MOLECULAR ANALYSIS OF THE GENOMIC DNA OF SIX VARIETIES OF COWPEA, *VIGNA UNGUICULATA* L. WALP.

BY

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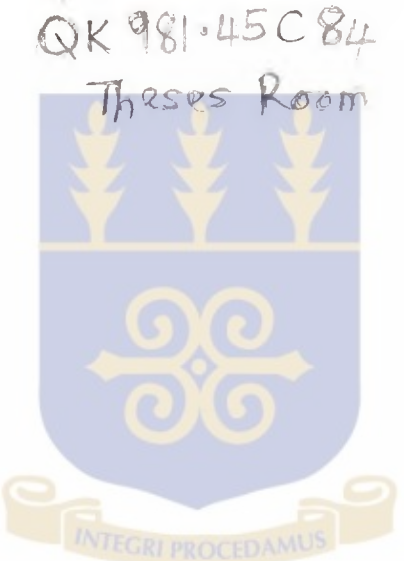

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DECLARATION

I certify that this work has not been submitted to any other university for any degree. The experimental work was done by me. Due acknowledgement has been given for all the guidance received.

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DEDICATION

To all those third world scientists sweating to a headway in their chosen carriers.

There is always a way out.

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ABSTRACT

Three non-chemical conditions of preservation, storage at room temperature (that is benchtop), in a freezer and at simulated herbarium were examined to determine which is most suitable for the preservation of cowpea leaves for molecular studies. Under each storage condition, the effect of duration of storage and the difference in leaf age (that is flush or mature) on DNA yield and purity were assessed. Periodically, DNA was extracted from both flush and mature leaf samples from 6 cowpea (*Vigna unguiculata* L. Walp.) varieties, Amantin, Asontem, Ayiyi, Bengpla, Ejura Red and Soronko beginning from the day of harvest (day 1), through day 7, 21, 35 and 49 after storage. At room temperature and simulated herbarium conditions, the yield and purity of the DNA extracted from both flush and mature leaves decreased during the period of the study. Time was found to correlate inversely with the purity and yield of DNA in all cases. Under these conditions, the yield of DNA extracted from day 1 samples (fresh samples) of both leaf types was significantly different from the DNA yield from day 7 to 49 samples. Also, no significant difference was found in DNA yield from day 7 to 49 samples. However, in most cases, more DNA was obtained from flush leaves than from their corresponding mature leaves. Of the three conditions, leaves stored at room temperature yielded the least amount of DNA. Samples kept under simulated herbarium conditions yielded more DNA than kept in the freezer but the difference was not significant. Also, a fairly constant yield and purity was obtained for DNA extracted from frozen samples. Frozen samples yielded relatively purer DNA than their

corresponding samples stored either at room temperature or at simulated herbarium though the observed differences were not significant. Therefore, storage in a freezer provided the best non-chemical preservation condition among the three assessed.

Only 2 (GA and AR) out of 15 random primers assayed using the technique of Random amplified polymorphic DNA-polymerase Chain Reaction, (RAPD-PCR) amplified segments of the genomic DNA extracted from the 6 varieties. Further analysis (Restriction Fragment Length Polymorphism studies) was done on the GA-primed 1200 bp PCR product using eight restriction endonucleases, *Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hinf*I, *Kpn*I *and Pvu*I. Four enzymes, *Alu*I, *Hae*III. *Hinf*I *and* *Kpn*I digested the product. However, for each enzyme, identical banding patterns were observed on both agarose and polyacrylamide gels in all the six varieties Single stranded conformation polymorphism analysis (SSCP) was conducted on the two AR-primed products, 369 bp and 545 bp. Only the 545 bp was denatured but the profiles of the bands in all the six varieties were also identical. Therefore, these molecular techniques could not be used to identify the individual varieties.,

CHAPTER ONE

1.1 GENERAL INTRODUCTION

The current explosion in global human population has led to an urgent search for rapid means of increasing the world's food supply. A major concerted approach aimed at meeting this growing food requirement is through the improvement of staple food crops, that is, crops identified as major components of diets of populations. This approach demands developing new plant varieties which would produce higher yields of better nutritional quality without any adverse effects on the surrounding environment.

Cowpea, *Vigna unguiculata* (L.) Walp., is an important food grain legume that is widely grown in the developing world, notably in Africa, India and Brazil (Rachie and Singh, 1985). In Africa, it represents the major source of dietary protein for her population and a feed for a large number of livestock. It is cheap, easily available and versatile in food preparations and, its consumption cuts across all social, religious and economic barriers.

In most parts of tropical Africa and in India, it is eaten in the form of dry seeds and in East Africa, cowpea is grown for its seeds, pods and leaves (Dovlo, 1976). It is also used as fodder and as quick growing cover-crop (Rachie, 1985). It improves soil fertility because it is able to fix large quantities of nitrogen (up to 240 kgN per hectare) in soils symbiotically with the bacterium *Rhizobium* and in the process it can leave a fixed nitrogen deposit in the soil of up to 60-70 kg per hectare for the succeeding crop (Rachie, 1985).

1.2 Origin and Distribution of Cultivated Cowpeas

Africa and Asia are centres of genetic diversity of cowpea that suggest ancient origin in these regions. However, the current view held by plant taxonomists is that cowpeas originated in Africa because many wild forms, which are resistant to cultivated cowpea pests and diseases exist in abundance. Faris (1965), Rawal (1975), and Steele (1976) have referred to it as a West African Neolithic domesticate. This view is supported by the oldest archaeological evidence in Africa - a carbon dating (ca.1450-1400 BC) of cowpea remains discovered in a Kintampo rockshelter in central Ghana (Flight, 1976). Mehra and Steele (1980) also have reported of limited archaeological evidence from stone implements in the Niger river that suggest that domestication might have taken place around 3000 BC. The exact location of domestication of wild cowpeas in Africa however remains unknown.

The wide distribution of cowpeas in Asia asserts to an early introduction of the crop in this region from Africa, probably between 2000 and 3500 BC. Cowpeas might have reached India over 2000 years ago from East Africa along with other crops. From Asia cowpeas were introduced into southern Europe where the Romans cultivated it under the respective names *Phaseolos* and *Phaseolus*. By the seventeenth century, the Spanish explorers had introduced it to the West Indies. More cultivars might have reached the New World from West Africa during the Slave Trade in the eighteenth century. This was followed by its introduction into the southern parts of the United States of America (Rachie and Singh, 1985).

1.3 Nutritional Status of Cowpeas

The details of proximate composition of cowpeas are given in Table 1.

Table 1: Chemical Composition of Cowpeas (%)

Component (g/100g)	a	b	c
Crude Protein	24.2±0.1	23.5	24.80±0.48
Available Carbohydrate	54.4±0.91	-	63.60±2.40
Fat (Ether Extract)	1.2	1.4	1.90±0.62
Ash	3.5	3.5	3.60±0.17
Crude Fibre	ND	ND	6.30±0.64
Thiamine (mg/100g)	0.77	0.8	0.74±0.22
Riboflavin (mg/100g)	0.25	0.2	0.42±0.14
Niacin (mg/100g)	3.48	2.8	2.81±0.26

This data on the proximate composition of cowpeas was undertaken by [a]Uzogara *et al.*, (1988, 1991); [b]Soucci *et al.* (1986) and [c]Bressani (1985). ND Not determined

Like other legumes, cowpeas are a good source of energy, amino acids, vitamins, essential minerals and dietary fibre (Walker, 1981). Protein content is usually between 20 and 30% (Onigbinde and Akinyele, 1989), with soluble carbohydrates making up 50-65% (Longe, 1980). These two components constitute the major source of energy that is derived from cowpeas. However, cowpeas suffer from a low protein digestibility of 52%, a low net protein utilization of 50.6% and a biological value of about 58.17% (Bressani, 1985). These limitations notwithstanding, the nutritional value of cowpeas is highly rated by nutritionists because they provide supplementary amino acids to traditional diets that are mainly cereals, starchy roots and tubers and have been used to fortify infant weaning foods (Aykroyd *et al.*, 1982). Matthews (1989) has attributed this property to their high lysine content (and other essential amino acids) which make them excellent improvers of

the protein quality of foods low in lysine such as cereals (Uzogara and Ofuya, 1992). But a drawback to these quality characteristics is a low level of the sulphur-containing amino acids, methionine and cysteine (Rachie and Singh, 1985).

In addition, cowpeas are rich sources of thiamin and niacin, and contain reasonable amounts of the water-soluble vitamins, riboflavin, pyridoxine and folic acid. They are also a good source of the essential minerals, calcium, magnesium, potassium, iron, zinc and phosphorus (Aykroyd *et al.*, 1982). They contain low levels of fat, cholesterol and sodium, thus making them invaluable source of food to hypertensive individuals. The immature seeds provide good sources of vitamin A, β-carotene and vitamin C (Eheart and Sholes, 1948).

High consumption of cowpeas has been found to be associated with reduced incidence of the so-called 'diseases of affluence'- colon cancers, diverticular diseases, obesity, coronary heart disease, diabetes mellitus and dental caries (Walker, 1982). Such diseases are less commonly observed in developing countries where legumes and other high fibre plant foods are common staples (Burkitt and Trowell, 1975). Consumption of dietary fibre is reported to have a hypo-cholesterolemic effect (Shutler *et al.*, 1987), a hypoglycemic effect (Jensen and Jepsen, 1982), and decreases intestinal transit time while increasing faecal bulk. Dietary fibre binds bile acids thus, enhancing their degradation into short chain fatty acids in the intestines. It also increases viscosity and retards digestion (Passmore and Eastwood, 1986).

Despite the foregoing nutritional attributes of cowpeas, the presence of indigestible oligosaccharides - raffinose, stachyose, and verbascose (Phillips and Abbey, 1989) reduce the nutritional status of cowpeas. These oligosaccharides are largely unavailable for human nutrition due to the absence of specific degrading enzymes, namely α-galactosidases and β-fructosidases. Following ingestion, these oligosaccharides, as well as phytate salts which are not hydrolyzed by the gastro-intestinal enzymes, pass to the lower gut. Here, a variety of micro-organisms of the colon hydrolyze and ferment the former producing gas or flatus as well as free fatty acids. These flatulence factors alter water retention and faecal bulk. Flatulence can be uncomfortable especially for infants and old people. It can be accompanied by frequent belching, abdominal distention, diarrhoea and weakness.

1.4 The Systematics of *Vigna unguiculata* (L.) Walpers

Systematics has been defined by Monger and Songer (1988) as the scientific study of diversity of living things and the relationships among them. It embraces taxonomy, that is, the study of the principles, rules and procedures of classification, and in many instances taxonomy and systematics are used interchangeably. Presently, systematics aims at providing the most natural classification possible. Three approaches, the phenetic, phylogenetic and cladistic are being used. In the phenetic approach, organisms are classified on the basis of characters without attempting to reconstruct any evolutionary relationships. The phylogenetic approach is based on evolutionary relationships and it considers the sequence of branching of new groups from a common ancestor (that is, the genealogy) and the degree to which the groups have continued to change ever since. The

cladistic approach on the other hand considers the closer common ancestry only to be important and not the degree to which groups have subsequently diverged.

In all these approaches, the principles (or characters) of classification have been the same. These characters have come principally from structural and biochemical features. Structural characters provide the major source of evidence by which living organisms are classified. The morphology and anatomy of higher plants and animals, including that of embryos and various developmental stages as well as of mature adults provide characters which are easy to observe and they provide comparative data base for classification. But in recent times, the advent of techniques for studying cellular and molecular organization of cells from different organisms have generated massive comparative data from the analysis of biochemical characters such as proteins and nucleic acids.

