et al., 2001; Rahman and Rajora, 2001), which significantly inferred that plants derived even from organised meristem culture may not always be genetically true-to-type.

The value of the somaclonal variants observed in *Curcuma* with respect to tree improvement of this species is not recognized since the relationship between RAPD markers and somaclonal variation and various traits of interest is not known. The results of this study demonstrate that somaclonal variation has occurred in the micropropagated plants of *C. longa* even though organized meristem tissues of vegetative buds were used as the explant source, and that this variation could be detected using RAPD markers. Rani and Raina (2000) stated that the existence of somaclonal variation among micropropagated plants derived through the culture of organized meristems has been revealed for various morphological, cytological, biochemical, and molecular traits. This study demonstrated that the clonal fidelity of micropropagated plants of *C. longa* cannot always be assured even when organized tissues of vegetative buds are used as the explant source and that RAPDs are useful markers for determining the clonal integrity and somaclonal variation of *in vitro* regenerated *C. longa* germplasm.

6. CRYOPRESERVATION

6.1. Introduction

In the tropics, numerous vegetatively propagated crop plants traditionally play a vital role in the rural economy as potential sources of agricultural and horticultural commodities, industrial raw materials, and indigenous medicines. These important crop genetic resources are always under threat of extinction since they do lack sexual reproduction and eventually lack genetic diversity within their entire gene pool. Most of the germplasm of vegetatively propagated species is mainly conserved in field gene banks. Increasing numbers of accessions require intensive labour and cost only to maintain a small proportion of diversity in the field conditions. This method of conservation, however, also presents certain drawbacks, which limit its efficiency and threaten the security (Takagi et al., 1998). Owing to this, there is a mounting demand for stable long-term storage of the germplasm of vegetatively propagated crop plants. It is currently well recognized that cryopreservation of plant germplasm is a preferable option that has obvious advantages over *in vitro* storage (Engelmann, 1997; Wilkinson et al., 2003). Long-term conservation of such vegetatively propagated plants is fundamental because *in vitro* technique can only be used for the medium term conservation of germplasm, which is not only time consuming and labour-intensive, but in addition may not ensure good genetic stability of *in vitro* micropropagated plantlets (Sarkar and Naik, 1998). Cryogenic storage has therefore been recognized as a key method for long term storage of the base collection of vegetatively propagated crop plants since these plants have been yet received a little attention (Engelmann, 1991 and 1997; Sarkar and Naik, 1998; Takagi et al., 1998).

It is already described earlier that Curcuma species are the important tropical plants reproduce extremely by means of vegetative underground rhizomes. This plant used as spices, medicines, dyeing agents cosmetics and ornamentals in the tropical Asia (Salvi et al., 2000; Shirgurkar et al., 2001). It is also mentioned earlier that, the chemical composition of *C. longa* has thoroughly been investigated and a number of different active substances that exhibit germicidal, aromatic, carminative, antihelmentic, antioxidant, anti-tumour, cholesterol lowering activities were confirmed (Cao et al., 2001; Jitoe et al., 1992; Kikuzaki and Nakatani, 1993; Masuda et al., 1993; Sasaki et al., 2002). Despite, this plant has also attracted

attention because of their anti-inflammatory (Yoshioka et al. 1998), antihepatotoxic (Matsuda et al., 1998), neuroprotective activity (Sasaki et al., 2002) and cytotoxic activity against human ovarian cancer cells (Syu et al., 1998). Recently *Curcuma* has also received attention and generated worldwide commercial demands as ornamental plants (Maciel and Criley, 2003; Paisooksantivatana, et al., 2001a and 2001b). This important species encompasses low genetic variation as a result of sexual incompatibility due to its triploid nature. Due to the above reason a continuous loss of genetic variability in this species results serious threats to extinction. Development of appropriate methods of long-term conservation by means of cryostorage of varieties and landraces *C. longa* and related species is requisite to safeguard these species for future generations.

To the best of knowledge, there is no work that has been published on the cryopreservation of *C. longa* species. The present study was therefore undertaken to establish an effective cryopreservation technique based on the vitrification approach using *in vitro* grown axillary buds. Most of the previous studies on vitrification procedures so far suggested that the key to successful vitrification of cells or small tissues is the effective increase in the concentration of cellular solutes achieved by treating with chemicals e.g. sucrose prior to the PVS2 treatment for vitrification. One of the main objectives of the present study was therefore, to achieve optimal pre-culturing conditions before treating with PVS2 solution. Finally, an optimal PVS2 incubation condition in terms of time period and concentration was also investigated aiming to establish a successful cryopreservation procedure for *C. longa* species.

6.2. Results

6.2.1. Effects of different vitrification solutions

In a preliminary experiment, excised axillary buds were preconditioned for a week and precultured over night. Precultured buds were then incubated into three vitrification solutions of PVS, PVS2 and Steponkus solution for different time periods. It was observed that PVS and Steponkus solution were less or even not suitable for cryopreservation of the axillary buds of *C. longa* L., whereas PVS2 solution was found to be an effective vitrification solution. Buds incubated into PVS2 solution for 20 min were able to recover for up to 60 (± 3.85) % while, 51.11 (± 5.88) % buds treated for 30 min recovered. Recovery rates of buds incubated for 10 and 40 min were considerably lower which were 26.67 (± 3.85) % and 28.89 ((± 4.44) % respectively (Table 6.1). Further experiments were therefore, conducted using PVS2

solution to optimise the different cryopreservation conditions including preconditioning with enriched sucrose medium and vitrification.

6.2.2. Effects of preconditioning with sucrose

The effects of preconditioning treatment with sucrose were investigated. A significantly varied success was achieved from *in vitro* cultures with elevated sucrose concentrations varying from a concentration of 0.10 - 0.50 M (Fig 6.1) and also from various time periods of preconditioning (Fig 6.2) on survival rates of axillary buds. Fig 6.1 demonstrated that there was no significant difference of the recovery rates among control buds (without plunging in LN) exposed to different PVS2 treatments. The survival rates varied from 62.22 to 88.88 %. Buds stored in LN showed more significant variation of the recovery rates in different concentrations of sucrose used for preconditioning treatments. Recovery rates varied between 24.44 and 53.33%, which was considerably lower than the survival rates of control buds. Fig 6.2 illustrates that there was also no significant differences of recovery rates between the PVS2 treated control buds for different periods of incubation in 0.30 M sucrose. In contrast, the buds exposed to LN demonstrated a significant variation for different time periods. A high percentage of survival was achieved from the treatments with incubation periods of 5 and 7 days. Survival rates in these cases were 55.55 ± 8.01 % and 53.33 ± 3.85 % respectively. However, the survival rates obtained from 3 – 15 days were not statistically different.

Table 6.1. Survival rate (%) of axillary buds of *C. longa* in different vitrification solutions for different time periods (after treatment in loading solution).

Duration	PVS (± SE)*	PVS2 (± SE)*	Steponkus soln.**
10 min	0.00 %	26.67 (3.85) %	0.00 %
20 min	15.55 (2.22) %	60.00 (3.85) %	0.00 %
30 min	22.22 (5.88) %	51.11 (5.88) %	0.00 %
40 min	20.00 (3.85) %	28.89 (4.44) %	0.00 %

Axillary buds were preconditioned for 5 days with 0.3 M sucrose and precultured isolated buds overnight; incubation in the loading solutions for 20 min; *Incubated in the loading solution-1 (MS components supplemented with 2 M glycerol and 0.4 M sucrose); **Incubated in the loading solution-2 (MS medium components supplemented with 1.5 M ethylene glycol); 20 buds were treated in each of three repeated experiments; (± standard error)

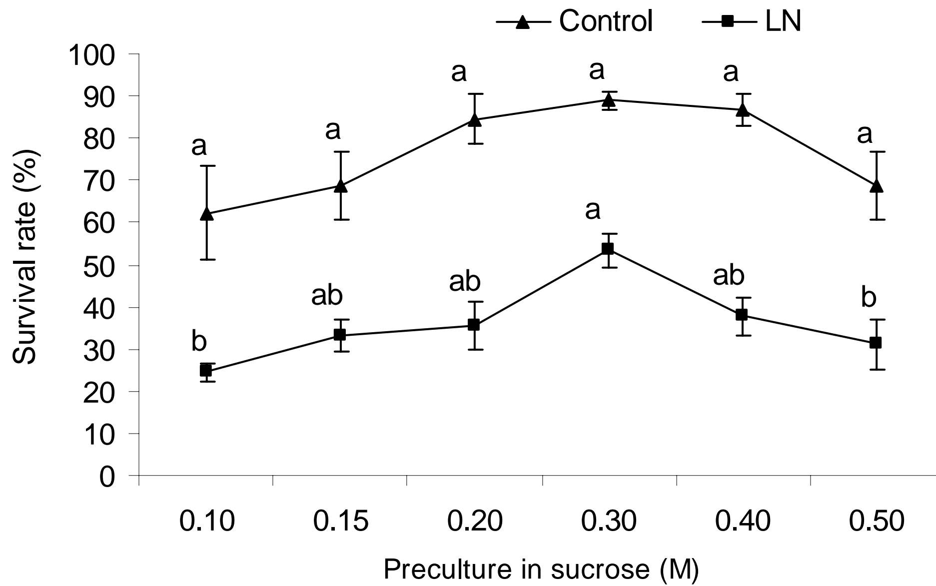


Fig 6.1. Effects of different concentrations of sucrose in the preconditioning treatment; buds were preconditioned for 3 days; treated with loading solution 1 for 20 minutes and incubated in PVS2 solution for 20 min.; 15 - 20 buds were treated in each of three repeated experiments; letters a and b in the diagram indicate points used for Tukey`s grouping test for significance.

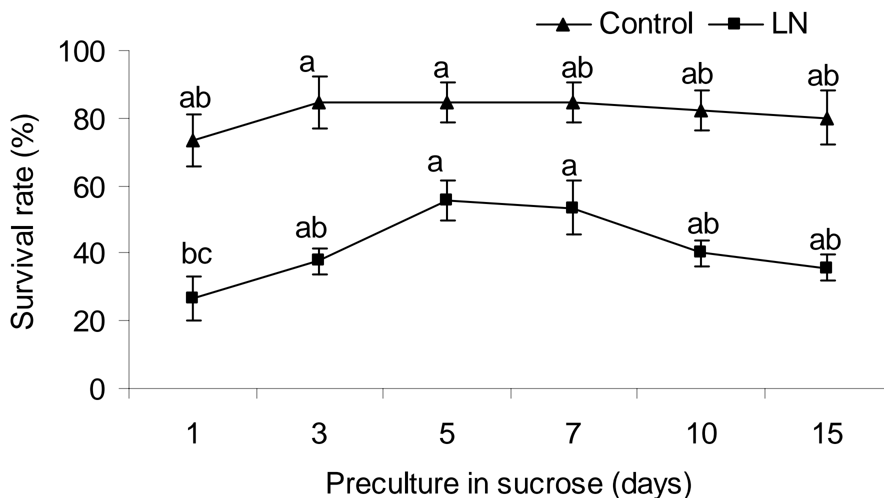


Fig 6.2. Effects of different preculture periods; buds were cultured in MS medium supplemented with 0.3 M sucrose; treated with loading solution 1 for 20 min; 15 - 20 buds were treated in each of three repeated experiments; Bars are standard errors bars; letters in the diagram indicate points used for Tukey`s grouping test for significance.

6.2.3. Effects of different treatments with PVS2 solution

The optimum strength of PVS2 solution and incubation periods were also investigated and it was observed that both of these greatly affected survival of the axillary buds of *C. longa*. In case of incubation periods, there was no significant difference among the control treatments, but a gradual decrease of the percentage of recovery was found with an increase of incubation periods (93.33 ± 4.41 % – 68.33 ± 7.26 %) in PVS2, while significant differences of the survival rate were observed among different treatments when buds were plunged in LN. The highest survival rate was achieved (51.67 ± 6.01 %) from the buds that were incubated for a period of 20 min., however, the survival rates for 15 min (33.33 ± 7.26 %) and 30 min (48.33 ± 4.41 %) incubation time did not statistically vary from the survival rate obtained for 30 min incubation (Fig 6.3).

The strength of PVS2 solution (Fig 6.4) was also tested and significant differences were obtained for three different concentrations of PVS2. The highest survival rate was achieved for the full strength (53.33 ± 4.41 %), whereas, a very low percentage of survival rates were achieved from 0.60x (10.00 ± 2.89 %) and 0.80x (23.33 ± 3.34 %) strength of PVS2. In case of the control experiment, there was no significant difference of recovery rates between treatments; however, full strength PVS2 solution slightly decreased the survival rate (81.67 ± 1.67 %) compared to 0.06x (93.33 ± 4.41 %) and 0.80x strength (85.00 ± 2.89 %).