However, in most plants morphology and agronomic characters are still widely used in the traditional manner for classification (Rachie and Singh, 1985). The morphological features that are usually used include the style and floral forms but seed morphology, size, yield and weight, pigmentation, and disease resistance are considered priority agronomic criteria for the identification of plant genotypes and in the development of genetic linkage maps. However, in some cases, they do not generate enough comparative data base for identifying individual varieties.

Classification of the Leguminosae has however seen much improvement up to the generic and tribal levels. This is because the principles or criteria for classification have undergone much improvement. Beginning with the traditional system of classification,

plants are identified on the basis of simple morphological criteria such as style and floral morphology. At the generic level, difficulties arising from the former system of classification are resolved in accordance with the actual course of evolution of the genus (Rachie and Singh, 1985). This incorporates information derived from biochemical analysis, anatomy, and cytology which are of taxonomic value. Presently, the geographical and ecological location, and style morphology are also being employed in the elucidation of evolutionary relationships among plant genera which is a further deviation from the traditional system of classification (Baudet, 1978). Furthermore, molecular biology and serological techniques have been applied in some instances. These have generated valuable new character correlation which have improved classification and given more insight into the taxonomy of the Leguminosae at the generic, tribal and family levels. Classification of the Leguminosae at these levels is therefore without controversy, although it is incomplete (Rachie and Singh, 1985).

The controversy in taxonomy and classification commences at the intraspecific level. However, the current classification is based on Harlan and de Wet's concept (1971) of reproductive affinity among species. In this concept, races and related species are assigned to primary, secondary and tertiary gene pools. The primary gene pool corresponds to species of the taxonomic hierarchy. This includes both cultigen and the wild forms, all hybridizing freely. The secondary gene pool comprises other species that are relatives of the crop and are suitable for interspecific hybridization. They are isolated from the species partly by chromosomal and gene barriers. The tertiary gene pool involves still greater barriers to hybridization and it embraces taxa that display either inviable or sterile hybrid with the cultivated plant and do not permit gene flow by normal

introgression. The range of total gene pool varies with each crop (Rachie and Singh, 1985).

1.4.1 Taxonomy within *Vigna*

Two schemes of classification have emerged for *Vigna*. The first is by Verdcourt (1970) and the second is a revision of Verdcourt's classification by Maréchal *et al.*, (1978). These schemes have been compared in Table 2.

Table 2 Comparison of Classification and Nomenclature of the taxa within Section Catiang of the sub-genus *Vigna* (Savi)

Marechal *et al.* (1978)	**Verdcourt (1970)**
V. unguiculata	*V. unguiculata*
Sub. sp. unguiculata	
cv-gr Unguiculata E. Westphal	sub. sp. unguiculata (L.) (Walp.) Verdcourt
cv-gr Biflora E. Westphal	sub. sp. cylindrica (L.) Van Eseltine
cv-gr Sesquipedalis E. Westphal	sub. sp. sesquipedalis (L.) Verdcourt
cv-gr Textilis E. Westphal	
Sub. sp. dekindtiana	sub. sp. dekindtiana (Harms) Verdcourt
var. dekindtiana	
var. mensensis (Sechweinf.) M., M. & S.	sub. sp. mensensis (Sechweinf.) Verdcourt
	Vigna unguiculata (L.) Walp. var. protracta (E. Mey) Verdcourt
var. protracta (Wilczek) M., M. & S.	
var. pubenscens (Wilczek) M., M. & S.	var. pubenscens (Wilczek) M., M. & S.
Sub. sp. stenophylla (Harv.) M., M. & S.	*V. anguistifoliata* Verdcourt

Sub. sp. tenuis (E. Mey)	*V. tenuis* (E. Mey) Dietr.
V. nervosa Markotter	*V. nervosa Markotter*

Both schemes agree that cowpea belongs to the family Leguminosae, subfamily Papilionaceae; is a member of the tribe Phaseolae, subtribe Phaseolineae and found within the genus *Vigna*. Cowpeas belong to the section Catiang.

1.4.2 Taxonomy within *Vigna unguiculata*

Classification of the cowpeas at the intraspecific level has been provisional (Rachie and Singh, 1985). Verdcourt (1970) subdivided *V. unguiculata* into five (5) subspecies of which three (3) are cultivated and two (2) are closely related wild species. These cultivated subspecies are *unguiculata, cylindrica* and *sesquipedalis*. Verdcourt (1970) refers to the subspecies unguiculata as the common African domesticated cowpeas which he assigned the new name *Vigna unguiculata* (L.) Walpers. He named the two wild species as *dekindtiana* and *mensensis*. Maréchal *et al*. (1978) on the other hand, did not consider the cultivated subspecies *unguiculata, cylindrica* and *sesquipedalis* as distinct. They lumped them together as the intraspecific category "cultigroups" - *Unguiculata, Biflora (*for *cylindrica),* and *sesquidepedalis.* The authors assigned the cowpeas to the cultigroup Unguiculata and renamed them *Vigna unguiculata* subspecies unguiculata; cultigroup (cv-gr.) Unguiculata E. Westphal. In addition, they included cultigroup *Textilis*, a cultivar grown in northern Nigeria (Westphal, 1974). The wild species *dekindtiana* and *mensensis* were lumped under a single subspecies dekindtiana. These were differentiated by varietal means.

1.4.3 Classification of Cowpea Varieties

Several cowpea varieties have been described (Longe 1980, 1983; Akinyele and Adesina, 1986; Aletor and Aladetimi, 1989; De Mooy and De Mooy, 1990). Presently, about 15,000 accessions of cowpea are being kept in the Germplasm Unit of the International Institute of Tropical Agriculture (IITA) in Nigeria (Thottapilly *et al.*, 1992) but the exact number of cultivated cowpeas is yet to be established because varietal differences vary from country to country. De Mooy and De Mooy (1980) reported on the cooking time and quality of seven diverse cowpea varieties. Similarly, Longe (1980) reported on the composition of carbohydrate in different varieties of cowpea. He evaluated also differences in varieties in the chemical characteristics related to cooking quality of cowpeas (Longe, 1983). It is worth mentioning that the available literature reviewed were silent on the varietal differences of cowpeas. In Ghana, seven varieties have been identified on the basis of their morphological and agronomic traits (Twumasi-Afriyie & Arias, 1990). There appears to be significant similarity in the details of overlaps of these traits therefore, identification of every individual variety by this method is not possible.

1.4.4 Methods of Identification of Cowpeas

Several methods and techniques have been applied to identify cowpeas. These include identification by morphological and agronomic features, biochemical methods such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and some molecular techniques like cytotaxonomy.

Presently, cowpeas are identified on the basis of simple morphological criteria as represented in the traditional classification of plants and some agronomic features. Morphological features which form the basis of identification are growth habits, seed, and flower patterns (Rachie and Singh, 1985). Growth habits vary from erect, semi-erect through climbing to prostrate forms. The seeds differ in shape, colour, eye pattern and length (size). Seed length varies from 8 mm to 100 mm long and they may be kidney-shaped, rhomboid or ovoid. Eye patterns may or may not be present and, when present, they may be very small, thin or mottled. Some agronomists combine seed colour and eye pattern in the description of seed colour. Porter *et al.,* (1974) have described sixty-two (62) eye colorations. However, the spectrum of seed coat colours range from red, light red, black, grey, blue, white, coffee, brown to purple. The seeds may be contained in pods which are narrow, coiled or slightly curved, crescent or linear and vary from 8 cm to 100 cm long but they are not more than 10 mm wide. Six different patterns of pigmentation of pods have also been described and six different floral patterns of pigmentation have been reported. The length of the peduncles vary from less than 5 cm to more than 50 cm (Rachie and Singh, 1985).

Typical agronomic features employed in describing cowpeas are maturity time and group, yield per bag, and yield per hectare. Maturity time is defined as the number of days between sowing and pod maturity. In general, maturity time has been found to vary from 53 days to more than 120 days (Rachie and Singh, 1985).

Biochemical characterization of cowpea variants has also not yielded enough comparative data base for classification and identification. It has however provided some

insights into differences between subspecies and species. Pedalino *et al.*, (1990) reported light qualitative and quantitative variations in seed protein banding patterns in 35 cultivated *V. unguiculata*. In all, two major total seed protein banding patterns were observed on the basis of the absence or presence of the 23 and 32kDa subunits in eight out of the thirty-five accessions analyzed. They reported that seed albumin patterns varied in the number and molecular weight of the major subunits. Two major globulins (CP1 and CP2) were also observed in all the cowpea accessions analyzed. Using two-dimensional electrophoresis Pedalino *et al.*, (1990) showed that the CP1 consists of three (3) major subunits 63, 58, and 49kDa. The 58 and 49kDa subunits were found also in the CP2 globulin. These three major subunits proved to be heterogenous and were made of similar multiple charge forms in the pH range 4.6-5.1. Three other globulin subunits of molecular weights 94.5, 87 and 40kDa, were found to be composed of disulphide-linked subunits. The 87kDa subunit consists of both acidic (63kDa) and basic (22kDa) disulphide-bonded polypeptides. This subunit was identified as an 11S legumin-like protein component. SDS-PAGE revealed further that the CP2 globulin is composed of three minor components besides the major subunits (49 and 58kDa). They suspected that the CP2 could be a 7S vicilin-like protein and suggested that different subunit types could be assembled to form the CP2 as reported for 7S vicilin-like proteins.

A similar investigation has been conducted by Paino *et al.* (1988) to determine variability in the major seed proteins in thirteen (13) *Vigna* species. Seventy (70) accessions of *Vigna* species were studied. From this analysis, the authors detected great variability in the molecular weight and the relative intensity of Seed Gobulin 1 (G1) fraction components among the different species since G1 profiles permitted the 13 analyzed to be

distinguished. Observations made from this investigation permitted accessions belonging to the subspecies *unguiculata, stenophylla*, and *dekindtiana* in *V. unguiculata* to be distinguished. The report did not make reference to any differences in the major seed proteins of the cultivars within subspecies unguiculata.

This notwithstanding, Ahedor (1993) employed SDS-PAGE to investigate the possibility of characterizing six local cowpea varieties. He reported that only one variant, *Bengpla,* lacked a common major seed protein band (molecular weight 70.79 kDa) which was present in the other five.

Another mode of classification which has been employed in the identification of cowpeas is the study of its chromosome morphology (or cytotaxonomy). Chromosomes are classified on the basis of their total length, arm ratio and the pattern of chromosome distribution. Also the presence of any nucleolar chromosome is considered (Thottapilly *et al*., 1992).

The chromosomal characterization of *V. unguiculata* using karyomorphology has been carried out by a number of workers. Mukherjee (1968) used both conventional and C-and-H techniques to study pachytene chromosomes, whereas Kurata and Omura (1978) and Pignone *et al*., (1990) used chromosomes in the mitotic prometaphase and metaphase respectively. Others like Saccardo and Barone (1990) have kayrotyped *V. unguiculata* using an automatic image analysis system. These workers agree that *V. unguiculata* is diploid with a chromosome number of 22. Barone and Saccardo (1990) identified 11 bivalents and classified them on the basis of the total length of the chromosome, the arm

ratio, and the pattern of chromomere distribution. With respect to size, they recognized one very long (85.5μm) and a very short one (14.1μm); the chromosomes of intermediate length were allocated to three classes, 51.5-45.8μm; 39.2-30.5μm; 26.0-22.0μm). These findings have been corroborated by Pignone *et al.*, (1990). In addition, the presence of a nucleolar chromosome not mentioned by earlier karyotyping work on *V. unguiculata* has been reported (Mukherjee, 1968).

Karyomorphology of *Vigna unguiculata* has been used to distinguish between *Vigna unguiculata* and *Vigna vexillata*. However, karyomorphology on other *V. unguiculata* varieties have not been carried but this could be useful in studying differences between species but not at the level of sub-species, variety or population.

In Ghana, simple morphology and agronomic features still form the bases for identification of cowpeas. Studies on cowpeas from station variety trials conducted at six locations throughout the country by Twumasi and Arias (1990) provide the criteria for identifying seven Ghanaian commercial varieties. These varieties are known locally as AMANTIN, ASONTEM, AYIYI, BENGPLA, EJURA RED, SORONKO AND VALLENGA. Of these seven, the Crops Research Institute of Ghana has, under the Ghana-CIDA Grain Project, identified and classified four as varieties of high commercial value. These are represented in Table 3.

Table 3 Characteristics of Four Commercial Cowpea Varieties

Variety	*Seed Colour*	*Maturity Time/Days*	*Maturity Group*	*Yield per Hectare*	*Yield (Bags per acre)*
Amantin	*Mottled Red*	*65-70*	*Early*	*1.4*	*6*
Asontem	*Light Red*	*60-65*	*Medium*	*1.2*	*5*
Soronko	*Light Red*	*60-65*	*Extreme*	*1.7*	*7*
Vallenga	*Brown*	*70-80*	*Extreme*	*1.7*	*7*

Four varieties of cowpea identified on the basis of agronomic and morphological features. This classification was done using data from station variety trials conducted by the Crops Research Institute of Ghana at 4-6 locations throughout Ghana (Tumasi-Afriyie & Arias, 1990).

To conclude, various techniques including biochemical, chromosomal karyotyping, morphological and agronomic criteria have been applied to study the taxonomy of cowpeas with partial success. However, molecular methods have not been applied and DNA being the genetic material itself, and also displaying varying degrees of polymorphism in the genome, makes it the ideal candidate for detecting differences at all levels of taxonomy.

1.5 DNA POLYMORPHISM IN THE EUKARYOTIC GENOME

1.5.1 Eukaryotic Genome Structure and Organization

The genome is defined as the genetic constitution of an organism and, in terms of nucleotides, it is the total number of coding and non-coding sequences of nucleotides. These sequences can either be nonrepetitive, moderately repetitive or highly repetitive. Nonrepetitive sequences are unique in that they occur once in the genome. They include most structural genes coding for polypeptides and other sequences of no known functions

scattered randomly within the genome. Moderately repetitive sequences are repeated a small number of times in the form of related but not identical copies. These sequences are grouped into families. The members of each family consist of a set of nucleotide sequences that are sufficiently similar to renature with another. The differences between the individual members are the result of base substitutions, insertions, and deletions, all creating points within the related sequences at which the complementary strands cannot pair. The members of these families are often interspersed in a more or less regular way with longer stretches of nonrepetitive DNA. Thus, they are usually regarded as introns found within interrupted genes. Highly repetitive sequences generally are short and usually repeated as a tandem array. These repeats vary in length from a few nucleotides to several thousands. Because of its short repeating unit, it is sometimes described as simple sequence DNA. The very short sequences that are repeated in highly repetitive DNA are referred to as microsatellites or simple sequence repeats (SSR). This type of DNA sequences unlike the moderately repetitive sequences are not dispersed within the genome but are concentrated in the constitutive heterochromatin domain and their role remain obscure.

Arrangement and Organization of Genes

A gene can be defined as a sequence(s) of nucleotides within the genome that code for a protein or a non-protein product. The whole gene sequence includes flanking sequences, regulatory sequences, promoter site and the transcription unit itself. Most structural genes are located in nonrepetitive DNA. In most of these genes, however, their coding sequences are interrupted by long intervening stretches of bases of unknown function

transforming them into alternating series of coding (exons) and noncoding (introns) units. Within these interrupted genes, the order of the parts is the same in the genome as in its mature mRNA product. Also the interrupted gene retains the same structure in all tissues, including germ lines and somatic tissues in which it is or is not expressed, making the presence of an intron an invariant feature; and introns have no coding function. In addition, between species, positions of introns are conserved in homologous genes though the sequences of introns may differ and may be unrelated altogether. The exon sequences may however remain well related and conserved.

Genetic Variation and Repetitive DNA Sequences

The nature of these two classes of repetitive sequences, the moderately repetitive and the highly repetitive sequences, of the genome make them the most variable regions of any eukaryotic genome. Moderately repetitive DNA found within genes have conserved positions but their sequences and their sizes in any two homologous genes in two species may vary or may not be related altogether. An example is the ribosomal RNA genes (rRNA). These are arranged in tandem arrays of units, with each unit consisting of 18S, 5.8S and 25S rRNA coding regions separated by internal transcribed spacers (ITS). These units in turn are separated by an intergenic spacer (IGS) extending from the 3' end of the 25S rRNA coding region to the 5' end of the 28S rRNA coding region. The IGS varies in size from a few kilobase pairs (kb) to more than 30 kb. This length heterogeneity is observed in both plants and animals, and can occur between and within species. Length heterogeneity within a species is generally due to variation in the number of copies of subrepeats (repetitive elements of approximately 100-400 bp) present within the IGS.

These regions show high levels allelic polymorphism among species and represent an attractive source of genetic polymorphisms. Similarly, microsatellites or simple sequence repeats (SSRs) that consist of tandemly repeated sequence motifs 1-6 bp in length are in abundance and randomly distributed throughout the genome of higher eukaryotes besides the euchromatin. Microsatellites vary in length and in the number of the repeat unit even at a particular locus and consequently, they also show high levels of allelic polymorphism. This variation can be detected and efficiently analyzed with the polymerase chain reaction by selecting as primers the conserved DNA sequences flanking the SSRs. They therefore provide an attractive source of genetic polymorphisms for both plants and animals (Weber and May, 1989). Therefore, variable regions of the genome can be targeted for systematics.

Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphisms (RFLPs) are genetic markers based on cloned fragments of DNA (Young, 1992). RFLP assays detect DNA polymorphisms through restriction endonuclease digestions coupled with DNA blot hybridizations. It relies on the principle that interfertile organisms tend to have similar DNA sequences, though there are still likely to be many changes in DNA sequence scattered throughout the genome (Young, 1992) and exploit these differences to describe the genotype of an individual. Thus, employing restriction endonucleases, these enzymes recognize and cut DNA at short, specific sequences of nucleotides. Restriction enzyme digestion leads to the production of a distinctive set of DNA fragments which define an organism's RFLPs genotype.

However, since higher plants and animals have large genomes, restriction enzyme digestion generates many smaller fragments. These short DNA sequences distributed throughout the genome are individually examined for their restriction fragment pattern. Each fragment is inserted into a vector and cloned, and labeled with radioactive nucleotides or with chromogenic substances. Simultaneously, DNA fragments of different sizes are separated as they migrate to different positions on a gel electrophoretic matrix. Through 'Southern blotting', a technique involving transferring digested DNA onto nylon membranes, immobilizing them and making them accessible to labeled DNA clones, these labeled DNA clones are to be used as probes to characterize corresponding restriction fragments from the plant's DNA. Fragments complementary to the labeled cloned sequence bind, and only those fragments become associated with the chromogenic substance or with radioactivity are visible after autoradiography. By this means, the fragments derived from the specific region of the genome complementary to a cloned DNA can be visualized, while all other portions of the genome remain invisible. Repeating this procedure with several different DNA clones leads to a set of RFLP genetic markers that define the genotype of an organism at several short segments throughout its genome.

This ability to detect changes in the locations of restriction sites within a cloned DNA segment provides the essential key for RFLP analysis. Such restriction sites changes are apparent when the restriction fragment patterns are compared. Dissimilar fragment patterns can then be treated as alleles at a locus and used for segregation analysis and genetic mapping (Young, 1992).

RFLPs have been used extensively in population genetics and in systematics because their level of allelic variation in natural plant populations is much greater than that for morphological markers (Helentjaris *et al.,* 1985). As a result, one can take advantage of natural variation in existing populations eliminating the need to construct special genetic stocks. RFLPs have therefore become useful tools in determining relationships among various taxa. They have been used successfully to study genomic relationships in *Arachis* (Kochert *et al.,* 1991), *Brassica* (Song *et al.,* 1988), *Lycopersicon* (Miller and Tanksley, 1990) and *Solanum* species (Debener *et al.,* 1990). Nuclear RFLPs, in particular, are powerful in elucidating phylogenetic relationships because they have the added advantage of being a virtually unlimited source of characters that can effectively discriminate between genotypes, subspecies and species (Debener *et al.*, 1990). Thus, on the basis of RFLP data subjected to phylogenetic analysis, it was possible for Song *et al.,* (1988) to assert that an ascending order of chromosome numbers played a major role in the evolution of *Brassica* and related species.

In crop improvement progammes, RFLP has gained immense popularity because unlike other genetic markers, such as isozymes, unlimited numbers of RFLPs potentially exist for every plant species (Young, 1992). Moreover, RFLPs are available in most crosses and scoring one RFLP does not interfere with the ability to score others. This means that large numbers of RFLPs can be analyzed in a single cross, which makes it possible to construct a unified linkage map composed of many genetic markers. Similarly, because RFLP analysis is based on purified DNA, plants grown in any environment can provide starting material for RFLP analysis without destroying the plant. Using RFLPs, high

density linkage maps can be constructed and used to locate genes of economic importance (Young, 1992).

Young (1992) has stated that RFLP is the technology of choice for many species. However, RFLP technology is expensive, in terms of time involved in identifying target sequences, labour and logistics. Restriction enzymes, by their nature, are rare and their detection can require the use of 20-30 different enzymes. Blotting and hybridization protocols are time consuming. In addition, in instances where the species under study shows low levels of diversity at the DNA sequence level, the construction of a RFLP-based genetic map has, in most cases, proved very difficult and tedious (Akkaya *et al.*, 1992).

Variable Number of Tandem Repeats (VNTRs)

VNTRs or minisatellites are multiallelic loci consisting of repeated core sequences which are widely distributed in the genome of all organisms. These repeat units which are in the range of 11 -60 bp in length, occur as tandem arrays flanked by conserved endonuclease restriction sites. The length of the restriction fragment produced by this type of genetic locus is proportional to the number of core units it contains. This characteristic makes VNTR DNA extremely polymorphic. Assaying for minisatellites has two main advantages: firstly, minisatellites are multiallelic, and as a result, they are considered to be more useful than standard, diallelic restriction fragment length polymorphisms (RFLP); and secondly, microsatellites are also amenable to PCR analysis. However, because the repeat unit can be up to 50 bp and the number of repeats large, the utility of

PCR as a way of analyzing VNTR length may be limited (Boerwinkle et al., 1989; Lynch, 1988)

Random Amplified Polymorphic DNA (RAPDs)

The prerequisite for DNA sequence information has been a limitation in the application of the PCR technology and this led to the development of the Random Amplified Polymorphic DNA (RAPD) assay. RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or of an insertion or deletion within the amplified region (Paran *et al.,* 1991). Polymorphisms are thus usually noted by the presence or absence of an amplification product from a single locus (Wilde *et al.,* 1992). Generally, RAPD assay detects nucleotide sequence in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. The primer binds to the DNA at two different sites on opposite strands of the DNA template and if these priming sites are within an amplifiable distance of each other, a discrete DNA product is synthesized through thermocyclic amplification. The presence of each amplification product identifies complete or partial nucleotide sequence homology between the target DNA and the oligonucleotide primer, at each end of the amplified product (Tingey *et al.,* 1994).