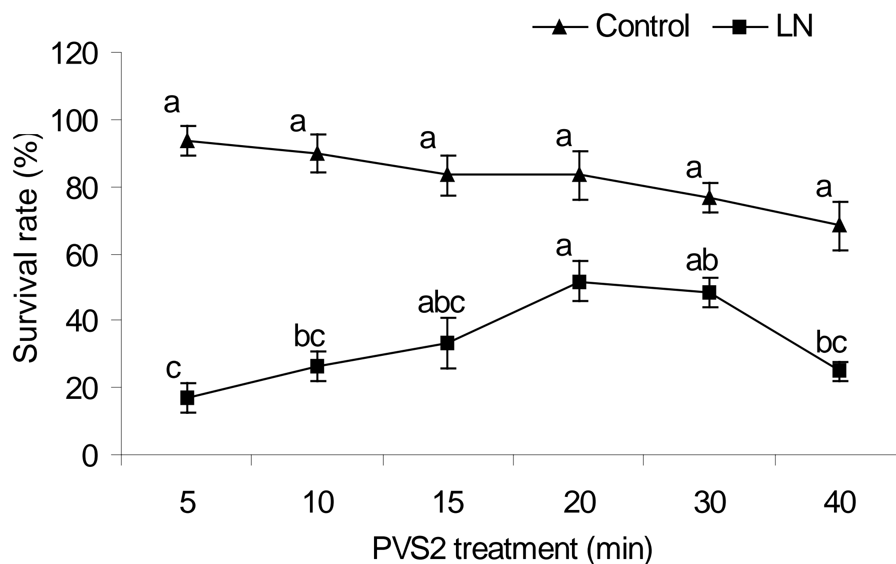


Fig 6.3 Effects of the incubation period in PVS2 solution; buds were cultured in MS medium supplemented with 0.3 M sucrose; treated with loading solution 1 for 20 min; 15 - 20 buds were treated in each of three repeated experiments; Bars are standard errors bars; letters in the diagram indicate points used for Tukey`s grouping test for significance.

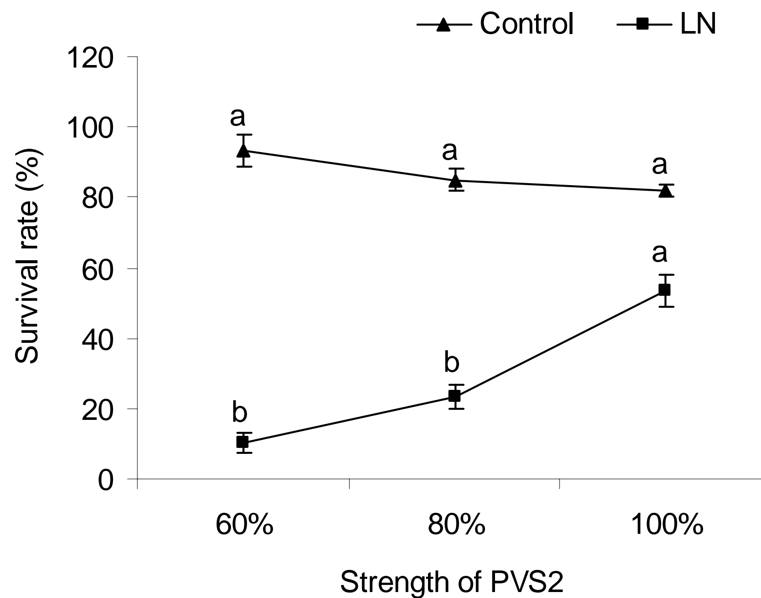


Fig 6.4. Effects of the strength of PVS2 solution used for incubation; buds were cultured in MS medium supplemented with 0.3 M sucrose; treated with loading solution 1 for 20 min; 15 - 20 buds were treated in each of three repeated experiments; Bars are standard errors bars; letters in the diagram indicate points used for Tukey`s grouping test for significance

6.2.4. Effects of the size of axillary buds

Fig 6.5 represents an image containing different size of axillary buds that were commonly found during this study. Effects of different size of axillary buds were tested. Fig 6.6 inferred the effects of different size of isolated axillary buds. Among three different sizes, medium sized buds (3-4 mm) showed the highest survival rates after cryopreservation (58.33 ± 6.01 %). However the survival rate achieved (40.00 ± 7.64 %) from the bigger size (>4 mm) did not differ statistically from the survival rate of medium size buds, whereas the smaller size of buds demonstrated a significant decrease of survival rate (30.00 ± 5.77 %).

6.2.5. Survivability of *C. longa* after recovery from freezing

Under optimum freezing conditions about 80% of the meristems were found to be capable to recover from freezing condition which were finally proficient to grow into intact plants when they were transferred to the multiplication medium (Fig 6.7). This result indicates the suitability of the vitrification method of long-term storage of *C. longa* germplasm.

Fig 6.5. Buds of *Curcuma longa* representing different size classes that used for cryopreservation experiments; 1) big : > 4 mm, 2) medium : 3 - 4 mm, and 3) small : < 3 mm

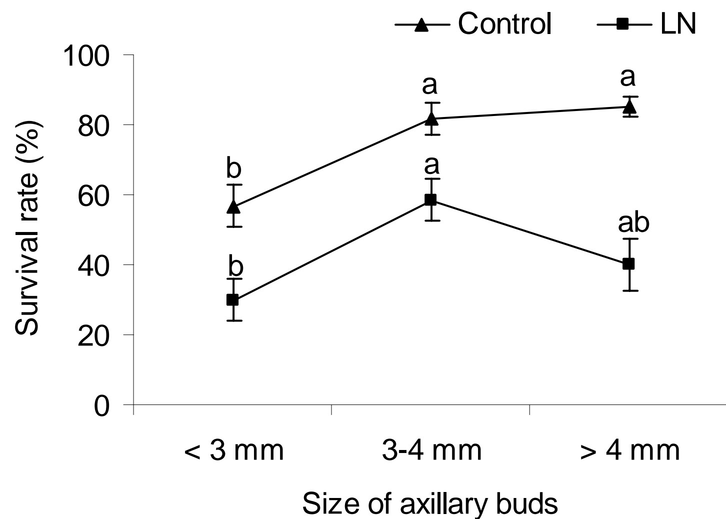


Fig 6.6. Survival rates of different size of buds; buds were preconditioned in the MS medium supplemented with 0.3 M sucrose; treated with loading solution- 1 for 20 minutes and incubated in full strength PVS2 solution for 20 min; 15-20 buds were treated in each of three repeated experiments; *Bars* standard error; letters in the diagram indicate points used for Tukey`s grouping test for significance

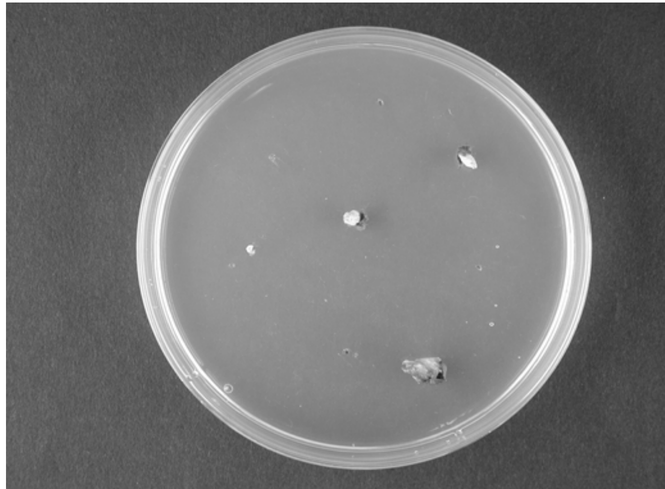


Fig 6.7. Recovered buds of *Curcuma longa* regrowing in multiplication medium after cryostorage

6.3. Discussions

It is currently well accepted that the successful cryopreservation of vegetatively propagated tropical crop plants is essential (Takagi et al., 1998; Thinh et al., 1998), because they always have a great chance of dwindling genetic variability as they do not have a sexual genetic exchange that ultimately lack genetic variability of plants. Suitability of cryopreservation system for long-term storage of plant germplasm has also inspected by Wilkinson et al. (2003). The results presented in this study demonstrate the efficacy of a vitrification procedure for the cryopreservation of *C. longa* that might be applicable also to other economically important *Curcuma* species or even other related species of the family Zingiberaceae. Vitrification was also reported as a suitable technique for other vegetatively propagated crop plants (Halmagyi et al., 2004; Helliot et al., 2003; Leunufna and Keller, 2003; Sarkar and Naik, 1998; Takagi et al., 1998). In this study, *C. longa* was considered as a model species since an effective *in vitro* multiplication system has already been established in this study and also previously by others (Balachandran et al., 1990; Nadgauda et al., 1978; Salvi et al., 2002; Shirgurkar et al., 2001; Sunitibala et al., 2001; Yasuda et al., 1988; Yusuf et al., 2001).

Among different vitrification solutions, PVS2 was found to be more effective for successful cryopreservation of axillary buds of *C. longa*. Many recent reports also described the PVS2 as an effective vitrification solution (Halmagyi et al., 2004; Helliot et al., 2003; Hirari and Sakai, 1998; Leunufna and Keller, 2003; Sarkar and Naik, 1998; Turner et al., 2001). In *C. longa* the

size of the axillary buds was found to be a substantial factor for successful recovery of the cryopreserved buds. In this study, it was observed that medium sized buds (3-4 mm) are more suitable to recover after cryostorage than bigger or smaller ones. Although the larger buds (> 4 mm) were found to be more tolerant to the PVS2 control treatment, the recovery rate after cryostorage was lower than for medium size buds. On the other hand smaller explants were found to be very sensitive in both conditions of control and LN treatment. The reason may be an incomplete penetration of the vitrification solution into the big buds leading to suboptimum vitrification of the inner cell layers. This suboptimum vitrification of bigger buds may result in the intracellular ice formation, which is a leading factor associated with physical damage of the tissue (Helliot et al., 2003). The same incomplete penetration of the vitrification solution into bigger buds may on the other hand reduce the toxic effects of the vitrification procedure and thereby cause a higher recovery among the control buds not exposed to LN. Beak et al. (2003) also reported that the size of explants had a significant effect on regeneration of cryopreserved apices of garlic. They observed that the explants of 1.5 mm diameter displayed higher regeneration than large ones of 4.5 mm diameter.

The present study showed that preconditioning of axillary buds was essential for successful cryopreservation of *C. longa* by vitrification. It was found that the preconditioning of the axillary buds had a significant effect on recovery of cryostored buds of *C. longa*. The optimal sucrose concentration for preconditioning was 0.3 M for the duration of 5-7 days. Beak et al. (2003) reported an optimal sucrose concentration of 0.3 - 0.5 M in the case of cryopreservation of garlic shoot apices. It is well recognised that sucrose is an important pre-growth additive for acquisition of desiccation tolerance during cryopreservation by vitrification (Sarkar and Naik, 1998). This hardening treatment leads to the mitigation of the subsequent injurious effects of the PVS2 solution resulting in numerous ultrastructural changes like the fragmentation of vacuoles into smaller ones, the differentiation of proplastids into amyloplasts containing starch and the swelling of organelles like the endoplasmic reticulum (Helliot et al., 2003; Takashi et al., 1997). Sucrose uptake in cultured shoots of sugar beet was measured by Vandenbussche et al. (1999) after a 1-week preculture on a medium enriched with 0.3 M sucrose. They found an uptake of free sugars by the apical dome, accumulating in parenchyma cells. Similar results were also observed with shoot tips of *Musa.* The role of sucrose preculture in the reduction of the amount of freezable water was emphasised by Panis et al. (1996). A sequential loading of preconditioned axillary buds with different concentrations of the PVS2 solution before vitrification by application of

concentrated PVS2 could also be useful to further improve the survival rate of vitrified buds as investigated by Sarkar and Naik (1998) in potato. Direct exposure of precultured explants to concentrated solutions could be detrimental. This harmful effect may be due to excessive osmotic stress or chemical toxicity exerted by concentrated PVS2 (Matsumoto et al., 1994). It is particularly important that cryopreserved axillary buds are capable of regenerating shoots without intermediary callus formation. The optimum preconditioning and vitrification conditions established in this study induced direct regeneration from vitrified axillary buds. Post-thaw culturing of vitrified axillary buds under dark condition and on medium containing an elevated level of sucrose (0.3 M) for overnight was found to be beneficial for the survival rate and the regeneration of multiple shoots. A high-concentration of sucrose medium might be favourable for osmotic adjustment that allows the cryopreserved buds from the trauma of cryo-shock (Sarkar and Naik, 1998). Developed vitrification procedure can be employed for long-term cryostorage of *C. longa* L. and possibly also for related varieties and species. Mix-Wagner et al. (2002) investigated the recovery of potato apices after several years of storage in liquid nitrogen and observed that after several years' storage in liquid nitrogen, there were no major changes in either survival rate or recovery of frozen apices. The AFPL (Amplified Fragment Length Polymorphisms) was employed by Wilkinson et al. (2003) to detect genetic variation of cryopreserved *Cosmos atrosanguineus*. Not any genetic variation was detected after 12 months cryostorage.