Many advantages have been observed in the use of the RAPD assay. Little knowledge of the biochemistry or molecular biology of the species is required. Also, RAPD markers are easy to develop and less expensive since no prior target DNA sequence information for the design of amplification primers is required. Moreover, because it is a PCR-based

assay, it is also simple and fast and can be applied to any species for which DNA can be prepared (Tingey *et al.*, 1994). But unlike conventional PCR, only single primers of arbitrary nucleotide sequence are required. In addition, each random (or arbitrary) primer on the average is capable of directing the amplification of DNA at several discrete loci in the genome. This property has made RAPD assay an efficient way to screen for nucleotide sequence polymorphism between individuals. Similarly, using single arbitrarily chosen primers, simple and reproducible fingerprints of complex genomes can be generated. The polymorphisms detected can be used as genetic markers, and can thus be used to construct genetic maps. RAPD markers can therefore be used for large-scale genetic mapping applications. However, RAPD markers are usually genetically dominant (Williams *et al.*, 1990) and the degree of polymorphism is low.

The RAPD assay has been used by several workers (Arnold *et al.*, 1991; Hu and Quiros, 1991; Welsch and McClelland, 1990,1991; Cregan *et al.*, 1994; Ballinger-Crabtree *et al.*, 1992, Caeteno-Anolles *et al.*, 1991; Hadrys *et al.*, 1992; Kreosovich *et al.*, 1992; Wilde *et al.*, 1992 & Williams *et al.*, 1990). Each exploited the efficiency of detecting DNA sequence-based polymorphisms in the RAPD assay. These include development of genetic maps, targeting genetic markers, pooling strategies and in population genetics. RAPD markers have now been used extensively to create DNA fingerprints for the study of individual identity and taxonomic relationships in eukaryotes and prokaryotes. Several groups have reported on the utility of DNA markers as sources of phylogenetic information. Arnold *et al.* (1991) were successful in using RAPD markers to test for interspecific nuclear gene between *Iris fulva* and *I. hexagona*, and to study the presumed hybrid origin of *I. nelsoni*. Hu and Quiros (1991) were able to show that the amplification

products from only 4 random primers were sufficient to discriminate between 14 different broccoli and 12 different cauliflower cultivars (*Brassica oleracea* L.). RAPD markers have also been used to assess the amount of genetic diversity in germplasm collections.

Simple Sequence Length Polymorphism or Microsatellites (SSLP)

Microsatellites or Simple Sequence Repeats (SSRs) can be defined as relatively short stretches of DNA that consist of tandemly repeated sequence motifs 1-6 bp in length (Morgante *et al.*, 1994). They exist in abundance and are randomly distributed throughout the genome of higher eukaryotes (Braaten *et al.*, 1988; Hamada *et al.*, 1982). Microsatellites vary in length and in the number of the repeat unit even at a particular locus and consequently, they show high levels of allelic polymorphism (Morgante *et al.*, 1994). This variation can be detected and efficiently analyzed with the polymerase chain reaction (Litt and Luty, 1989) by selecting as primers the conserved DNA sequences flanking the SSRs (Akkaya, 1992). They therefore provide an attractive source of genetic polymorphisms for both plants and animals (Weber and May, 1989).

SSRs have been employed in the construction of saturated genetic maps in humans, rats and for several other mammalian species (Dietrich *et al.*, 1992; Weissenbach *et al.*, 1992). In some cases, genetic linkage maps based solely on SSR variants have been constructed for several mammalian species (Saghai-Maroof *et al.*, 1994).

Different repeat motifs have been identified in both plants and animals (Morgante and Olivieri, 1993). Of these, dinucleotide repeats are the most abundant microsatellites in

both mammals (Litt and Luty, 1989) and plants (Morgante and Olivieri, 1993). The predominant type of dinucleotide repeat however vary between animals and plants (Morgante and Olivieri, 1993). The main difference that has been observed between plant and mammalian microsatellites, based on the sequences present in data bases, is that $(AT)_n$ appears to be the most frequently observed type of repeat in plants (Morgante and Olivieri, 1993) whereas $(GC)_n$ repeat unit predominates in mammals. In mammals, $(CA)_n$ repeats are reported to occur with a frequency of about 5-10 x 10^4 per genome in mammals (Stalings *et al.*, 1991).

SSRs have also been reported in several species of plants including soybean (Cregan 1992, Akkaya *et al.*, 1992), wheat (Devos *et al.*, 1995), grape (Thomas and Scott 1993), barley (Saghai-Maroof *et al.*, 1994), maize (Senior and Heun, 1993), rice (Wu and Tanksley 1993; Zhao and Kochert, 1993), sunflower (Brunel, 1994), avocado (Lavi *et al.*, 1995), *Arabidopsis thaliana* (Bell and Ecker, 1994) and *Brassica* (Lagercrantz *et al.*, 1993). Screening of DNA libraries for the presence of dinucleotide repeats revealed that there are about 5 x 10^3 to 3 x 10^5 $(AC)_n$ and $(AG)_n$ sites per genome in maize and in tropical tree species (Condit and Hubbell, 1991). It is estimated that $(GA)_n$ repeats occur on the average about once every 225 kb and that $(GT)_n$ repeats occur about once every 480 kb in the rice genome (Wu and Tanksley, 1993). These high estimates suggest that SSR variants may be useful for genomic mapping in plants and also in marker-directed plant breeding (Saghai-Maroof *et al.*, 1994). For example, Akkaya *et al.* (1992) reported microsatellite loci in soybean with as many as eight alleles. Jiang *et al.* (1994) have also reported microsatellites ranging from 11 to 26 alleles per locus with gene diversity values

ranging from 0.71 to 0.95. These values contrast to an average gene diversity of 0.30 for 132 RFLP probes reported by Keim *et al*. (1992).

However, one drawback to SSRs utilization has been the relative difficulty of their development compared with other classes of genetic markers but their relative abundance, the high information content and the ease of automating assays for SSRPs make them genetic markers of choice in mammalian genetics (Rafalski and Tingey, 1993a).

Furthermore, Wu and Tanksley (1993) have developed a technique 'Random Amplified Polymorphic DNA (RAMPs)' in order to compensate for the weaknesses of both SSR and RAPDs (i.e. to overcome the difficulty involved in the development of microsatellite primers and the problem associated with low degree of polymorphism among RAPD markers). The method is based on the random distribution of the nucleotide sequences immediately flanking the SSRs. This program was designed to switch between the high and low annealing temperature during PCR reaction. Since the melting temperatures of the anchored primers are usually 10-15^{0}C higher than those of the RAPD primers, in the PCR cycles with higher annealing temperature only the anchored primer should anneal efficiently, whereas at low annealing temperature cycles of both anchored microsatellite and RAPD primers should anneal. Thus, DNA sequences from microsatellites loci are preferentially amplified. This technique was used to detect many genetic markers in soy bean (Wu and Tanksley, 1993) The reference is for genetic mapping of rice" not soybean

1.6 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method for the enzymatic synthesis of nucleic acids by which a target DNA fragment is exponentially replicated (Saiki *et al.*, 1988 and Mullis *et al.*, 1986). It is a method of cloning DNA without the use of micro-organisms and has become a versatile technology. As a result, it is widely employed in many fields to amplify both double stranded and single-stranded DNA. It allows the rapid selection and amplification of segments of DNA and permits rapid analysis or characterization of sequences of interest. Moreover, since PCR is an amplification-based assay, extremely small amounts of starting biological material (nanogram quantities of DNA) are required. This property of PCR has been applied in the examination of nucleotide sequences from day-old samples to ancient preserved specimens which have been frozen, dried and hidden in anaerobic sediments, or soaked in alcohol or formalin (Paabo, 1989). It is being applied also in population biology and molecular systematic studies.

In general, PCR makes use of a pair of flanking sequences as oligonucleotide primers, one complementary to each of the strands. The oligonucleotide primers, oriented with their 3' ends pointing towards each other, hybridize to opposite strands of the target sequence so that the intervening DNA region is amplified by DNA polymerase. The reaction takes place in three basic steps: template denaturation, primer annealing, and new strand extension and it involves repeated thermal cycles of denaturing DNA at high temperature, reannealing in the presence of a large excess of the oligonucleotides (as primers) and enzymatic extension along the nucleic acid template in the presence

of excess of the four deoxyribonucleotides by a thermostable DNA polymerase (*Taq Polymerase)* isolated from *Thermus aquaticus.* Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies theoretically doubles at every cycle. Consequently, these reactions over a number of cycles lead to the selective amplification of specific DNA sequences.

In a typical standard PCR protocol, the first step involves raising the temperature rapidly to 92-96°C and holding it at this temperature for 15 seconds to 1 minute. This causes the double stranded template to dissociate. Usually, this first step is preceded by an initial denaturation for 2 minutes which improves the efficiency of the reaction. For the second step, the temperature is rapidly lowered to 50°C to enable the annealing of the oligonucleotide molecules. The excess of primers ensures that the former competes successfully with the double strands of the original template molecules for annealing to the DNA. The two priming oligonucleotides determine the specificity of the reaction. This specificity can also be controlled by varying the annealing and extension times and temperatures, the magnesium ion and enzyme concentrations in the reaction mix. Each set of oligonucleotide primers has its own optimal conditions and the length of the primers usually range from 10 and 23 bp. The base composition of the primer determines its annealing temperature. Generally, primers with a high GC content require higher annealing temperatures (Wilson, 1994). Under standard conditions, the annealing temperature in a reaction should be 5°C lower than its melting temperature (T_m). This is calculated using the following relation:

$$T_m = [4(G + C)] + 2(A + T)$$

Primer annealing temperature determines the stringency of the reaction, the lower the annealing temperature the less specific the annealing and higher the chances of mispriming. In the third phase, that is new strand extension, the temperature is raised rapidly to 70-75°C for the maximum efficiency of the *Taq Polymerase.* Similarly, the number of cycles necessary varies between 25 and 40 depending on the number of target molecules in the template DNA.

There are two main types of PCR, symmetric (double stranded) and asymmetric (Single stranded) PCR. In symmetric PCR, equimolar concentrations of the two primers, both forward and reverse, are added to generate double-stranded products. Assymmetric PCR uses unequal concentrations of the primers. One primer is usually added in much excess so that double stranded products are generated during the initial cycles of the reaction. As the reaction progresses, the primer in low concentration becomes depleted leaving the one in excess to synthesize single-stranded products. Assymmetric PCR is, however, considered inefficient because it requires higher concentrations of template than conventional reactions (Gyllensten and Erlich, 1988) and this multiplies the problems associated with mispriming of templates and the amplification of non-specific products. This type of PCR is usually employed in the preparation of templates for DNA sequencing.

Genetic assays based on the PCR are amenable to automation than conventional techniques. They are also fast and simple to perform, highly specific, reliable and are preferable in experiments where the genotype of a large number of individuals is to be

determined at a few genetic loci. A drawback to its versatility is the prerequisite for DNA sequence information. This former limitation has been overcome with the development of RAPDs technology.

The sizes of PCR products are estimated by comparison with the mobility of standards of known molecular sizes (or weights) on agarose gel. In principle, the mobility of a DNA molecule is related to its size. The larger the molecule the lower its rate of migration. The rate of migration of DNA molecules on agarose gels are inversely proportional to the logarithms of the molecular weights and this is expressed as below:

$$D = a - b(\log M)$$

where D is the distance moved by the DNA molecule on the gel: M is its molecular weight and a and b are constants. In practice, the sizes of the DNA molecules were estimated by interpolation from a plot of the logarithm of molecular weight against distance on gel.

The combination of PCR and restriction enzyme analysis of the PCR product provides an alternative and cost effective approach in studying variation among species and varieties. Amplified DNA sequences (PCR products) are subjected to restriction enzyme digestion in order to detect restriction site differences of the amplified DNA sequences of the varieties within cowpea. An advantage of using amplified sequences is that these are small (in size) as such, their fragments are more likely to be separated and visualized as individual bands on gels unlike the digested bands of their parent genomic DNA which when fractionated are observed as smears on gels.

1.7 Objectives of the Project

With the elucidation of the molecular basis of inheritance, biological macromolecules have assumed an increasingly important role in evolutionary studies. Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and variation in their quality and quantity provide a set of heritable markers that can be used to study the genetic structure of populations or to estimate relationships among taxa. Such molecular studies have generated massive comparative data bases which are being used in molecular systematics (Hillis & Moritz, 1990).

Major advances in the manipulation and analysis of nucleic acids have intensified the study of DNA and RNA variation. Among these, techniques such as hybridization and dissociation of DNA, use of restriction endonucleases to assay DNA sequences from the nucleus, the mitochondrion, and the chloroplast, and comparison of primary sequences. have revealed various degrees of polymorphisms. Polymorphism is the result of base substitutions, deletions, and rearrangements in the DNA among species and subspecies.

These DNA markers are sequences of DNA that can be genetically mapped through traditional linkage analysis. They (as DNA-based diagnostics) exploit the differences in sequences of DNA scattered throughout genomes to define the genotype of each individual. DNA markers are numerous, selectively neutral, and (frequently) co-dominantly expressed. They provide similar information about change in DNA sequence (as polymorphisms). As a technology, they are now well established as a means to assay

diversity at the locus, chromosome and the whole genome (Tingey *et al.*, 1994). Moreover, with the aid of the polymerase chain reaction (PCR), these analyses have been accelerated generating in the process, immense molecular information which have refined classification especially at the subspecies and varietal levels of some plants. Notable among these markers are restriction fragment length polymorphism (Young, 1992), random amplified polymorphic DNA (Tingey *et al.*, 1994), microsatellite or simple sequence repeat polymorphism (Cregan *et al.*, 1994), and variable number of tandem repeats (Nakamura *et al.*, 1987; Jeffreys *et al.*, 1985 and 1988). This has led to the development of a number of DNA diagnostic assays and markers which have allowed an almost limitless supply of genetic loci for identification and mapping.

The principal goal of this project was to analyze the genomic DNA of the six cowpea varieties found in Ghana for variation and to exploit the variation found for their identification.

It was clear from the literature that there was no information on the best means of storage of plant material for molecular work. Most molecular researchers beside forensic scientists prefer fresh samples. Storage in liquid nitrogen is a common practice in many plant molecular biology laboratories. However, the scarcity and more importantly the high cost of this chemical in Ghana has resulted in the need to identify simple but readily available means of storing/preserving plant leaves for further molecular work. Thus, the first objective of this study was to find the best possible non-chemical means of storing plant leaves for molecular studies.

It was also evident from the literature that classification of cowpeas at the intraspecies level is ambiguous and also no simple criteria of morphological characters can be used in the traditional system of classification to identify all the six varieties. It was also observed that protein characterization has not provided enough comparative data base for identification purposes, and the use of karyomorphology is also limited. Thus, the second objective of this work was to use available molecular methods and techniques to provide some comparative data base for identification of the six varieties.

In summary, the specific objectives of this project are twofold:

1. to determine the best non-chemical means of preserving cowpea leaves for further molecular work in the laboratory in the absence of liquid nitrogen; and then
2. to develop molecular biology techniques such as PCR, RAPD, PCR-RFLP and SSCP that can be used to detect DNA polymorphism for the identification of six varieties of cowpea found in Ghana namely; Amantin, Asontem, Ayiyi, Bengpla, Ejura Red, and Soronko.

CHAPTER TWO

MATERIALS AND GENERAL MOLECULAR METHODS

2.0 INTRODUCTION

This chapter provides the materials and general molecular methods used in the present study. Details of modifications in each method would be found under the respective sections.

2.1 BIOLOGICAL MATERIALS

Homozygous seeds of six varieties of Cowpea, *Vigna unguiculata* (L.) *Walp.*, Amantin, Asontem, Ayiyi, Bengpla, Ejura Red and Soronko were obtained from the Department of Crop Science, University of Ghana. These were planted on experimental beds in the University of Ghana Agricultural Farm in Legon and the leaves harvested after two weeks.

2.2 GENERAL REAGENTS AND SOLUTIONS

Reagents

The details of reagents used, with names and addresses of suppliers are listed as appendix C.

Enzymes

The following enzymes were used in the present work: Ribonuclease A, Taq DNA Polymerase (5U/µl). The Taq polymerase was supplied as a set with its accompanying buffer, 50 mM $MgCl_2$ and 1.0% (w/v) W-1 detergent. The restriction enzymes *Alu*I, *Da*I, *Eco*RI, *Eco*RV, *Hae*III, *Hinf*I, *Kpn*I, and *Pvu*I and their accompanying buffers. The names and addresses of suppliers are listed as appendix C.

Deoxyribonucleotide triphosphates (dNTPs)

The four deoxyribonucleotides namely; deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) as well as Bacteriophage Lambda (λ) DNA were employed in this study.

2.2.1 DNA Molecular Weight Markers

The following DNA molecular weight size markers were used at various stages of the present study:

100 bp DNA ladder

This was obtained from GIBCOBRL (Life Technologies) and had the following sizes: 100 bp, 200 bp, 300 bp, 400 bp, 500 bp,600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1100 bp, 1200 bp, 1300 bp, 1400 bp, 1500 bp, 1600 bp, 2002 bp

λ/HaeIII molecular weight marker

Digestion of λ DNA with *Hae*III generates fragments of thefollowing lengths: 130 bp, 232 bp, 288 bp, 316 bp, 331 bp, 400 bp, 459 bp, 489 bp, 600 bp, 721 bp, 829 bp, 1243 bp, 1619 bp, 2070 bp.

2.2.2 Standard Solutions

Details of all solutions prepared can be found in Appendix D.

2.3 THE EXTRACTION OF GENOMIC DNA FROM LEAVES OF COWPEA VARIETIES

Fresh leaves obtained from young plants were first washed in copious volumes of tap water and disinfected for 30 minutes in a 15% chlorex solution to take care of contaminating bacteria and fungi. They were subsequently washed in copious volumes of sterile double distilled water, drip dried and weighed to obtain their fresh weight.

2.3.1 The Isolation of Genomic DNA from Cowpea Leaves

The method used throughout this study was a modification of the protocol used by Hoelzel.

(1992). Each sample was completely homogenized in a pre-chilled sterile mortar. The paste was transferred into a 40-ml centrifuge tube, and both the mortar and pestle were then washed with 10 ml of the extraction buffer and added to the content in the centrifuge tube. The content was mixed thoroughly and incubated at 65°C for 30 minutes in an Eyela Water Bath while shaking constantly. The tubes were then centrifuged at 20 000 g for 10 minutes at temperatures ranging between 0°C and 4°C using a Tomy Refrigerated centrifuge. The supernatant was transferred into a fresh 40-ml centrifuge tube and1/3 volume of 8M KAc (pH 4.8) added. The contents were gently mixed and left to stand on ice for 5 to 10 minutes. It was then centrifuged again at 20 000 g for 20 minutes but this time the supernatant was transferred into a clean 30-ml centrifuge tube to which was added 0.6 volumes of isopropanol. The contents were gently stirred to mix and kept at -20°C for 6 hours and then centrifuged at 20 000 g for 20 minutes to precipitate nucleic acids. The supernatant was discarded and the pellet left to dry at room temperature. To the pellet was added 2 ml of Tris- EDTA (TE), buffer and the tube then kept on ice until dissolved. When dissolution was complete the tube was then centrifuged at 20000 g for five minutes to pellet any buffer insoluble debris that might be present in the extract. The supernatant was transferred into a fresh 4-ml polypropylene centrifuge tube and 200 µl of 3M sodium acetate (NaAc, pH 7.6) and 0.6 volumes of isopropanol added. The contents were mixed gently and frozen at - 20°C for one hour and centrifuged again at 12 000 g for twenty minutes. The pellet was washed with 500 µl 70% EtOH for 10 minutes and centrifuged at 12 000 g. The supernatant was discarded and the tubes left to dry at room temperature. To each tube 480 µl of TE buffer and 20 µl of Ribonuclease A (10 mg/ml) were added and the tubes left to stand on ice to dissolve the pellet. The contents were then transferred into 1.5-ml eppendorf tubes and stored at -20°C until ready for use.

2.3.2 Purification of Nucleic Acids

The Phenol method of extraction of DNA was used in another round to purify the isolated genomic DNA. An equal volume of phenol was added to the DNA solution in a 1.5 ml eppendorf tube. It was vortexed briefly and centrifuged at 10 000 g for 5 minutes. The upper aqueous phase was transferred into a fresh eppendorf tube and two volumes of absolute ethanol added. The contents of the tube were mixed gently and kept at -20°C for thirty minutes and then spun at 10 000 g for 5 minutes. The supernatant was discarded and the pellet left to dry at room temperature. Two hundred microlitres (200 µl) of TE buffer was then added.

2.3.3 Determination of the Concentration and Purity of Extracted DNA

For each sample, 50-µl of the extracted DNA was taken and diluted 200 fold for the spectrophotometric analysis. The purity of DNA in the original samples were estimated as below

Ratio of DNA:Protein, Purity (protein) = $(D_{OD260} - E_{OD300}) \times P_{OD280}^{-1}$

where:

D_{OD260}- Absorbance measured at 260 nm to estimate the quantity of nucleic acids present in the extract.

E_{OD300}- Absorbance measured at 300 nm to estimate the quantity of proteins present in the extract which also absorb at 260 nm.

P_{OD280}- Absorbance measured at 280 nm to estimate the quantity of proteins present in the extract.

The concentration of DNA in each sample was estimated from the relation that an OD of 1 at 260 nm corresponds to approximately 50 µg/µl of double- stranded DNA (Maniatis *et al.*, 1982). This is represented below:

DNA yield (µg/ml) = $50\mu g(D_{OD260} - E_{OD300}) \times A \times B^{-1}$

where:

A: Total volume (µl) of sample prepared, 500 µl

B: Aliquot (µl) of sample taken, 50 µl

2.4 POLYMERASE CHAIN REACTION (PCR)

A typical PCR reaction mix for a 100 µl reaction contains the following: 1X PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 100 µg/ml gelatin), 1.5 mM $MgCl_2$; 200 µM each of Deoxyribonucleotides (dATP, dCTP, dGTP & dTTP), 2.5 Units *Taq* DNA Polymerase, 25 µM Primer; 20 ng sample DNA Template.

For the present study the reaction volume of 25 µl was used and the amplification reaction was carried out in 0.5 ml Eppendorf tubes. The contents were thoroughly mixed and overlaid with 25 µl of mineral oil . The mineral oil was added to avoid evaporation and refluxing of the reaction mix during thermocycling.

A typical PCR reaction is carried out as follows: An initial melt at 94°C for 2 minutes followed by 30 cycles of 94°C for 20 seconds denaturation step, 55°C for 20 seconds annealing step and 72°C for 30 seconds extension step. Each amplification reaction was concluded with a final extension step of 72°C for 5 minutes to ensure the completion of all synthesized DNA strands. The details of modifications to this protocol can be found in the chapter four.

2.5 ANALYSIS OF AMPLIFIED DNA SEQUENCE

2.5.1 Estimation of Sizes of PCR products

The sizes of PCR products were estimated by comparison with the mobility of standards of known molecular sizes (or weights) on agarose gel. The sizes of the marker used are listed in section 4.3.3.1.