This report is certainly a step forwards towards an efficient cryopreservation system for *C. longa* L. Nevertheless further experimental efforts are still required to improve this preliminary cryopreservation system for the species. So far optimisation trials have been carried out on the basis of survival of buds detected 8 - 10 weeks after thawing. An optimisation concerning regeneration of plants and not only survival of buds seems to be necessary and may improve results. Another question not touched so far is whether the method is applicable also to other genotypes without major adjustments. At least four varieties namely Surma, Roma, Ranga and Rasmi are popular all over the sub-continent. In addition some wild relatives, like *C. aromatica* and *C. amada* are also important for curcumin production and cultivated in many regions in Bangladesh as well as other neighbouring countries. Before final application of any cryopreservation method for germplasm storage it has also to be investigated if the method provides genetic stability. Methods under development to monitor the status of biological diversity may also be used to investigate genetic stability after cryopreservation.

7. GENERAL DISCUSSIONS

It is thought that the estimated rates of species extinction of recent years is thousand times higher than in the past which is only comparable to some of the catastrophic mass extinction events of the past (Novacek and Cleland, 2001). Serious consequences are associated with the loss of biological diversity as a result of environmental degradation, excessive use of resources and the mounting pressures from the world's growing population. An estimation of the world population was made by Cincotta et al. (2000), which inferred that more than 1.1 billion people, nearly 20% of world population, were living within the biodiversity hotspots areas in 1995. This implies that substantial human-induced environmental changes are prone to persist the biological diversity in the hotspots regions and eventually the demographic change remains an important issue in global biodiversity conservation.

The current massive fragmentation of habitat and extinction of many of the species is taking place on a devastatingly in a short timescale. Their effects will essentially reset the future evolution of the planet's biota (Novacek and Cleland, 2001). Unfortunately the process of extinction as well as complexity of gene erosion is ever-increasing (Hammer et al., 2003). This dramatic loss of biodiversity has become a severe event all over the world in recent times particularly in developing countries of the tropics. It is assumed that a substantial number of species extinction will also have taken place by 2050 and the existing large blocks of tropical forest will be much reduced and fragmented. These changes will not only be restricted to themselves but also threaten the survival of humans as a biological species (Jenkins, 2003).

Consequently, the irreversible loss of cultivated and wild species that comprise the loss of subspecies, landraces and former varieties during the past 100–150 years is of major concern to gene banks and plant professionals (Hammer et al., 2003). The basic element of plant genetic resource conservation is the genes within gene pools of a target species. For successful conservation these gene pools should as whole be considered and conserved. Genetic conservation is the process that enthusiastically retains and manages the diversity of the gene pool with a view to definite and prospective utilisation. Maxted et al. (1997) projected a model, which is an attempt to construct unambiguous and fundamental elements of genetic resource conservation, as well as to determine the interrelations among them. This

model discriminates two primary complementary conservation strategies of *ex situ* and *in situ*, each of which includes a range of different techniques that can be implemented to achieve the aim of the conservation scheme. Indeed, no one alone can effectively conserve the gene pool, though, biodiversity safeguard results only from the application of a range of *ex situ* and *in situ* techniques applied in a complementary manner. One technique performs as a backup to the others. Furthermore, genetic polymorphism is the central issue of plant genetic conservation since it is the primary source for variation of the morphological and physiological appearance of plants. This leads to evolutionary changes within and among species, and to genetic diversity (Hammer et al., 2003). Therefore, it presents a basis to adapt actively to changing environmental conditions and finally the genetic makeup allows them to respond to the challenges of the future. For this reason a successful conservation programme solely depends on the strategies that consider the genetic polymorphism of plants and subsequent events in terms of sustainable environmental adaptation.

Genetic resources of *Curcuma* are currently taken into account as one of the conservation concerned genera in tropics since most of the species of *Curcuma* including cultivated ones are thought to be declining their genetic variations since they do not have any sexual reproduction, even though, some diploids and tetraploids have been reported. It is largely due to incompatibility and high pollen sterility that is very common in the family Zingiberaceae (Joseph et al., 1999). Generally such clonally propagated plants comprise low allelic diversity within the species and are always in risk of extinction. Clonal plants are characterised by the ability to produce genetically identical genet that can produce potential independent ramets. Owing to this life history trait, low genetic diversity and gene flow between populations are expected in clonal plants (Auge et al., 2001; Eckert et al., 2003). Since understanding the genetic structures of the species is a prerequisite to undertake any successful conservation program, because species that lack adequate genetic variations are at greater risk of extinction and the existing levels of genetic variations and the maintenance of these variations are the major issues for plant genetic diversity conservation.

This study concerned several key areas of *Curcuma* genetic diversity to support further applied research on genetic diversity analysis, crop improvement and long-term conservation programmes. To achieve that *in vitro* regeneration and micropopagation techniques as well as development of long-term *in situ* conservation thorough cryopreservation of *Curcuma* germplasm were also investigated, since the genus *Curcuma* is an important genus of the

tropical plant family of Zingiberaceae composed of about 70 -80 species annual or perennial herbs (Purseglove, 1974; Sirirugsa, 1999). Within this genus many species are economically important in terms of spice, medicines and ornamentals that play a vital role in the tropical Asia including Bangladesh. *C. longa* is one of the important species, which yields turmeric, one of the important colouring and aromatic ingredients of curry powder that is enormously used in Asian cuisines (Purseglove, 1974; Apavatjrut et al., 1999) and pharmaceutical industries since it has been considered as an extremely important medicinal plant (Majeed et al., 1995). A number of literatures described the importance of the genus *Curcuma* as source of spices, medicines and horticultural as well as industrial products (Apavatjrut et al., 1999; Cao et al., 2001; Cao and Komatsu, 2003; Joe et al., 2004; Maciel and Criley, 2003; Majeed et al., 1995; Paisooksantivatana et al., 2001a and 2001b; Purseglove, 1974; Sasaki et al., 2002; Sasaki et al., 2004; Yusuf et al., 2001). The most important components of turmeric are curcuminoids that refer to a group of phenolic compounds, which chemically related to its principal ingredient of curcumin. The three main curcuminoids that were isolated from turmeric are curcumin, demethoxycurcumin and bisdemethoxycurcumin (Jayaprakasha et al., 2002; Lechtenberg et al., 2004; Majeed et al., 1995).

In order to analyse genetic diversity within and among species and populations of the genus *Curcuma*, RAPD technique was utilised. Up till now, a huge number of studies on genetic diversity have followed this popular marker (Nybom, 2004; Torimaru et al., 2003; Uptmoor et al., 2003). However, RAPD markers undergo with some shortcomings such as reproducibility and few analytical problems that gene diversity and inbreeding coefficient cannot be estimated without assuming Hardy-Weinberg equilibrium due to the dominant nature, but the simplicity of the technique and the unlimited number of markers that can be generated from RAPD technique encouraged to utilise RAPD. Furthermore, assumption of Hardy-Weinberg equilibrium is not problematic in the present study since *Curcuma* species are vegetatively propagated plants that do not possess selfing and inbreeding events in their life history. Since these events can generate inaccuracy in plant genetic diversity analyses (Lacerda et al., 2001; Lynch and Milligan 1994; Stewart and Excoffier, 1996). A number of recent publications have recognized that RAPD technique is a powerful tool to analyse population genetic consequences because RAPD usually displays a large number of polymorphic (di-allelic) loci that can be obtained relatively easily even for species for which no prior genetic information is available and at a relatively low cost (Eckert et al., 2003; Fu et al., 2003; Hardy, 2003; Jordano and Godoy, 2000; Kjølner et al., 2004; Masumbuko et al., 2003; Torimaru et al.,

2003). In fact, some authors have already compared RAPD markers with other popular markers and revealed a similar and comparable patterns such as AFLP (Díaz et al., 2001; Kjølner et al., 2004; Nybom, 2004; Uptmoor et al., 2003), ISSR (Nybom, 2004; Uptmoor et al., 2003) and allozyme (Buso et al., 1998; Waycott, 1998) which led us to believe that the present results would be comparable to other dominant as well as co-dominant markers based studies.

In this study it was surprisingly observed that some *Curcuma* species have displayed a low genetic diversity within the species. It is possibly due to the fact of vegetative reproduction that does not have any sexual exchange of genetic materials. In contrast some species, however, maintain considerable amounts of genetic variation within the species (Table 3.4). It was also projected from this study that genetic clustering based on RAPD data supports the morphological grouping of the species in some extent (Fig 3.7). Three cultivated species of *C. longa, C. amada* and *C. aromatica* were found to be closely related species which supports their important feature of containing high amount of curcumin in the underground rhizomes with orange yellow colour. The close association of the cultivated species that contain high amount of curcumin suggest that these cultivated species might be monophyletic. Among the wild species one of the closely associated species groups in the dendogram contained several species of *C. angustifolia*, *C. elata*, *C. latifolia, C. zanthorrhiza* and *C. zedoaria* (Fig 3.2 and 3.7). These observations are in agreement with the results of Apavatjrut et al. (1999) as they described a close association of the most of the above species. These species along with *C. aeruginosa* were reported to be triploid with 2n = 3x = 63 (Apavatjrut et al., 1996). This is to be mentioned that this large morphologically and genetically similar group along with some others e.g. *C. aeruginosa, C. amarissima* are common and frequent in Bangladesh. In addition, *C. australasica* and *C. viridiflora* were two newly reported species in Bangladesh, which were found to be associated closely with the above species group. The close association of *C. aeruginosa* and *C. amarissima* supports their unique morphological trait of blue colour of the rhizome, however the rhizomes of *C. aeruginosa* are comparatively smaller and containing blue circle in the central portion only while the *C. amarissima* rhizomes are throughout prominent blue and larger in size. One distinct species in the tree was *C. latifolia*, which was also morphologically diverse comprising a very wide and large ovate shaped leaf containing light purple band along the mid rib. From this study a decisive comment on their evolutionary history cannot be made since their sequence data were not available. Recently however, preliminary molecular research on the genus has been carried out in The Royal

Botanic Garden Edinburgh, UK. This revealed that the percentage of sequence divergence in the nuclear DNA internal transcribed spacer 2 (ITS2) among species of subgenus *Curcuma* was very low. It might indicate that the species have recently diverged. Due to this evolutionary fact along with their reproductive feature of clonal propagation intermingle many morphological characters. Eventually, taxonomic identity of the species within the genus hitherto remains problematic. However, further detailed investigation on relatedness based on molecular sequence data will help to solve this taxonomic enigma of the genus, since very recently Sasaki et al. (2004) suggested the used of the single-nucleotide sequence of the *trn*K gene to identify *Curcuma* species of *C. longa*, *C. phaeocaulis*, *C. zedoaria*, and *C. aromatica*.

Population genetic structure of *C. zedoaria* inferred that the zedoary populations maintain considerable amount of genetic diversity within and among populations (Table 3.9). Among the populations, Srimangal, Chittagong and Sitakundu were found to be close to each other while Savar and Birganj populations are genetically rather distinct (Fig 3.5, 3.6 and 3.7). This observation supports their eco-geographic distribution and other environmental features that influence their genetic makeup and distribution. Populations of hilly areas (Srimangal, Chittagong and Sitakundu) that comprise a higher number of individuals are attributed to comparably higher level of genetic diversity than those of plain land (Savar) and plateau land (Birganj) populations comprising a lower number of individuals. The high level of genetic diversity in hilly areas located in the eastern part of the country also supports the hypothesis of the close relation and influence of its Indo-Malayan centre of origin comprising greater diversity as described by many authors (Apavatjrut et al., 1999; Maciel and Criley, 2003; Purseglove, 1974; Sirirugsa, 1999). Moreover, it is remarkable that these hilly populations are reasonably undisturbed and occupy different ecological conditions ranging from open to shady places of forest margins, which cover altitudes from near sea level to 500 m above the mean sea level. In contrast, the plain land and plateau land populations are ecologically rather homogeneous and experienced with a higher disturbance due to intensive agricultural practices. This observation also agrees with the findings of Paisooksantivatana et al. (2001a) in *C. alismatifolia* as they found a higher genetic variation in highland populations. Still some questions are there, why the plateau land population is more distinct and why it shows low genetic diversity? The most plausible explanation for this fact is that this population thought to be colonized and established as a natural population in the past when the area was covered by preliminarily '*Sal'* forest (*Shorea rubusta*) that was gradually fragmented due to habitat

loss through occupying areas by modern agricultural crops and ultimately reduced the number of individuals from the natural populations in their evolutionary histories. Collection rate of zedoary plant from these populations by the local inhabitants are much higher than the hilly populations that might be another important reason of genetic loss of these populations.