2.5.2 Restriction Enzyme Digestion

Restriction enzyme digestions of the PCR products were carried out using the incubation buffers recommended by the various manufacturers. All incubations were conducted in water baths at temperatures also recommended by manufacturers. To avoid partial digestions, excess enzymes were used and the reactions were carried out for a minimum of six hours. A final volume of 20-µl reaction volumes were carried out in individual 0.5-ml Eppendorf tubes. The digestion reaction mix in volumes of 20 µl each was prepared using 2 µl 10x enzyme reaction buffer, 1 µl PCR Product (as sample DNA), and about 5U of restriction enzyme (volume depends on initial enzyme concentration). The reaction volume was made up to 20 µl with sterile double distilled water (sddw). In the case of *Eco*RI, an equal volume of Bovine Serum Albumin (BSA) and the enzyme were added to make up the final volume of 20 µl.

2.5.3 Restriction Fragment Length Polymorphism sudies

Restriction enzymes were used in attempts to detect site differences of PCR amplified DNA sequences of the cowpea varieties. The digestions were carried out as described in section 2.5.2 and the products analyzed by electrophoresis.

2.5.3.1 Agarose Gel Electrophoresis

Electrophoresis of the PCR and its digested products were carried out through 1-2% agarose gel containing 0.5 µg/ml ethidium bromide and ran in 1x Tris-Acetate-EDTA (TAE) buffer using the Minigel system (Biorad). Horizontal gels were used in this study.

Five to fifteen microlitres (5-15 µl) of the PCR products were mixed with 1-2 µl of the gel loading buffer orange G (5X) and loaded into wells on the gel. The gels were usually ran at 80V for two hours in the Biorad Minigel Submarine electrophoretic kit and were visualized and photographed over a UV transilluminator (UPC, USA), at short wavelength using a Polaroid camera fitted with an orange filter and a Polaroid type 667 film.

2.5.3.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In this study, discontinuous phase SDS-PAGE was the method of choice for the separation of DNA fragments generated by digesting the GA-primed PCR product (1200 bp in size) with the nine restriction enzymes. This technique was used also to compare the PCR products of GA and AR primers in all the six varieties. It was also employed to separate denatured DNA generated in the single stranded conformation polymorphism (SSCP) studies.

A discontinuous slab gel of 5% (stacking gel) and 12.5% (resolving gel) were made and electrophoresis performed using the Mini-Protean Dual Slab Cell System (Biorad). The glass plates sandwiches and the equipment were assembled and operated in accordance with protocols supplied by the manufacturer. 0.75 mm spacers and combs were used in all cases.

Twenty mililitres of resolving gel was prepared as follows: 2.50 ml of 0.5 M Tris-HCl, pH 8.8, 10 % (w/v) SDS, 8.335 ml of 12.5% running gel solution, 20 mg APS and 13.34 µl TEMED were each measured into a beaker and ddw added to make a final volume of 20 ml.

The stacking gel was prepared by adding 3.35 ml ddw to 2.50 ml of 0.375M Tris-HCl, pH 8.8, 100 µl of 10% (w/v) SDS, 2 ml of 12.5% running gel solution, 5 µl TEMED and 5 mg ammonium persulphate (APS).

The gel was pre-run at 120V in 1x TBE for about 30 minutes. 10 µl of samples in 1x orange gel loading buffer were mixed, loaded and the gel run at 120V for about 2 hours. The system was then disassembled, the gel was removed and prepared for silver staining. The gel was then placed on a visible light box and photographed.

2.5.3.3 Silver Staining

The protocol below was followed in cases where silver staining was applied in the detection of DNA on SDS polyacrylamide gels.

The polyacrylamide gel was fixed in the fixative (10% Ethanol:0.5%Acetic acid) for 30 minutes. The fixative was then poured off and the staining solution of 11 mM $AgNO_3$ poured over it and left for another 30 minutes. The staining solution was drained and the gel washed thoroughly with tap water. It was then developed in a 0.75 M NaOH solution containing 0.8% (v/v) formalin. When the bands were conspicuous the reaction was quenched by the addition of a solution of 10% glacial acetic acid. The resulting solution was subsequently drained off and replaced with tap water.

2.6 Nonisotopic Discontinuous Phase Single Strand Conformation Polymorphism (DP-SSCP)

This technique was employed with the view of detecting slight differences that might be present in the PCR products of the six cowpea varieties owing to the failure to observe such minute migratory differences on the SDS-PAGE gel.

2.6.1 Re-extraction of RAPD PCR products from Agarose Gel

PCR product bands (about 369 and 545 bp respectively) generated using primer *AR* were sliced into two different 1.5-ml eppendorf tubes. They were frozen for one hour at -40°C and spun subsequently at 12 000 g for 10 minutes. The supernatants were transferred into fresh eppendorf tubes and one-third volume of KAc (8 M) was added. Two volumes of ethanol was then added to the resulting solution. They were gently mixed and kept at -20°C for 2 hours. The tubes were then centrifuged at 12 000 g for 10 minutes. The supernatant

were discarded and the tubes were vacuum-dried under running water to get rid of any remaining ethanol. The pellet was then re-dissolved in 5 µl sddw and stored at -20°C until needed.

2.6.2 Re-PCR of Re-extracted PCR products from Agarose gels

Three microlitres (3 µl) of each re-extracted PCR product was used as template DNA for reamplification of the product. The Reaction was carried out under PCR thermocycling conditions as stated previously. The reaction mixture of 25 µl contained 12.3 µl sddw, 1x PCR Buffer, 2 mM $MgCl_2$, 0.4% w-1(detergent), 25 µM each of dATP, dCTP, dGTP, dTTP, 1U Taq DNA Polymerase, 800 µM Primer with 3.0 µl template DNA.

Following PCR, 8 µl of each product was electrophoresed on an ethidium bromide stained (1%) agarose gel. The profile of the bands on the gel were visualized with the aid of the UV transilluminator and the gels were photographed.

2.6.3 DP SSCP Analysis of reamplified PCR Products

Ten microlitre (10 µl) of each re-amplified PCR product was used for DP SSCP analysis. The denaturation mix in 13.0 µl volumes contained 0.7 µl ddw, 10.0 µl PCR Product, 1.3 µl Denaturant (500 mM NaOH-20 mM EDTA), and 1.0 µl Gel Loading Buffer (0.5 % Bromophenol in formamide).

The reagents were mixed well, vortexed and then span down. The resulting mix was denatured by boiling in water for ten minutes and snap cooled on ice. All the mix was then loaded and electrophoresed on 4-12% gradient SDS polyacrylamide gel at 120V for 5.5 hours. The gel was developed using silver staining and photographed.

The 12% (w/v) polyacrylamide:bisacrylamide (29:1 ratio) lower resolving gel (in a total volume of 10 ml) comprised the following: 3.05 ml ddw, 1.25 ml of 0.375 M Tris-HCl (pH 6.8), 50 µl 10% (w/v) SDS, 625µl 0f 30% Acrylamide: Bisacrylamide, 25 mg Ammonium persulphate (APS), 5 µl Tetramethylethylenedi-ammie (TEMED).

The 4% (w/v) Polyacrylamide:bisacrylamide upper stacking gel (in a total volume of 5 ml) comprised the following: 3.35 ml distilled water, 2.50 ml 0.375M Tris-HCl (pH 8.8), 100 µl 10% SDS, 2.00 ml 30% acrylamide: Bis-acrylamide, 25 mg Ammonium persulphate (APS), 5 µl Tetramethyl-ethylenediammie (TEMED). The solution was allowed to polymerize for 30 minutes at room temperature and gel pre-run for 30 minutes at 120V.

CHAPTER THREE

STUDIES ON PRESERVATION METHODS OF PLANT LEAVES FOR MOLECULAR ANALYSIS

3.1 INTRODUCTION

One of the most important considerations with regards to molecular studies of plants is the method of collection and preservation of samples. Although DNA has been successfully isolated from herbarium sheets and fossils (Doyle and Dickson, 1987; Golenberg *et al.,* 1990), the yield and quality of DNA can be greatly affected by the condition of the original tissue and means of preserving it prior to extraction (Pyle and Adams, 1989). This is particularly true of tissues that contain large quantities of tannins, phenolics or other secondary metabolites that interfere with successful extraction of DNA (Hoelzel *et al.,* 1992). To overcome this problem, many plant molecular biologists recommend extracting plant DNA from freshly harvested material. It is generally acknowledged that a relatively good yield and high quality of plant DNA is obtained from new growth early in the season (Hoelzel *et al.,* 1992). Temporary conditions of storage including cool and moist conditions for freshly harvested material has been suggested (Hoelzel *et al.,* 1992). For long term preservation, two methods, either non-chemical or chemical can be used. The non-chemical methods include freezing, refrigeration and drying. The most applied chemical method is rapid drying of leaves in individual bottles containing anhydrous $CaSO_4$ (Liston *et al.,* 1990). Ethanol, methanol, propanol, sulphuric acid, Carnoy's solution (acetic acid-absolute ethanol, 1:3), formal saline and liquid nitrogen (-70°C) have also been used in the preservation of biological material (Post *et*

al., 1993). Chemical preservation of plant material however generally results in highly degraded DNA (Pyle and Adams, 1989).

In order to identify the most appropriate preservation methods for biological samples for molecular work, a number of studies have been conducted to assess the effects of different storage conditions on DNA preservation. Similar studies have been carried out on vertebrate tissues (Goelz *et al.*, 1985; Arctander, 1988; and Bramwell and Burns 1988), and on insects (Post *et al.*, 1993). These studies, in general, have been rather selective in the methods of preservation examined and this has made it difficult to draw general conclusions regarding the reliability of the different preservation procedures. However, no firm conclusions can be drawn from these studies as to which of the different methods of preservation, and the duration of storage prior to extraction give the maximum yield and significantly affect the yield and the quality of the isolated DNA from plants. This information is important because special consideration needs to be given to the preservation and subsequent maintenance of samples which will be used for subsequent DNA studies. In particular, establishing the conditions that optimize conservation of the primary structure of DNA is critical, since it is the comparison of nucleotide sequence data that constitutes a major part of modern molecular systematics. Most molecular studies involve cloning of DNA sequences of genes and this requires a good yield of genomic DNA which is not sheared into very small fragments (Post *et al.*, 1993).

In reviewing the literature on the methods of preserving biological samples it was not clear which method is ideal for the preservation of leaves for further molecular studies. Since the main aim of the present study was to seek differences at the DNA level between different varieties of cowpea, it was important that good quality DNA was isolated for analysis. Therefore, for the present study, the effect of three non-chemical preservation methods on the quality and yield of the genomic DNA extracted from cowpea leaves were assessed. These were preservations at room temperature or on benchtop (between 25-30°C), simulated herbarium conditions and deep freeze (-20°C). These methods of preservation were chosen because facilities are readily available and the methods are also economical. Similarly, consideration was given to the high cost and irregular supply of chemicals such as liquid nitrogen and ethanol used in preserving plant samples.

3.2 Objective

The objective of the present study was to determine the effect of three non-chemical means of storage of cowpea leaves for molecular studies.

Under each storage condition, the duration of storage prior to DNA extraction, the age of the leaves (that is leaf type), and probable differences due to variety were assessed. For the first part of the study four varieties of cowpea, *Vigna unguiculata* (L.) Walp., Amantin, Asontem, Ejura Red and Soronko were used. The varieties Bengpla and Ayiyi were used for the second part and Ejura Red and Soronko for the third. The study was conducted over a period of seven weeks beginning from the day of harvesting.

3.3 MATERIALS AND METHODS

3.3.1 Biological Material

Cowpea leaves were harvested two weeks after sowing from their respective varieties on experimental beds in the University of Ghana Agricultural Farm in Legon. For each variety, two types of leaves, that is flush and mature, were harvested separately. Young leaves included both flush and two-day old leaves; and much older leaves (mature) included all the other leaves. The leaves of Amantin and Asontem were kept at room temperature; leaves of Ejura Red and Soronko were divided into two: one set was kept at room temperature and the other set was stored in the deep freezer. Mature leaves of Ayiyi and Bengpla were placed between plain sheets of paper in a folder to simulate herbarium conditions.

All the samples were first washed in copious amounts of tap water and then disinfected in a 15% (v/v) chlorex solution for 30 minutes to rid them of bacteria and viruses. Thereafter, the leaves were washed again four times in copious amounts of sddw and drip dried. All the samples were weighed initially to obtain their fresh weights (see appendix A).

3.3.2 Isolation of Genomic DNA

The method used to isolate DNA has been described in section 2.3.2. Each sample was treated in duplicate.

3.4 RESULTS

3.4.1 Estimation of DNA Purity and Concentration

The purity and yield of DNA of the individual samples were estimated using the procedures described under sections 2.3.2 and 2.3.3 respectively. The methods used for these estimations have been reported in tables 4 to 12.

3.4.2 Analysis of Results

DNA was extracted from the sampled leaves on days 1, 7, 21, 35 and 49. The composite sample, in this instance, was described by the total number of indices of all the varieties stored under a specified condition at a given time. The mean yield[1] and mean purity[2] of the composite sample were designated as mean yield and mean purity respectively to differentiate it from the means of the yield and purity of individual varieties. The results were analyzed statistically using the SPSS computer software. The procedure of Simple ANOVA was employed for this purpose and all analyses were reported at a significant level of 95% ($p \leq 0.05$) , and correlation coefficients as Pearson coefficients (r).

3.4.3 The Effect of Storage at Room Temperature on DNA Concentration and Purity

The leaves of four varieties of cowpea, Amantin, Asontem, Ejura Red and Soronko, were used for this study. The results are presented in Tables 4 to 7. The results of the effect of storage on the mean DNA yield and mean purity obtained from flush leaves are shown in Tables 4 and 6 respectively, whilst the respective mean DNA yield and mean purity obtained from mature leaves are presented in Tables 5 and 7.

Table 4: The Effect of Storage at Room Temperature on DNA Concentration of Flush Leaves

	Mean DNA Yield (mg/g)				Composite
Time (Days)	Amantin	Asontem	Ejura Red	Soronko	Mean Yield[1]
1	5.334	6.187	1.909	1.711	3.785
7	1.564	2.899	0.693	0.710	1.467
21	1.970	0.822	0.593	0.640	1.006
35	0.745	2.149	0.706	1.724	1.331
49	0.691	0.865	0.638	0.986	0.795

It was observed from Table 4 that generally the DNA yield from flush leaves decreased with time. The highest DNA yield obtained for all the different varieties were on day 1 (fresh samples) and these values were found to be significantly different from yields obtained from the 7-day and 49 day samples. However, between day 7 and day 49 samples no significant differences was found in DNA yields for all varieties.

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Table 5: The Effect of Storage at Room Temperature on DNA Concentration of Mature Leaves

	Mean DNA Yield (mg/g)				Composite
Time (Days)	Amantin	Asontem	Ejura Red	Soronko	Mean Yield[1]
1	0.365	2.089	2.149	2.214	1.704
7	0.653	2.783	0.735	0.855	1.256
21	0.515	0.754	0.695	0.680	0.661
35	0.728	0.761	0.862	0.755	0.777
49	0.264	0.275	0.422	0.484	0.361

The results obtained revealed that, just as in the case of flush leaves, the DNA yield from mature leaves decreased with time for the composite sample. Significant difference has been found between DNA yield over time. The highest DNA yields were obtained from day 1 samples in all but the Amantin variety. However, the pattern between the mean yield and time was the same as that observed with the flush leaves (see Table 4).

Analysis of the data revealed that between any two varieties differences in variety significantly affected the mean yield at a given time between Amantin and Asontem, Amantin and Ejura Red, and Amantin and Soronko but not between Asontem and Ejura Red, Asontem and Soronko, and Ejura and Soronko when the mean yields of these varieties were compared.

Between leaf types, more DNA was obtained from flush leaves than from mature leaves but the difference was significant in Amantin and in the composite sample only ($p \leq 0.05$).

Table 6: The Effect of Storage at Room Temperature on DNA Purity of Flush Leaves

	Mean Purity of Extract				Composite
Time (Days)	Amantin	Asontem	Ejura Red	Soronko	Mean Purity[2]
1	1.965	1.465	1.975	1.900	1.826
7	1.575	1.495	1.390	1.300	1.440
21	1.555	1.225	1.255	1.240	1.319
35	1.310	1.695	0.835	0.820	1.165
49	1.310	1.065	1.375	1.460	1.303

The results revealed that for flush leaves, the purity of the DNA extract generally decreased with time in the composite sample, Amantin and in Asontem varieties. However, no clear pattern was observed in Ejura Red and Soronko varieties (see Table 6). The highest DNA purities obtained for all varieties (except Asontem) and the composite sample were with day 1 samples. The purity of the extracts was significantly reduced by the 7th day after harvesting. However, no significant differences were found in the DNA purities of extracts obtained from day 7, 21, 35, and 49.

Table 7: The Effect of Storage at Room Temperature on DNA Purity of Mature Leaves

	Mean Purity				Composite
Time (Days)	Amantin	Asontem	Ejura Red	Soronko	Mean Purity[2]
1	1.740	1.630	1.860	1.695	1.731
7	1.730	0.970	1.000	1.150	1.213
21	1.215	0.810	0.830	0.990	0.961
35	1.390	1.255	1.295	1.555	1.374
49	1.135	1.215	1.410	1.105	1.216

Table 7 revealed that the highest mean purity obtained for extracts from mature leaves in each variety was on day 1. However, no clear pattern could be observed between the variation of mean purity with time in the composite sample or in any of the varieties. On the other hand, ANOVA analysis revealed that the duration of storage significantly affected the purity of the DNA extract in the composite sample ($p \leq 0.05$), Amantin ($p \leq 0.05$), and Asontem and Ejura Red ($p \leq 0.05$).

Analysis of the data revealed that between any two varieties, differences in varieties did not significantly affect the purity of extracts during the period of the study at any given time ($p \leq 0.05$) However, between any pair of leaf types, DNA extracts from flush leaves were relatively purer than those obtained from their corresponding mature leaves at any given time though this difference was insignificant ($p \leq 0.05$).

3.4.4 The Effect of Cold Storage at -20°C on DNA Concentration and Purity of Flush Leaves

Reresults obtained on the effect of storage at -20°C on DNA concentration and purity from flush leaves are presented in Table 8 and that of mature leaves in Table 9.

Table 8: The Effect of Storage at -20°C on DNA Concentration and Purity of Flush Leaves

	Mean DNA Yield (mg/g)			Mean Purity of Extract		
Time (Days)	Ejura Red	Soronko	Composite Mean Yield[1]	Ejura Red	Soronko	Composite Mean Purity[2]
1	1.909	1.711	1.810	1.975	1.900	1.938
7	2.130	2.035	2.083	1.895	1.485	1.690
21	3.460	2.930	3.210	1.735	1.735	1.735
35	2.294	2.406	2.350	1.705	1.275	1.490
49	2.448	2.271	2.399	1.828	1.599	1.714

The DNA yields of the extracts from flush leaves remained fairly constant over the period of the study (Table 8). The differences in the DNA yields of the different samples were found to be insignificant. Furthermore, Pearson's correlation revealed no significant correlation between mean yields and time, and between mean purities and time of samples stored at -20°C.

Table 9: The Effect of Storage at -20°C on DNA Concentration and Purity of Mature Leaves

	Mean DNA Yield (mg/g)			Mean Purity of Extract		
Time (Days)	Ejura Red	Soronko	Mean Yield[1]	Ejura Red	Soronko	Mean Purity[2]
1	2.149	2.214	2.182	1.860	1.695	1.778
7	1.200	1.270	1.235	1.890	1.575	1.733
21	2.080	4.216	3.374	1.680	1.740	1.710
35	2.532	0.362	1.675	1.550	0.795	1.550
49	1.990	2.062	2.026	1.745	1.670	1.708

As observed with flush leaf samples the DNA yield and purity remained fairly constant over the period of 49 days of storage at -20°C (Table 9). However, ANOVA analysis indicated duration of storage of the leaf samples significantly accounted for the differences observed in the mean DNA yields of extracts from the composite sample and from Soronko but not from Ejura red. The duration of storage also accounted for the significant differences observed between purities in the composite sample but in none of the two varieties. Differences in leaf type were found to be insignificant.

3.4.5 The Effect of Storage at simulated Herbarium Conditions on DNA Concentration and Purity

Only mature leaves of two varieties, Ayiyi and Bengpla, were used for this part of the study.

The results obtained are presented in Table 10.

Table 10: The Effect of Storage at simulated Herbarium Conditions on DNA Concentration and Purity of Mature Leaves

	Mean DNA Yield (mg/g)			Mean Purity of Extract		
Time (Days)	Ayiyi	Bengpla	Mean Yield[1]	Ayiyi	Bengpla	Mean Purity[2]
1	6.216	5.706	5.959	1.985	1.980	1.983
7	1.983	2.909	2.191	1.500	1.660	1.580
21	2.736	1.899	2.333	1.500	1.415	1.506
35	1.360	1.420	1.460	1.440	1.572	1.480
49	1.675	1.568	1.372	1.225	1.280	1.253

In both varieties, the highest mean yields of DNA extracted and their purities were obtained from day 1 samples, that is on the day of harvesting. It was generally observed that the yield of DNA and purity decreased with time (Table 10). Individual varieties also showed a progressive decline in the DNA yield and purity over the period of the study though the pattern was not so clear after day 7. However, differences in the mean yield of DNA and mean purity extracted at a given time from each variety were also observed.

The analysis of the data revealed that there were significant differences in mean yield and mean purity within any variety. Also, a significantly negative correlation between the mean yield over the time of storage ($r = -0.6583$) and the mean purity and duration of storage ($r = -0.7792$) of the DNA extract. However, difference in variety was a insignificant factor. Independent t-test showed that the mean DNA yield on day 1 was significantly different from those obtained during the rest of the study.

3.4.6 Effect of Different Conditions of Storage on DNA Concentration and Purity

The effects of the three different non-chemical conditions of storage, namely: benchtop (room temperature), freezer and simulated herbarium were examined to determine which is most suitable for preserving cowpea leaves for further molecular analysis in terms of quantity and quality (purity) of the isolated DNA. The mean DNA yields and the purities of the extract making up composite samples were used in this study.

From Fig. 1 it is observed that with the exception of extraction done on day 1, relatively higher yields of DNA were obtained from frozen samples than their corresponding samples kept at room temperature during the period of the study . These differences were found to be significant. Also it was observed that mean yields of DNA extracts from benchtop samples significantly decreased during the period of the study whereas that of extracts from frozen samples remained fairly stable. Summarised details of these results can be found in table B. 1 (appendix B).

Similarly, between purities, relatively purer DNA were obtained from frozen samples than from their corresponding benchtop samples (Fig. 2) though the differences were found to be insignificant.