Further investigation on chromosome numbers and ploidy levels of the genus *Curcuma* was made and some new reports were made in this study, for example chromosome numbers of *C. latifolia* and *C. viridiflora* were counted for the first time. A wide range of poloidy levels and chromosome numbers of 2n = 40, 42, 63, 57 and 84 were observed in this study (Table 4.1). Similar results were also reported by previous authors (Apavatjrut et al., 1996; Beltran and Kiew, 1984; Chakravorti, 1948; Chattarjee et al., 1989; Chen et al., 1984; Darlington and Wylie, 1955; Das et al., 1999; Eksomtramage et al., 1996 and 2002; Raghavan and Bhattacharya, 1943; Ramachandran, 1969; Roy et al., 1999; Sharma and Bhattacharya, 1959; Sirirugsa, 1999; Weerapakdee and Krasaechai, 1997; Venkatasubban, 1946). The basic number n= 21 found to be more frequent in different species with three different ploidy levels of diploid, triploid and tetraploid. However, basic number of chromosomes n = 20 was also obtained from some accessions (Table 4.1, Fig 4.1 and 4.2). Basic numbers of some species are yet to be confirmed since they comprise different numbers of chromosomes that are in fact confusing. It could be a possible explanation for this feature that, either they are independent species originated from different occasions or they are aneuploids that decreased or increased the chromosome numbers. Variation in 2n chromosome numbers i.e. aneuploidy was reported in the genus *Curcuma* by Beltran and Kiew (1984) and Eksomtramage et al. (2002).

Flow cytometry technique was also utilised for analysing interspecific and interpopulational genome size variations (Table 4.3 and 4.4). A number of recent studies have also reported that flow cytometry can be employed successfully for determination of genome size and ploidy levels of plant species (Bennett and Smith, 1991; Bennett and Bennett, 1992; Bennett and Leitch, 1995; Bennett et al., 2003; Bureš et al., 2004; Lysák et al., 2000; Obermayer et al., 2002; Rayburn et al., 1997; Tuna et al., 2001). 2C DNA amounts of different species also support different ploidy levels of *Curcuma*. The accessions of *C. rubescens* showed diploid (Chittagong population) and triploid (Birganj population) ploidy levels whereas *C. aeruginosa* found to be more diverse containing triploid, tetraploid and pentaploid individuals within the species (Table 4.3). Detailed genome size analysis and estimation of nuclear DNA amounts of *Curcuma* species has not been determined so far. In this study 2C values of a number of

species were determined for the first time. Only the 2C DNA values of *C. zanthorrhiza* (Bharathan et al., 1994) and 4C DNA amounts of *C. amada, C. caesia, C. longa* var. Suroma and *C. longa* var. TC-4 (Das et al., 1999) are available in the literatures. In contrast, a considerable amount of variation in the genome size within and among *C. zedoaria* populations was observed. Genome size variations among the individuals among the populations varied significantly.

A detail investigation was made on different *in vitro* conditions of growth and multiplication of *C. longa* and a simple protocol is recommended that can be used routinely for high frequency regeneration of *C. longa* var. Surma in Bangladesh. Optimum amounts of 12 µM BA and 0.3 µM NAA were found to be efficient (Table 5.3 and Fig 5.1). It is assumed that the internal growth regulators (especially auxin) are very active in *C. longa*, hence it does not require higher amounts or any other PGR for *in vitro* regeneration. Moreover, the entire procedure can be completed without any callus induction since it requires very low amount of PGR. This fact has an advantage since callusing may produce comparatively more variability among the regenerated plants. This regeneration system could also be used as a source of genetically identical germplasm for long long-term *in vitro* preservation. It is to be mentioned that, incorporation of the wetting agent Tween-20 likely to be helpful during establishment of contamination free explants as it allows better surface sterilization. High percentage of survivability and successful establishment in the soil indicates the efficacy of the offered regeneration system. In this study, field level evaluation could not be made as turmeric is a tropical crop for which an appropriate natural condition is not available in the glasshouse in Hannover, however Salvi et al. (2002) have already investigated and evaluated the field level efficiency of micropropagated turmeric plants and observed that micropropagated plants were superior to the conventionally propagated turmeric plants.

Besides the achievement of high frequency shoot regeneration, *in vitro* microrhizome induction in *C. longa* was also investigated. A remarkable success was achieved in this study that can efficiently produce a higher amount of large microrhizomes. Produced *in vitro* microrhizomes would be a suitable source of disease free seed rhizomes that could be stored and transported easily which are advantageous over plantlets production (Fig 5.5). In addition to that, *in vitro* microrhizomes can eliminate the necessity of acclimatization in the field. Under *in vivo* conditions microrhizomes can successfully germinate shoots after transferring them into soil. Germination, survival rate, and morphological characters were varied among

the different sizes of microrhizomes. Similarly, Shirgurkar et al. (2001) reported that bigger microrhizomes were more efficient and vigorous in the field and grew faster. In this case, higher survival rate was achieved because average weight of the produced microrhizome was higher which inferred that increasing the bigger microrhizome is a key factor in achieving commercial success in microrhizome induction in *C. longa* and the presented protocol is one step forwards to that of commercial need. Sharma and Singh (1995) reported that microrhizomes can be stored under moist conditions at room temperature for several months and that more than 80% of the sprouted microrhizomes developed shoots and roots after they had been successfully transferred directly to the field.

Genetic instability of the micropropagated plants was analysed using RAPD technique. The results of this study demonstrated that somaclonal variation has taken place in the micropropagated plants of *C. longa* even though organized meristem tissues of vegetative buds were used as explants. Various factors are well recognised to induce somaclonal variations such as the *in vitro* process, duration, auxin and cytokinin concentrations, their ratio, and other nutritional conditions and *in vitro* stress, minor point mutations, rearrangement of nuclear or organellar DNA, polyploidy and epigenetic changes are all known to induce somaclonal variation (Devarumath et al., 2002; Rahman and Rajora, 2001). Genetic variations of *in vitro* regenerated plants have also been investigated by several workers (Damasco et al., 1996; Devarumath et al., 2002; Gimenez et al., 2001, Rani et al., 1995, 2000 and 2001; Rival et al., 1998). It can be pointed out that the genetic constitution in each clone/variety which determined the stability/variability of the micropropagated plants under the given cultural conditions ((Devarumath et al., 2002). It would appear that *C. longa* var 'Surma' is inherently unstable under *in vitro* conditions which lead to the *in vitro*-induced somaclonal variation. Similar results have been obtained in micropropagated plants of *Camellia sinensis* (Devarumath et al., 2002), and poplars (Rani et al., 1995 and 2001). The findings of the present study in concurrence with earlier reports ((Devarumath et al., 2002; Rani et al., 1995, 2000 and 2001; Damasco et al., 1996; Rani and Raina, 1998 and 2000; Rival et al., 1998; Gimenez et al., 2001; Rahman and Rajora, 2001) have significantly emphasised that plants derived even from organised meristem culture may not always be genetically true-to-type. Determination of genetic stability of *in vitro* regenerated plants is therefore essential before utilising either in commercial propagation or genebank storage purposes. Hence, RAPD marker would be suitable for such investigation since it is rapid and cost effective.

It is well accepted that the successful cryopreservation of vegetatively propagated tropical crop plants is crucially important (Thinh et al. 1998; Takagi et al., 1998) since they always have great chance of diminishing genetic variations. Suitability of cryopreservation for the long-term storage of plant species has recently been investigated by Wilkinson et al. (2003). *C. longa* L. is such an important crop of the tropical Asia that reproduces by means of vegetative reproduction. This study revealed that the vitrification procedure was found to be suitable for cryopreservation of *C. longa* germplasm. This procedure was also reported as a suitable technique for other vegetatively propagated crop plants (Halmagyi et al., 2004; Helliot et al., 2003; Leunufna and Keller, 2003; Sarkar and Naik, 1998; Takagi et al., 1998). In addition to that, the size of explants was found to be a substantial factor for successful recovery of the cryopreserved buds (Fig 6.5 and 6.6). In this case it was observed that medium size of buds (3-4 mm) are suitable to recover after cryostorage. However, the larger buds (> 4 mm) were found to be more tolerant to the PVS2 treated control treatment but recovery rate reduced after cryostorage, whereas, smaller explants were found to be very sensitive in both conditions of control and LN treatment. It might be due to suboptimum vitrification of the buds in these cases results the intracellular ice formation, which is a leading factor associated with physical damage of the tissue (Helliot et al., 2003). Beak et al. (2003) also reported that the size of explants had a significant effect on regeneration of cryopreserved apices of garlic.

A proficient cryopreservation protocol was established for the first time that can be use for routine cryoprservation of *Curcuma* buds. The experimental results inferred that preconditioning of axillary buds was essential for optimum vitrification of *Curcuma* axillary buds, which is a prerequisite for a successful achievement in cryopreservation. It is well recognised that sucrose is an important pre-growth additive for acquisition of desiccation tolerance during cryopreservation by vitrification (Sarkar and Naik 1998). This hardening treatment leads to the mitigation of the subsequent injurious effects of the PVS2 solution resulted by numerous ultrastructural changes of the fragmentation of vacuoles into smaller ones, the differentiation of proplastids into amyloplasts containing starch and the swelling of organelles like the endoplasmic reticulum (Takashi et al., 1997; Helliot, 2003). Sucrose uptake in cultured shoots of sugar beet was measured by Vandenbussche et al. (1999) after a 1-week preculture on a medium enriched with 0.3 M sucrose and found that the uptake of free sugars by the apical dome which was accumulated into the parenchyma cells. Similar results were also observed with shoot tips of *Musa* cultivars and the role of sucrose preculture in the reduction of the amount of freezable water was described by Panis et al. (1996). The optimum

preconditioning and vitrification conditions established in this study induced direct regeneration from vitrified axillary buds. Previous studies on other plants indicated that there was no major change was found after long-term preservation in liquid nitrogen either in survivability (Mix-Wagner et al., 2003) or in genetic polymorphisms (Wilkinson et al., 2003). At least four *Curcuma* varieties namely Surma, Roma, Ranga and Rasmi are popular all over the sub-continent. In addition some wild relatives, like *C. aromatica* and *C. amada* are also important for curcumin production and cultivated in many regions in Bangladesh as well as other neighbouring countries. Before employing the offered cryopreservation method it is essential to test whether the method is also applicable to other varieties and species.

8. CONCLUSIONS

Concluded that the genus *Curcuma* is crucially important for its wide range of uses. The present study revealed that some of *Curcuma* species and particularly populations of *C. zedoaria* were found to be prone to declining genetic variability. It is largely due to high anthropogenic interferences and massive destruction of habitats. In addition, ecogeographical conditions and habitat distribution patterns are also influential for the genetic diversity of the populations. From conservation point of view these associated factors shall have to be considered prior to sampling the accessions for *ex situ* and *in situ* conservation programmes. The results of chromosomal variation and 2C DNA values that are reported here for the first time would certainly contribute to the effective samplings of conservation targeted species and populations. Furthermore, cytological and flow cytometry data could also be helpful for *Curcuma* genetic improvement and breeding programmes.

The established *in vitro* protocols are indeed one step forward to the improved micropropagation system for *C. longa*, which would undoubtedly be useful for large-scale production of *Curcuma* species both for commercial and *in vitro* conservation purposes. This study offered an efficient cryopreservation system for the first time that can be employed for long-term cryostorage of *C. longa* germplasm. However, the presented results give emphasis on detecting genetic stability prior to undertake long-term conservation of *Curcuma* germplasm through cryopreservation. RAPD would definitely be suitable for determining genetic integrity of *in vitro* regenerated as well as cryoconserved *Curcuma* germplam.

In spite of these substantial outcomes of this study, there are several areas where a considerable amount of research efforts are still required towards *Curcuma* genetic improvement and conservation. One of such area is to undertake an applied research project on *ex situ* and *in situ* conservation of *Curcuma* in Bangladesh before losing some of the valuable wealth of *Curcuma* biodiversity. Further, molecular marker-based studies can be extended to investigate detailed biological and ecological processes of the threatened *Curcuma* species to support *Curcuma* conservation in practice. Another question not touched in this study is whether the established cryopreservation method is applicable also to other genotypes or species without any major adjustment. Genetic stability of cryoconserved germplasm should be tested before employing routine cryopreservation of *Curcuma* accessions for long-term conservation. Additional molecular techniques are indispensable to identify some beneficial genes that are responsible for different bioactive substances like curcumin. Finally, a successful genetic transformation system of *C. longa* would be substantial for producing disease resistant lines as well as integrating beneficial genes towards *Curcuma* genetic improvement.

9. REFERENCES

Abbott, A. J. and Belcher, A. R. 1986. Potato tuber formation *in vitro*. **In**: *Plant Tissue Culture and It's Agricultural Application* (Withers, L. A. and Alderson, P. G. eds.). Butter-worths, London. Pp. 113-122.

Albertson, R. C. Markert, J. A., Danley, P. D. and Kocher, T. D. 1999. Phylogeny of a rapidly evolving clade: the cichlid fishes of lake Malawi, East Africa. *Proc. Natl. Acad. Sci. U.S.A.* **96**: 5107-5110.

Alizadeh, S., Mantell, S. H. and Viana, A. M. 1998. *In vitro* shoot culture and microtuber induction in steroid yam *Dioscorea composita* Hemsl. *Plant Cell Tiss. Org. Cult.* **53**: 107-112.

Apavatjrut, P., Anuntalabhochai, S., Sirirugsa, P. and Alisi, C. 1999. Molecular Markers in the identification of some early flowering *Curcuma* (Zingiberaceae) species. *Ann. Bot.* **84**: 529-534.

Apavatjrut, P., Sirisawad, T., Sirirugsa, P., Voraurai, P. and Suwanthada, C. 1996. Studies on chromosome number of seventeen Thai *Curcuma* species. *Proc. 2nd Natl. Conf. Fl. Orn.* **2**: 86-99.

Arora, J. S., Singh, K., Grewal, H. S. and Chanana, Y. R. 1996. *In vitro* cormel production from nodal buds and cormel tips in *Gladiolus*. **In**: *Plant Tissue Culture* (Islam, A. S., ed.). Oxford and IBH Publishing Co. Pvt. Ltd, New Delhi. Pp. 50-53.

Auge, H., Neuffer, B., Erlinghagen, F., Grupe, R. and Brandel, R. 2001. Demographic and random amplified polymorphic DNA analyses reveal high levels of genetic diversity in a clonal violet. *Mol. Ecol.* **10**: 1811-1819.

Avise, J. C. 1994. *Molecular markers, natural history, and evolution.* Chapman and Hall, New York, USA.

Balachandran, S. M., Bhat, S. R. and Chandel, K. P. S. 1990. *In vitro* clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). *Plant Cell Rep.* **8**: 521-524.

Beak, H.-J., Kim, H.-H., Cho, E.-G., Chae, Y.-A. and Engelmann, F. 2003. Importance of explants size and origin and of preconditioning treatments for cryopreservation of garlic shoot apices by vitrification. *CryoLett.* **24**: 381-388.

Behura, S., Sahoo, S. and Srivastava, V. K. 2002. Major constituents in leaf essential oils of *Curcuma longa* L. and *Curcuma aromatica* Salisb. *Curr. Sci.* **83**: 1312-1313.

Beltran, I. C. and Kiew, K. Y. 1984. Cytotaxonomic studies in the Zingiberaceae. *Notes R. B. G. E.* **41**: 541-559.

Bennett, M. D. and Leitch, I. J. 1995. Nuclear DNA amounts in angiosperms. *Ann. Bot.* **76**: 113-176.

Bennett, M. D. and Smith, J. B. 1991. Nuclear DNA amounts in angiosperms. *Phil. Trans. Royal Soc. London B.* **334**: 309-345.

Bennett, M. D., Bhandol, P. and Leitch, I. J. 2000. Nuclear DNA amounts in angiosperms and their modern uses- 807 new estimates. *Ann. Bot.* **86**: 859-909.

Bennett, M. D. and Smith, J. B. 1976. Nuclear DNA amounts in angiosperms. *Phil. Trans. Royal Soc. London B.* **274**: 227-274.

Bennett, M. D., Leitch, I. J., Price, H. J. and Johnston, J. S. 2003. Comparison with *Caenorhabditis* (~100 Mb) and Drosophila (~175 Mb) using flow cytometry show genome size in Arabidopsis to be ~157 Mb and thus -25% larger than the Arabidopsis genome initiative estimate of ~125 Mb. *Ann. Bot.* **91**: 547-557.

Bennett, S. T. and Bennett, M. D. 1992. Variation in nuclear DNA amount between wild and cultivated populations of *Milium effusum* (2n = 28). *Genome* **35**: 1050-1053.

Benson, E. E. 1999. *Plant Conservation Biotechnology*. Taylor and Francis, London.

Bharathan, G., Lambert, G. and Galbraith, D. W. 1994. Nuclear DNA content of monocotyledons and related taxa. *Amer. J. Bot.* **81**:381-386.

Borthakur, M. and Bordoloi, D. N. 1992. Micropropagation of *Curcuma amada* Roxb. *J. Spice Arom. Crops* **1**:154-156.

Brown, A. H. D. and Weir, B. 1983. Measuring genetic variability in plant populations. **In**: *Isozyme in Plant Genetics and Breeding, Part A.* (Tanksley, S. D. and Orton, T. J. eds.). Elsevier Science Publishers BV. Amsterdam, NL.

Bureš, P., Pavlíček, T., Horová, L. and Nevo, E. 2004. Microgeographic genome size differentiation of the Carob Tree, *Ceratonia siliqua*, at 'Evolutuon Canyon', Israel. *Ann. Bot.* **93**: 529-535.

Buso, G. S. C., Rangel, P. H. and Ferreira, M. E. 1998. Analysis of genetic variability of South American wild rice populations (*Oryza glumaepatula*) with isozymes and RAPD markers. *Mol. Ecol.* **7:** 107-117.

Bussell, J. D. 1999. The distribution of random amplified polymorphic DNA (RAPD) diversity amongst populations of *Isotoma petraea* (Lobeliaceae). *Mol. Ecol*. **8**: 775-789.

Caetano-Anolles, G., Bassam, G. J. and Gresshof, P. M. 1991. High resolution DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnolgy* **9**: 553-556.

Callow, J. A., Ford-Lloyd, B. and Newbury, H. J. 1997. *Biotechnology and plant genetic resources: conservation and use*. CAB International, NY.

Cao, H., Sasaki, Y., Fushimi, H., and Komatsu, K. 2001. Molecular analysis of medicinally-used Chinese and Japanese *Curcuma* based on 18S rRNA gene and *trn*K gene sequences. *Biol. Pharm. Bull.* **24**: 1389-1394.

Cao, H. and Komatsu, K. 2003. Molecular identification of six medicinal *Curcuma* plants produced in Sichuan: evidence from plastid trnK gene sequences. *Yao Xue Xue Bao*. **38**: 871-875.

Cardoso, M. A., Provan, J., Powell, W., Ferreira, P. C. G. and Oliveira, D. E. 1998. High genetic differentiation among remnant populations of the endangered *Caesalpinea echinata* Lam. (Leguminosae- Caesalpinioideae). *Mol. Ecol.* **7:** 601-608.

Carlson, J. E., Tulsieram, L. K., Glaubitz, J. C., Luk, V. W. K., Kauffeldt, C. and Rutledge, R. 1991. Segregation of random amplified DNA markers in F_1 progeny of conifers. *Theor. Appl. Genet*. **83**:194-200.

Cavallini, A. and Natali, L. 1991. Intraspecific variation of nuclear DNA content in plant species. *Caryologia* **44**: 93-107.

Chakravorti, A. K. 1948. Multiplication of chromosome numbers in relation to speciation in Zingiberaceae. *Sci. Cult*. **14**: 137-140.

Chalmers, K. J., Waugh, R., Sprent, J. I., Simons, A. J. and Powell, W. 1992. Detection of genetic variation between and within populations of *Gliricidia sepiun* and *G. maculata* using RAPD markers. *Heredity* **69**: 465-472.

Chatterjee, A., Ghosh, S. and Roy, S. C. 1989. A cytological survey of eastern Himalayan plants III. *Cell Chrom. Res.* **12**: 22-29.

Chen, Y., Bai. S., Cheng, K., Zhang, S. and Nian, L. 1999. Random amplified polymorphic DNA analysis on *Curcuma wenuujin* and *C. sichuanensis. Zhongguo Zhong Yao Za Zhi*. **24**: 131-133.

Chen, Z., Chen, S. and Huang, S. 1984. A report on chromosome numbers of Chinese Zingiberaceae (2). *Guihaia* **4**: 13-18.

Cincotta, R. P., Wisnewski, J. and Engelman, R. 2000. Human population in the biodiversity hotspots. *Nature* **404**: 990-992.

Clark, A. G. and Lanigan, C. M. S. 1993. Prospects for estimating nucleotide divergence with RAPDs. *Mol. Biol. Evol.* **10**: 1096-1111.

Cruzan, M. B. 1998. Genetic markers in plant evolutionary ecology. *Ecology* **79**: 400-412.

Damasco, O. P., Graham, G. C., Henry, R. J., Adkins, S. W. and Godwin, I. D. 1996. Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa* spp. AAA) bananas. *Plant Cell Rep.* **16**: 118-123.

Dantu, P. K. and Bhojwani, S. S. 1987. *In vitro* propagation of *Gladiolus*. *Gartenbauwissenschaft* **52**: 90-93.

Darlington, C. D. and Wylie, A. P. 1955. *Chromosome Atlas of Flowering Plants*. George Allen & Unwin Ltd., London. Pp. 345-346.

Das, A. B., Rai, S. and Das, P. 1999. Karyotype analysis and cytophotometric estimation of nuclear DNA content in some members of the Zingiberaceae. *Cytobios* **97**: 23-33.

Dawson, K., Chalmers, K. J. and Waugh, R. 1993. Detection and analysis of genetic variation in *Hordeum spontaneum* populations from Israel using RAPD markers. *Mol. Ecol*. **2**: 151-159.

Degen, B., Caron, H., Bandou, E., Dutech, C. and Maggia, L. 2001. Small scale spatial genetic structure of six tropical tree species in French Guiana. **In**: *Genetic Response of Forest Systems to Changing Environmental Conditions* (Müller-Starck, G. and Schubert, R. eds.). Kluwer Academic Publishers, Dordrecht. Pp. 75-92.

Dekkers, A. J., Rao, A. N. and Goh, C. J. 1991. *In vitro* storage of multiple shoot cultures of gingers of ambient temperatures of 24-29^{0}C. *Sci. Hort.* **47**: 157-168.

Devarumath, R. M., Nandy, S., Rani, V., Marimuthu, S., Muraleedharan, N. and Raina, S. N. 2002. RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (Assam-India type). *Plant Cell Rep*. **21**: 166-73.

Díaz, V., Muňiz, M. and Ferrer, E. 2001. Random amplified polymorphic DNA and amplified fragment length polymorphism assessment of genetic variation in Nicaraguan populations of *Pinus oocarpa. Mol. Ecol.* **10**: 2593-2603.

Dice, L. R. 1945. Measures of the amount of ecological association between species. *Ecology* **26**: 297-302.

Doležel, J. Bartoš, J., Voglmayr, H. and Greilhuber, J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry* **51A**: 127-128.

Doležel, J., Greilhuber, J., Lucretti, S., Meister, A., Lysák, M. A., Nardi, L. and Obermayer, R. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. *Ann. Bot.* **82** (supplement A): 17-26.

Doyle, J. J. and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Eckert, C. G. 2002. The loss of sex in clonal plants. *Evol. Ecol.* **15:** 501-520.

Eckert, C. G., Lui, K., Bronson, K., Corradini, P. and Bruneau, A. 2003. Population genetic consequences of extreme variation in sexual and clonal reproduction in an aquatic plant. *Mol. Ecol.* **12:** 331-344.

Eksomtramage, L., Sirirugsa, P. and Mayakul, S. 1996. Chromosome numbers of some Thai Zingiberaceae. *Songklanakarin J. Sci. Technol.* **18**(2): 153-159.

Eksomtramage, L., Sirirugsa, P., Jivanit, P., Maknoi, C. 2002. Chromosome counts of some Zingiberaceae species from Thailand. *Songklanakarin J. Sci. Tech.* **24**: 311-319.

Ellsworth, D. L., Rittenhouse, K. D. and Honeycutt, R. L. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Bio Techniques* **14**: 214-217.

Engelmann, F. 1991. *In vitro* conservation of tropical plant germplasm- a review. *Euphytica* **57**: 227-243.

Engelmann, F. 1997. *In vitro* conservation methods. **In**: *Biotechnology and plant genetic resources: conservation and use* (Newbury, H. J. and Callow, J. A. eds.). Ford-Lloyd BV, Wallingford, CABI. Pp. 119-162.

Excoffier, L., Smouse, P. E. and Quattro, J. M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**: 479-491.

Exocoffier, L. and Smouse, P. E. 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: Molecular variance parsimony. *Genetics* **136**: 343-359.

Fay, M. F. 1992. Conservation of rare and endangered plants using *in vitro* methods. *In Vitro Cell. Dev. Biol.* **28P:** 1-4.

Fritsch, P. and Rieseberg, L. 1992. High outcrossing rates maintain male and hermaphrodite individuals in populations of the flowering plant *Datisca glomerata*. *Nature* **359**: 633-636.

Fritsch, P., Rieseberg, L. H. 1996. The use of random amplified polymorphic DNA (RAPD) in conservation genetics. **In**: *Molecular Genetic Approaches in Conservation* (Smith, T. B. and Wayne, R. K.). Oxford University Press, New York. Pp. 54-73.

Fu, C., Qiu, Y. and Kong, H. 2003. RAPD analysis for genetic diversity in *Changium smyrnioides* (Apiaceae)- an endangered plant. *Bot. Bull. Acad. Sini.* **44:** 13-18.

Gaiotto, F. A., Bramucci, M. and Grattapaglia, D. 1997. Estimation of outcrossing rate in a breeding population of *Eucalyptus urophylla* with dominant RAPD and AFLP markers. *Theor. Appl. Genet*. **95**: 842-849.

Galbraith, D. W., Harkins, K. R., Maddox, J. M., Ayres, N. M., Sharma, D. P. and Firoozabady E. 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* **220**: 1049–1051.

Garner, N. and Blake, J. 1989. The induction of potato microtubers *in vitro* on media free growth regulatory substances. *Ann. Bot.* **63**: 666-674.

Gerber, S., Mariette, S., Streiff, R., Bodenes, C. and Kremer, A. 2000. Comparison of microsatellites and amplified fragment length polymorphism markers for parentage analysis. *Mol. Ecol*. **9**: 1037-1048.

Gimenez, C., Garcia, E. D., Enrech, N. X. D. and Blanca, I. 2001. Somaclonal variation in banana: cytogenetic and molecular characterization of somaclonal variant CIEN BTA-03. *In Vitro Cell Dev. Biol. Plant* **37**: 217–222.

Gopal, J., Minocha, J. L. and Dhaliwal, H. S. 1998. Microtuberization in potato (*Solanum tuberosum* L.). *Plant Cell Rep*. **17**: 794-798;

Grewal, S. 1996. Microtubers from somatic embryos of *Bunium persicum*. *Indian J. Exp. Biol.* **34**: 813-815.

Grime, J. P. 1983. Prediction of weed and crop response to climate based on measurement of DNA content. *Aspects Appl. Biol.* **4**: 87-98.

Gustafsson, L. and Gustafsson, P. 1994. Low genetic variation in Swedish populations of the rare species *Vicia pisiformis* (Fabaceae) revealed with RFLP (rDNA) and RAPD. *Plant Syst. Evol.* **189**: 133-148.

Hadrys, H., Balick, M. and Schierwater, B. 1993. Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol*. **1**: 55-63.

Haig, S. M. 1998. Molecular contributions to conservation. *Ecology* **79**: 413-425.

Halmagyi, A., Fischer-Klüver, G., Mix-Wagner, G. and Schumacher, H. M. 2004. Cryopreservation of *Chrysanthemum morifolium* (*Dendranthema grandiflora* Ramat.) using different approaches. *Plant Cell Rep.* **22**: 371-375.

Hammer, K., Arrowsmith, N. and Gladis, T. 2003. Agrobiodiversity with emphasis on plant genetic resources. *Naturwissenschaften* **90**: 241–250.

Hamrick, J. L. and Godt, M. J. W. 1990. Allozyme diversity in plant species. **In**: *Plant population genetics, breeding, and genetic resources* (Brown, A. H. D., Clegg, M. T., Kahler, A. L. and Weir, B. S. eds.). Sinauer, Sunderland, Massachusetts, USA. Pp. 43-63.

Hamrick, J. L. and Loveless, M. D. 1989. The genetic structure of tropical tree populations, association with reproductive biology. **In**: *The Evolutionary Ecology of Plants* (Bock, J. H. and Linhart, Y. eds.). Westview Press, Boulder, Colorado. Pp. 129-146.

Hardy, O. J. 2003. Estimation of pairwise relatedness between individuals and characterization of isolation-by-distance processes using dominant genetic markers. *Mol. Ecol.* **12**: 1577-1588.

Harlan, J. R. and de Wet, J. M. J. 1971. Toward a rational classification of cultivated plants. *Taxon* **20**: 509-517.

Harris, S. A. 1995. Systematics and random amplified polymorphic DNA in the genus *Leucaena* (*Leguminosae Mimosoideae*). *Plant Sys. Evol.* **197**: 195-208.

Helliot, B., Swennen, R., Poumay, Y., Frison, E., Lepoivre, P. and Panis, B. 2003. Ultrastructural changes associated with cryopreservation of banana (*Musa* spp.) highly proliferating meristems. *Plant Cell Rep.* **21**: 690-698.

Henshaw, G. G. 1975. Technical aspects for tissue culture storage for genetic conservation. **In**: *Crop Genetic Resources for Today and Tomorrow* (Frankel, O. H. and Hawkes, J. G. eds.). Cambridge University Press, New York. Pp. 349-358.

Heun, M. and Helentjaris, T. 1993. Inheritance of RAPDs in F_1 hybrids of corn. *Theor. Appl. Genet*. **85**: 961-968.

Heywood, V. H. 1985. *Flowering plants of the world*. Croom Helm, London, Sydney. Pp. 297-298.

Hill, W. G. and Weir, B. S. 2004. Moment estimation of population diversity and genetic distance from data on recessive markers. *Mol. Ecol.* **13**: 895-908.

Hirari, D. and Sakai, A. 1998. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. **In**: *Cryopreservation of tropical plant germplasm - current research progress and application* (Engelmann, F. and Takagi, H. eds.), Proceedings of the JIRCAS/IPGRI Joint International Workshop. Tsukuba, Japan. Pp. 205-211.

Holsinger, K. E. and Gottlieb, L. D. 1991. Conservation of rare and endangered plants: principles and prospects. **In**: *Genetics and Conservation of Rare Plants*. (Falk, D. A. and Holsinger, K. E. eds.). Oxford University Press, New York, USA. Pp. 195-208.

Holsinger, K. E. Lewis, P. O. and Dey, D. K. 2002. A Bayesian approach to inferring population structure from dominant markers. *Mol. Ecol*. **11**: 1157-1164.

Hoque, M. I., Islam, M. A., Sarker, R. H. and Islam, A. S. 1996. *In vitro* microtuber formation in potato (*Solanum tuberosum* L.). **In**: *Plant Tissue Culture* (Islam, A. S. ed.) Oxford and IBH Publishing Co. Pvt. Ltd, New Delhi. Pp. 221- 228.

Huff, D. R., Peakall, R. and Smouse, P. E. 1993. RAPD variation within and among natural populations of outcrossing buffalograss (*Buchloea dactyloides* (Nutt.) Engelm.). *Theor. Appl. Genet.* **86**: 927-934.

Isabel, N., Beaulieu, J., Thériault, P. and Bousquet, J. 1999. Direct evidence for biased gene diversity estimates from dominant random amplified polymorphic DNA (RAPD) fingerprints. *Mol. Ecol*. **8**: 477-483.

Jaccard, P. 1908. Nouvelles recherches surla distribution florale. *Bull. Soc. Vaud. Sci. Nat.* **44**: 223-270.

Jacquemyn, H., Honnay, O., Galbusera, P. and Roldán-Ruiz, I. 2004. Genetic structure of the forest herb *Primula elatior* in a changing landscape. *Mol. Ecol.* **13**: 211-219.

Jayaprakasha, G. K., Rao, L. J. M. and Sakariah, K. K. 2002. Improved HPLC method for the determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin. *J. Agr. Food Chem*. **50**: 3668-72.

Jenkins, M. 2003. Prospects for biodiversity. *Science* **302**: 1175-77.

Jitoe, A., Masuda, T., Tengah, I. G. P., Suprapta, D. N., Gara, I. W. and Nakatani, N. 1992. Antioxidant activity of tropical ginger extracts and analysis of the contained curcuminoids. *J. Agr. Food Chem.* **40**: 1337-1340.

Joe, B., Vijaykumar, M., Lokesh, B. R. 2004. Biological properties of curcumin-cellular and molecular mechanisms of action. *Crit. Rev. Food Sci. Nutr*. **44**: 97-111.

Jordano, P. and Godoy, J. A. 2000. RAPD variation and population genetic structure in *Prunus mahaleb* (Rosaceae) an animal –dispersed tree. *Mol. Ecol.* **9**: 1293-1305.

Joseph, R., Joseph, T. and Jose, J. 1999. Karyomorphological studies in the genus *Curcuma* Linn. *Cytologia* **64**: 313-317.

Kikuzaki, H. and Nakatani, N. 1993. Antioxidant effects of some ginger constituent. *J. Food Sci.* **58**: 1407-1410.

Kim, E. K., Hahn, E. J., Murthy, H. N. and Peak, K. Y. 2003. High frequency of shoot multiplication and bulblet formation of garlic in liquid cultures. *Plant Cell Tiss. Ogr. Cult.* **73**: 231-236,

King, L. M. and Schaal, B. A. 1989. Ribosomal-DNA variation and distribution in *Rudbeckia missouriensis*. *Evolution* **43**: 1117-1119.

Kjølner, S., Såstad, M., Taberlet, P. and Brochmann, C. 2004. Amplified fragment length polymorphism versus random amplified polymorphic DNA markers: clonal diversity in *Saxifraga cernua*. *Mol. Ecol.* **13**: 81-86.

Klein-Langhorst, R. M., Vermunt, A., Weide, R., Liharska, T. and Zabel, P. 1991. Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). *Theor. Appl. Genet*. **83**: 108-114.

Kress, W. J., Prince, L. M. and Williams, K. J. 2002. The phylogeny and a new classification of the gingers (Zingiberaceae): evidence from molecular data. *Amer. J. Bot.* **89**:1682-1696.

Kumar, S., Tamura, K, Jakobsen, I. B. and Nei, M. 2001. *MEGA2: Molecular Evolutionary Genetics Analysis software.* Arizona State University, Tempe, Arizona, USA.

Kwon, J. A. and Morden, C. W. 2002. Population genetic structure of two rare species (*Colubrina oppositifolia* and *Alphitonia ponderosa*, Rhamnaceae) from Hawaiian dry and mesic forests using random amplified polymorphic DNA markers. *Mol. Ecol*. **11**: 991-1001.

Lacerda, D. R., Acedo, M. D. P., Lemos, J. P. F. and Lovato, M. B. 2001. Genetic diversity and structure of natural populations of *Plathymenia reticulata* (Mimosoideae), a tropical tree from the Brazilian Cerrado. *Mol. Ecol*. **10**: 1143-1152.

Lamboy, W. F. 1994. Computing Genetic Similarity Coefficients from RAPD data: The effects of PCR artifacts. *PCR Meth. Appl.* **4**: 31-37.

Landry, P. A. and Lapointe F.-J. 1996. RAPD problems in phylogenetics. *Zoologica Scripta* **25:** 283-290.

Langis, R., Schnabel, B., Earle, E. D. and Steponkos, P. 1989. Cryopreservation of *Brassica campestris*. *Cryo-Letters* **10**: 421-428.

Lechtenberg, M., Quandt, B., Nahrstedt, A. 2004. Quantitative determination of curcuminoids in *Curcuma* rhizomes and rapid differentiation of *Curcuma domestica* Val. and *Curcuma zanthorrhiza* Roxb. by capillary electrophoresis. *Phytochem. Anal.* **15**: 152-158.

Leunufna, S., and Keller, E. R. J. 2003. Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). *Plant Cell Rep.* **21**: 1159-1166.

Lewontin, R. C. 1972. The apportionment of human diversity. **In**: *Evolutionary Biology 6* (Dobzhansky, T., Hecht, M. K. and Steere, W. C. eds.). Appleton-Century-Crofts, New York. Pp. 381-398.

Loveless, M. D. and Hamrick, J. L. 1984. Ecological determinants of genetic structure in plant populations. *Ann. Rev. Ecol. Syst.* **15**: 65-95.

Lynch, M., Milligan, B. G. 1994. Analysis of population genetic structure within RAPD markers. *Mol. Ecol.* **3**: 91-99.

Lysák, M. A. and Dolezel, J. 1998. Estimation of nuclear DNA content in *Sesleria* (*Poacea*). *Caryologia* **52**: 123-132.

Lysák, M. A., Rostková, A., Dixon, J. M., Rossi, G., and Doležel, J. 2000. Limited genome size of *Sesleria albicans*. *Ann.Bot.* **86**: 399-403.

Maciel, N. and Criley, R. A. 2003. Morphology Growth and Flowering Behavior of *Curcuma Zedoaria. Acta Hort. (ISHS)* **624**: 111-116.

Majeed, M., Badmeav, V., Shivakumar, U, Rajendran, R. 1995. *Curcuminoids: antioxidant phytonutrients*. Sabinsa Coorporation, NJ, USA.

Maknoi, C. and Sirirugsa, P. 2002. *Notes on the infra-generic classification of the genus Curcuma L*. Poster Abstract. 12th flora of Thailand meeting, Forest Herbarium, Phaholyothin Road, Chatuchak, Bangkok 10900, Thailand.

Martin, C., Gonzalez-Benito, M. E. and Iriondo, J. M. 1997. Genetic diversity within and among populations of a threatened species: *Erodium paularense* Fern. Gonz. & Izco. *Mol. Ecol*. **6**: 813-820.

Masuda, T., Jitoe, A., Isobe, J., Nakatani, N. and Yonemori, S. 1993. Anti-oxidative and anti-inflammatory curcumin-related phenolics from rhizome of *Curcuma domestica*. *Phytochemistry* **32**: 1557-1560.

Masumbuko, L. I., Bryngelsson, T., Mneney, E. E., Salomon, B. 2003. Genetic diversity in Tanzanian Arabica coffee using random amplified polymorphic DNA (RAPD) markers *Hereditas* **139**: 56-63.

Matsuda, H., Ninomiya, K., Morikawa, T., and Yoshikawa, M. 1998. Inhibitory effect and action mechanism of sesquiterpebes fro Zedoaria rhizome on D-galactosamine/ lipopolysaccharide-induced liver injury. *Bioorg. Med. Chem. Lett.* **8**: 339-344.

Matsumoto, T., Sakai, A. and Yamada, K. 1994. Cryopreservation of *in vitro*-grown apical meristems of wasabi (*Wasabia japonica*) by vitrification and subsequent high plant regenerations. *Plant Cell Rep.* **13**: 442-446.

Maxted, N., Ford-Lloyd, B. V. and Hawkes, J. G. 1997. Complementary conservation strategies. **In**: *Plant Genetic Conservation: The In Situ Approach* (Maxted, N., Ford-Lloyd, B. V., and Hawkes, J. G. eds.), Chapman & Hall, London. Pp. 15–40.

Maxted, N., Guarino, L., Myer, L. and Chiwona, E. A. 2002. Towards a methodology for on-farm conservation of plant genetic resources. *Crop Gen. Res. Evol*. **49**: 31-46.

Menini, U. G. 1998. *Policy issues for the conservation and utilisation of horticultural genetic resources fro food and agriculture*. World Conference on Horticultural Research, Rome, Italy.

Mikulík, J. 1999. Propagation of endangered plant species by tissue cultures. *Biologica* **37**: 27-33.

Mix-Wagner, G., Schumacher, H. M., and Cross, R. J. 2002. Recovery of potato apices after several years of storage in liquid nitrogen. *CryoLett.* **24**: 33-41.

Mohmand, A. S. and Nabors, M. W. 1990. Somaclonal variant plants of wheat derived from mature embryo explants of three genotypes. *Plant Cell Rep*. **8**: 558–560.

Morden, C. W. and Loeffler, W. 1999. Fragmentation and genetic differentiation among subpopulations of the endangered Hawaiian mint *Haplostachys haplostachya* (Lamiaceae). *Mol. Ecol.* **8**: 617-625.

Mueller, U. G. and Wolfenbarger, L. L. 1999. AFLP genotyping and fingerprinting. *Trends in Ecol. Evol.* **14**: 389-394.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* **15**: 473-497.

Nadgauda, R. S., Mascarenhas, A. F., Hendre, R. R. and Jagannathan, V. 1978. Rapid multiplication of turmeric (*Curcuma longa* L.). *Ind. J. Exp. Biol.* **16**: 120-122.

Nayak, S. 2000. *In vitro* multiplication and microrhizome induction in *Curcuma aromatica* Salisb. *Plant Growth Regul*. **32**: 41-47.

Nei, M. 1972. Genetic distance between populations. *The Amer. Natur.* **106**: 283-292.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. U.S.A.* **70**: 3321-3323.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics* **89**: 583-590.

Nei, M. 1987. *Molecular Evolutionary Genetics.* Columbia University Press, New York.

Nei, M. and Li, W.-H. 1979. Mathametical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci*. **76**: 5269-5273.

Nirmalbabu, K., Rema, J. and Ravindran, P. N. 1994. Biotechnology research in spice crops. **In**: *Advances in Horticulture vol. 9, Plantation and Spice crops, Part I.* (Chadha, K. L. and Rethinam, P. eds.). Malhotra Publishing House, New Delhi. Pp. 633– 653.

Novacek, M. J. and Cleland, E. E. 2001. The current biodiversity extinction event: Scenarios for mitigation and recovery. *Proc. Natl. Acad. Sci. U S A*. **98**: 5466-5470.

Nybom, H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Mol. Ecol.* **13**: 1143-1155.

Obermayer, R., Leitch, I. J., Hanson, L. and Bennett, M. D. 2002. Nuclear DNA C-values in 30 species double the familial representation in pteridophytes. *Ann. Bot.* **90**: 209-217.

Paisooksantivatana, Y., Kako, S. and Seko, H. 2001a. Genetic diversity of *Curcuma alismatifolia* Gagenp. (Zingiberaceae) in Thailand as revealed by allozyme polymorphism. *Gen. Res. Cro. Evol.* **48**: 459-465.

Paisooksantivatana, Y., Kako, S., and Seko, H. 2001b. Isozyme polymorphism in *Curcuma alismatifolia* Gagnep. (Zingiberaceae) populations from Thailand. *Sci. Hort*. **88**: 299-307.

Palomino, G., Doležel, J., Mendez, I. and Rubluo, A. 2003. Nuclear genome size analysis of *Agave tequilana* Weber. *Caryologia* **56**: 37-46.

Panis, B, Totté, N., Van Nimmen, K., Withers, L. A. and Swennen, R. 1996. Cryopreservation of banana (*Musa* spp.) meristem cultures after preculture on sucrose. *Plant Sci.* **121**: 95-106.

Peak, K. Y. and Murthy, H. N. 2002. High frequency of bulblet regeneration from bulb scale sections of *Fritillaria thunbergii*. *Plant Cell Tiss. Org. Cult.* **68**: 247-252.

Pence, V. C. and Soukup, V. G. 1993. Factors affecting the intiation of mini-rhizomes from *Trillium erectum* and *T. grandiflorum* tissues *in vitro*. *Plant Cell Tiss. Org. Cult.* **35**: 229-235.

Phunchaisri, B., Alisi, C., Anuntalabhochai, S. and Apavatjrut, P. 1998. Randomly amplified polymorphic DNA (RAPD) in *Curcuma* species. *Research and Development, The Institute of Science and Technology*. Chiang Mai University, Chiang Mai, Thailand.

Price, H. J. 1988. Nuclear DNA content variation within angiosperm species. *Evol. Trends in Plants* **2**: 53-60.

Prucksunand, C., Indrasukhsri, B., Leethochawalit, M. and Hungspreugs, K. 2001. Phase II clinical trial on effect of the long turmeric (*Curcuma longa* Linn) on healing of peptic ulcer. *Southeast Asian J. Trop. Med. Public Health* **32**: 208-215.

Purseglove, J. W. 1974. *Tropical crops monocotyledons.* Longman Group Ltd., London.

Purseglove, J. W., Brown, E. G., Green, C. L. and Robbins, S. R. J. 1981. *Spices. Vol. 2. Chapter 9. Tropical Agriculture Series*. Longman, New York.

Raghavan, T. S. and Venkatasubban, K. R. 1943. Cytological studies in the family Zingiberaceae with special reference to chromosome number and cyto-taxonomy. *Proc. Ind. Acad. Sci. Ser. B*. **17**: 118-132.

Rahman, M. H. and Rajora, O. P. 2001. Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). *Plant Cell Rep.* **20**: 531–536.

Ramachandran, K. 1969. Chromosome numbers in Zingiberaceae. *Cytologia* **34**: 213-221.

Rani, V. and Raina, S. N. 1998. Genetic analysis of enhanced axillary branching derived *Eucalyptus tereticornis* and *E. camaldulensis* plants. *Plant Cell Rep* **17**: 236-242.

Rani, V. and Raina, S. N. 2000. Genetic fidelity of organized meristem derived micropropagated plants: a critical reappraisal. *In Vitro Cell Dev. Biol. Plant* **36**: 319-330.

Rani, V., Parida, A. and Raina, S. N. 2001. Chromosome number dependent genome size and RAPD fingerprinting diagnostics for genetic integrity of enhanced axillary branching-derived plants of ten forest tree species. *Acta Hort.* **560**: 531-534.

Rani, V., Parida, A., and Raina, S. N. 1995. Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. *Plant Cell Rep.* **14**: 459-462.

Rani, V., Singh, K. P., Shiran, M., Nandy, S., Goel, S., Devarumath, R. M., Sreenath, H. L. and Raina, S. N. 2000. Evidence for new nuclear and mitochondrial genome organizations among high frequency somatic embryogenesis-derived plants of allotetraploid *Coffea arabica* L. (Rubiaceae). *Plant Cell Rep.* **19**: 1013-1020.

Rayburn, A. L., Birdar, D. P., Bullock, D. G., Nelson, R. L., Gourmet, C. and Wetzel, J. B. 1997. Nuclear DNA content diversity in Chinese soybean introductions. *Ann. Bot.* **80**: 321-325.

Reeves, G., Francis, D., Davies, M. S., Rogers, H. J. and Hodkinson, T. R. 1998. Genome size is negatively correlated with altitude in natural populations of *Dactylis glomerata*. *Ann. Bot.* **82** (Supplement A): 99-105.

Richards, A. J. 1986. *Plant Breeding Systems.* George Allen & Unwin, London.

Riedy, M. F., Hamilton, W. J. and Aquadro, C. F. 1992. Excess of non-parental bands in offspring from known primate pedigrees assayed using RAPD PCR. *Nucleic Acids Res.* **20**: 918.

Rival, A., Bertrand, L., Beale, T., Combes, M. -C., Trouslot, P. and Leshermes, P. 1998. Suitability of RAPD analysis for detection of somaclonal variation in oil palm (*Elaeis guineensis* Jacq.). *Plant Breed*. **117**: 73-76.

Rohlf, F. J. 2000. *NTSYSpc. Numerical Taxonomy and Multivariate Analysis System ver. 2.0.* Exeter Software, Setauket, New York.

Rout, G. R., Das, P., Goel, S. and Raina, S. N. 1998. Determination of genetic stability of micropropagated plants of ginger using Random Amplified Polymorphic DNA (RAPD) markers. *Bot. Bull. Acad. Sin.* **39**: 23-27.

Roy, J., Tessy, J. and Joseph, J. 1999. Karyomorphological studies in the genus *Curcuma* Linn. *Cytologia* **64**: 313-317.

Sakai, A., Kobayashi, S. S. and Ojima, I. 1990. Cryopreservation of nucellar cells of naval orange (*Citrus sinensis*) Osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Rep.* **9**: 30-33.

Salvi, N. D., George, L. and Eapen, S. 2000. Direct regeneration of shoots from immature inflorescence cultures of turmeric. *Plant Cell Tiss. Org. Cult.* **62**: 235-238.

Salvi, N. D., George, L. and Eapen, S. 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell Tiss. Org. Cult.* **66**: 113-119.

Salvi, N. D., George, L. and Eapen, S. 2002. Micropropagation and field evaluation of microprpagated plants of turmeric. *Plant Cell Tiss. Org. Cult.* **68**: 143-151.

Saralamp, P., Chuakul, W., Temsiririrkkul, R., Clayton, T. 1996. *Medicinal plants in Thailand*. Amarin, Bangkok.

Sarkar, D. and Naik, P. S. 1998. Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum* L.) clones by vitrification. *Ann. Bot.* **82**: 455-461.

Sasaki, Y., Fushimi, H., Cao, H., Cai, S. Q. and Komatsu, K. 2002. Sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trn*K gene and the application of amplification-refractory mutation system analysis for their authenticication. *Biol. Pharm. Bull.* **25**: 1593-1599.

Sasaki, Y., Fushimi, H., Komatsu, K. 2004. Application of single-nucleotide polymorphism analysis of the *trn*K gene to the identification of *Curcuma* plants. *Biol Pharm Bull*. **27**: 144-146.

Sato, T., Kwon, O. C., Miyake, H., Taniguchi, T. and Macda, E. 1995. Regeneration of plantlets from petiole callus of wild viola (*Viola patrinii* DC). *Plant Cell Rep.* **14**: 768-772.

Schmuths, H., Meister, A., Horres, R. and Bachmann, K. 2004. Genome size variation among accessions of *Arabidopsis thaliana*. *Ann. Bot.* **93**: 317-321.

Schneider, S. Roessli, D. and Excoffier, L. 2000. *ARLEQUIN, version 2.000. A software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Sharma, A. K. and Bahttacharya, N. K. 1959. Cytology of several members of Zingiberaceae. *Cellule* **59**: 229-349.

Sharma, T. R. and Singh, B. M. 1995. *In vitro* microrhizome production in *Zingiber officinale* Rosc. *Plant Cell Rep.* **15**: 274-277.

Shirgurkar, M. V., John, C. K. and Nadgauda, R. S. 2001. Factors affecting *in vitro* microrhizome production in turmeric. *Plant Cell Tiss. Org. Cult.* **64**: 5-11.

Shirin, F., Kumar, S. and Mishra, Y. 2000. *In vitro* plantlet production system for *Kaempferia galanga*, a rare Indian medicinal herb. *Plant Cell Tiss. Org. Cult.* **63**: 193-197.

Sirinivasan, K. R. 1953. Chromatographic study of the curcuminoids in *Curcuma longa*. *J. Pharm. Pharmacol.* **5**: 448-453.

Sirirugsa, P. 1999. *Thai Zingiberaceae: Species diversity and their uses*. International Conference on Biodiversity and Bioresources: Conservation and Utilization. Phuket, Thailand.

Slabbert, M. M., Niederwieser, J. G. 1999. *In vitro* bulblet production in *Lachenalia*. *Plant Cell Rep.* **18**: 620-624.

Srimal, R. C. 1997. Turmeric: a brief review of medicinal properties. *Fitoterapia* **LXVIII** (6): 483-492.

Stewart, C. N. J. and Porter, D. M. 1995. RAPD profiling in biological conservation, an application to estimating clonal variation in rare and endangered *Iliamna* in Virginia. *Biol. Conserv.* **74**: 132-142.

Stewart, C. N. J. and Excoffier, L. 1996. Assessing population genetic structure and variability with RAPD data: application to *Vaccinium macrocarpon* (American Cranberry). *J. Evol. Biol.* **9**: 153-171.

Sunitibala, H., Damayanti, M. and Sharma, G. J. 2001. *In vitro* propagation and rhizome formation in *Curcuma longa* Linn. *Cytobios* **105**: 71-82.

Syu, W. J., Shen, C. C., Don, M. J., Ou, J. C., Lee, G. H. and Sun, C. M. 1998. Cytotoxicity of curcuminoids and some novel compounds from *Curcuma zedoaria. J. Nat. Prod.* **61**: 1531-1534.

Takagi, H., Thinh, N. T. and Kyesmu, P. M. 1998. Cryopreservation of vegetatively propagated tropical crops by vitrification. *Acta Hort. (ISHS)* **461**: 485-494.

Takashi, S., Kaneko, M., Harada, T. 1990. Increase in freezing resistance of excised shoot tips of *Asparagus officinalis* L. by preculture on sugar-rich media. *Cryobiol.* **34**: 264-275.

Tanksley, S. D. and McCouch, S. R. 1997. Seed banks and molecular maps: unlocking genetic potential from the wild. *Science* **277**: 1063-1066.

Thinh, N. T., Takagi, H. and Sakai, A. 1998. Cryopreservation of *in vitro*-grown apical meristems of some vegetatively propagated tropical monocots by vitrification. **In**: *Cryopreservation of tropical plant germplasm - current research progress and application* (Engelmann, F. and Takagi, H. eds.), Proceedings of the JIRCAS/IPGRI Joint International Workshop. Tsukuba, Japan. Pp. 227-232.

Torimaru, T., Tomaru, N., Nishimura, N. and Yamamoto, S. 2003. Clonal diversity and genetic differentiation in *Ilex leucoclada* M. patches in an old-growth beech forest. *Mol. Ecol.* **12**: 809-818.

Torres, A. M., Millán, T. and Cubero, J. I. 1993. Identifying rose cultivars using random amplified polymorphic DNA markers. *HortScience* **28**: 333-334.

Travis, S. E., Maschinski, J. and Keim, P. 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax,* a critically endangered plant, using AFLP markers. *Mol. Ecol.* **5**: 735-745.

Tuna, M., Vogel, K. P., Arumuganathan, K. and Gill, K. S. 2001. DNA content and ploidy determination of bromegrass germplasm accessions by flow cytometry. *Crop Sci.* **41**: 1629-1634.

Turner, S. R., Senaratna, T., Bunn, E., Tan, B., Dixon, K. W. and Touchell, D. H. 2001. Cryopreservation of shoot tips from six endangered Australian species using a modified vitrification protocol. *Ann. Bot.* **87**: 371-378.

Uptmoor, R., Wenzel, W., Friedt, W., Donaldson, G., Ayisi, K. and Ordon, F. 2003. Comparative analysis on the genetic relatedness of *Sorghum bicolor* accessions from Southern Africa by RAPDs, AFLPs and SSRs. *Theor. Appl. Genet.* **106**: 1316-1325.

Uragami, A., Sakai, A., Nagai, M. and Takahashi, T. 1989. Survival of cultured cells and somatic embryos of *Asparagus officinalis* cryopreserved by vitrification. *Plant Cell Rep.* **8**: 418-421.

Uyoh, E. A., Nkang, A. E. and Eneobong, E. E. 2003. Biotechnology, genetic conservation and sustainable use of bioresources. *Afric. J. Biotech.* **2**: 704-709.

Vandenbussche, B., Leurdian, S., Verdoodt, V., Gysemberg, M., de Proft, M. 1999. Changes in sugar content and fatty acid composition of in vitro sugar beet shoots after cold acclimation: influence on survival after cryopreservation. *Plant Growth Regul.* **28**: 157-163.

Vekemans, X. and Jacquemart, A.-L. 1997. Perspectives on the use of molecular markers in plant population biology. *Belg. J. Bot.* **129**: 91-100.

Venkatasubban, K. R. 1946. A preliminary survey on chromosome numbers in Scitamineae of Bentham & Hooker. *Proc. Ind. Acad. Sci. Ser. B*. **23**: 281-300.

Vierling, R. A. and Nguyen, H. T. 1992. Use of RAPD markers to determine the genetic diversity of diploid wheat genotypes. *Theor. Appl. Genet*. **84**: 835-838.

Vilhar, B., Vidic, T., Jogan, N. and Dermastia, M. 2002. Genome size and the nucleolar number as estimators of ploidy level in *Dactylis glomarata* in the Solvenian Alps. *Plant Syst. Evol.* **234**: 1-13.

Villalobos, V. M., Ferreira, P. and Mora, A. 1991. The use of biotechnology in the conservation of tropical germplasm. *Biotechnol. Adv.* **9**: 197-215.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Le, T., Hornes, M., Frijiters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*. **23**: 4407-4414.

Vreugdenhil, D., Boogaard, Y., Visser, R. G. F. and Bruijn, S. M. 1998. Comparison of tuber and shoot formation from *in vitro* cultured potato plants. *Plant Cell Tiss. Org. Cult.* **53**: 197-204.

Waycott, M. 1998. Genetic variation its assessment and implications to the conservation of seagrasses. *Mol. Ecol.* **7**: 793-800.

Weerapakdee, W. and Krasaechai, A. 1997. Collection and development studies of certain *Curcuma* spp. *J. Agri.* **13**:127-136.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**: 7213-7218

Wilkinson, T., Wetten, A., Prychid, C., Fay, M. F. 2003. Suitability of cryopreservation for the long-term storage of rare and endangered plant species: a case history for *Cosmos atrosanguineus*. *Ann. Bot.* **91**:65-74.

Williams, J. G. K, Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18:** 6531-6535.

Wolfe, A. D. and Liston, A. 1998. Contributions of PCR-based methods to plant systematics and evolutionary biology. **In**: *Plant Molecular Systematics II* (Soltis, D. E., Soltis, P. S. and Doyle, J. J. eds.), Kluwer Academic Publishers, Boston. Pp. 43-86.

Wolff , K., El-Akkad, S. and Abbott, R. J. 1997. Population substructure in *Alkanna orientalis* (Boraginaceae) in the Sinai Desert, in relation to its pollinator behaviour. *Mol. Ecol.* **6**: 365-372.

Wright, S. 1978. *Evolution and the genetics of populations: variability within and among natural populations*, *Vol. 4*. University of Chicago Press, Chicago.

Yasuda, K., Tsuda, T., Shimizu, H. and Sugaya, A. 1988. Multiplication of *Curcuma* species by tissue culture. *Planta Medica* **54**: 75-79.

Yeh, F. C., Yang, R.-C., Boyle, T. B. J., Ye, Z.-H. and Mao, J. X. 1999. *POPGENE Ver. 1.32, The user-friendly shareware for population genetic analysis*. Molecular Biology and Biotechnology Centre, University of Alberta, Alberta, Canada.

Yoshioka, T., Fujii, E., Endo, M., Wada, K., Tokunaga, Y., Shiba, N., Hohsho, H. and Muraki, T. 1998. Antiinflammatory potency of dehydrocurdione a zedoary-derived sesquiterpene. *Inflamm. Res.* **47**: 467-481.

Yusuf, M. 2000. *Genetic diversity of the family Zingiberaceae in Bangladesh*. PhD Thesis, Jahangirnagar University, Dhaka.

Yusuf, N. A., Ibrahim, H. and Khalid, N. 2001. *Antibacterial evaluation and tissue culture studies of selected medicinal Curcuma species*. Proc. NSF Workshop 2001, Kuala Lumpur, Malayasia.

Zhivotovsky, L. A. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. *Mol. Ecol*. **8**: 907-913.

10. LIST OF PUBLICATIONS

Manuscripts submitted

1. Islam, M. A., Esch, E., and Kloppstech, K. Population genetic structure of *Curcuma zedoaria* (Christm.) Rosc. - a conservation prioritised medicinal plant in Bangladesh. Manuscript submitted to the *Conservation Biology*.

2. Islam, M. A., Schumacher, M. and Kloppstech, K. Cryopreservation of *in vitro*-grown axillary buds of *Curcuma longa* L. using vitrification dehydration technique. Submitted to the *CryoLetters*.

3. Islam, M. A., Meister, A., Schubert, V., Kloppstech, K. And Esch, E. Estimation of genome size and genetic diversity of the populations of *Curcuma zedoaria* (Chirsm.) Rosc. from Bangladesh. Manuscript submitted to the *Annals of Botany*.

4. Islam, M. A., Kloppstech, K. and Jacobsen, H. J. High frequency *in vitro* micropropagation of *Curcuma longa* var. Surma and evaluation of genetic instability using molecular marker. Manuscript submitted to the *Scientia Horticulturae*.

Manuscripts under preparation

5. Islam, M. A., Esch, E., and Kloppsstech, K. Genetic diversity of different *Curcuma* species (Zingiberaceae) in Bangladesh as revealed by RAPD markers. Manuscript is will be submitted to the *Theoretical and Applied Genetics*.

6. Islam, M. A., Meister, A., Schubert, V., Kloppstech, K. And Esch, E. Chromosome numbers and genome size variations of different *Cucurma* species occurred in Bangladesh. Manuscript will be submitted to the *Cytologia*.

7. Islam, M. A., Kloppstech, K. and Jacobsen, H. J. Efficient procedure for *in vitro* microrhizome induction in *Curcuma longa* L. (Zingiberaceae) – a medicinal plant of tropical Asia. Manuscript will be submitted to the *Journal of Plant Tissue Culture and Biotechnology*.

11. ERKLÄRUNG

Ich versichere, dass die Dissertation selbständig verfasst und die benutzten Hilfsmittel und Quellen, sowie gegebenenfalls die zu Hilfsleistungen herangezogenen Institutionen, vollständig angegeben wurden und die Dissertation nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

Hannover, 25 November 2004 **Md. Aminul Islam**

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