No comparisons could be made between benchtop and herbarium and herbarium and freezer conditions because data on flush leaves kept under herbarium conditions were not available.

fig.1 Effect of Different Storage Conditions on DNA Concentration of Flush Leaves

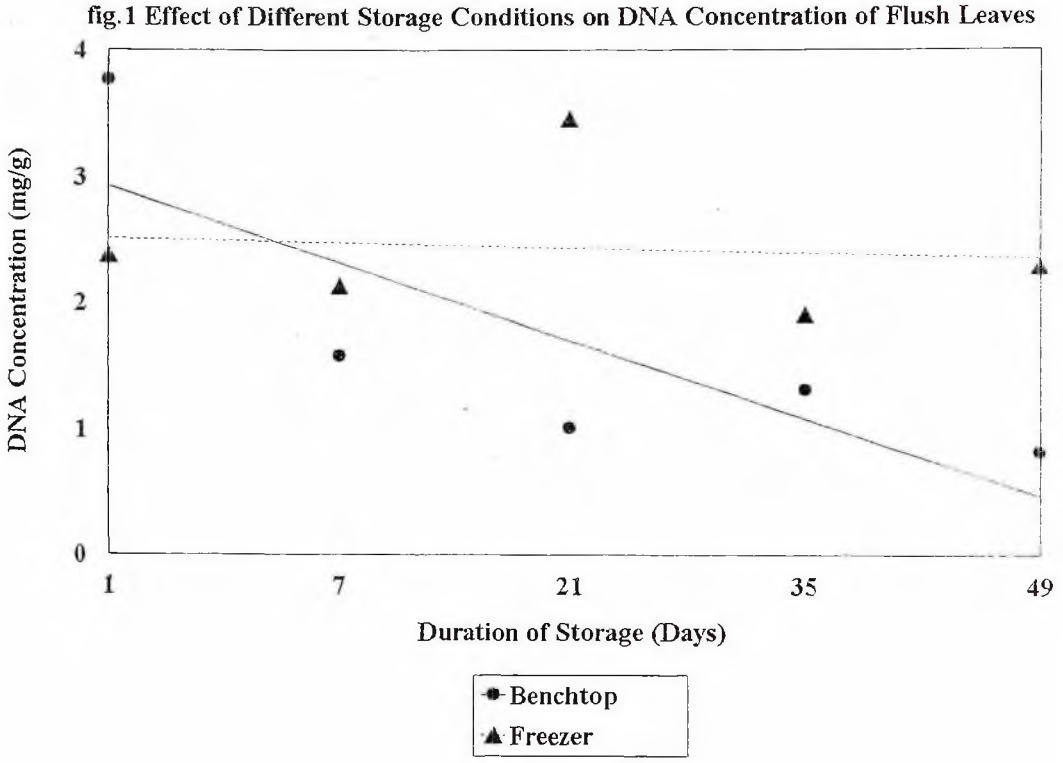


fig. 2: Effect of Different Storage Conditions on the Purity of DNA of flush Leaves

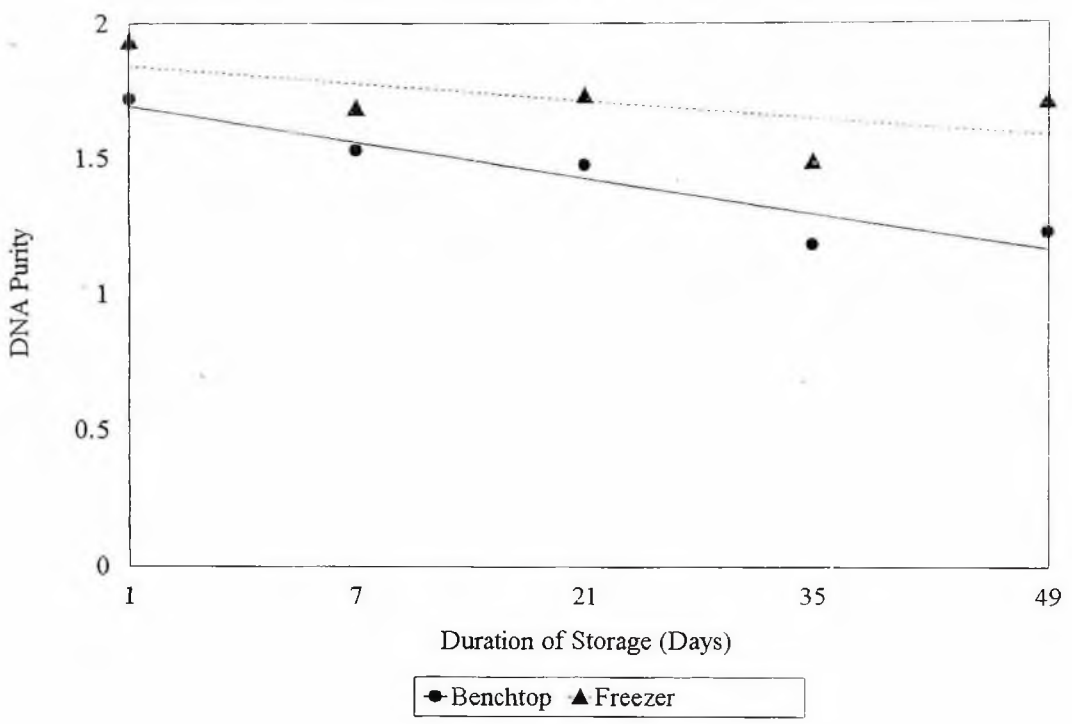


fig. 2: Effect of Different Storage Conditions on the Purity of DNA of flush Leaves

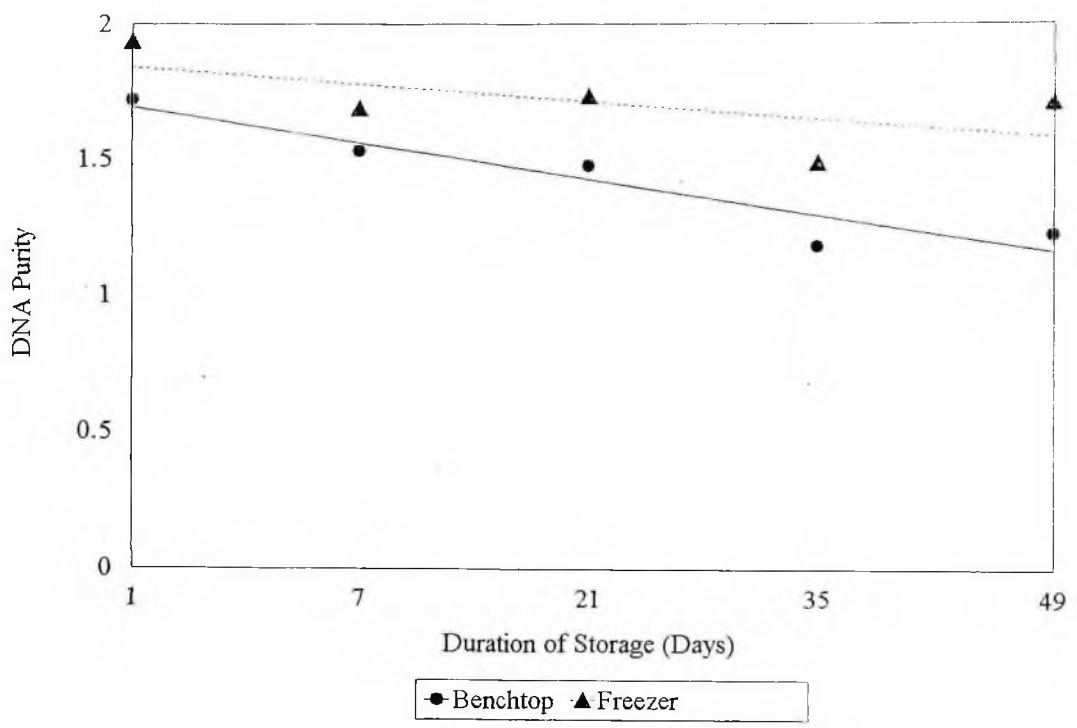


Figure 3 shows the mean DNA yield with time under the three conditions of storage. It was observed that the yields of samples kept under herbarium conditions declined most rapidly than in any of the other two conditions. The yield of samples kept in the freezer remained fairly constant but were higher than the yield of samples kept on benchtop at room temperature.

Figure 4 shows the mean purities of DNA over time under the three conditions of storage. The purities of DNA extracts from frozen samples were higher than either of the other two storage conditions, but also remained fairly constant during the period. Extracts from herbarium samples were relatively purer than from benchtop samples but thereafter decreased more rapidly with time than the latter.

With mature leaves, relatively higher yields with higher purities were obtained from frozen samples than from benchtop samples and the differences were found to be significant (Table B. 2, appendix B). Between benchtop and herbarium conditions, relatively higher DNA yields and purities were obtained for extracts of herbarium samples than benchtop samples and the differences were also significant. Between freezer and herbarium conditions, mean yields for extracts on day 1 were higher in herbarium samples than in frozen samples but these differences were not significant. Also, whereas the DNA yields from frozen samples were fairly constant over time the yields from herbarium samples significantly decreased with time. On the other hand, extracts from frozen sample relatively were purer than extracts from herbarium but the differences were not significant.

fig. 3: Effect of Different Storage Conditions on DNA Concentration of Mature Leaves

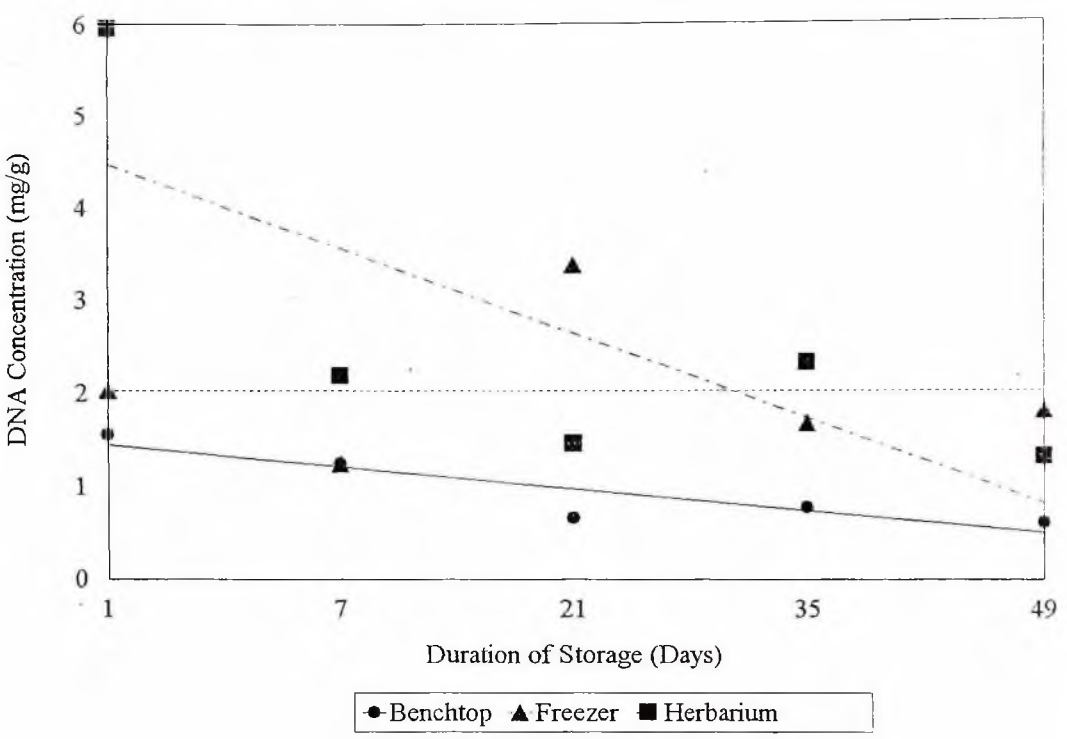
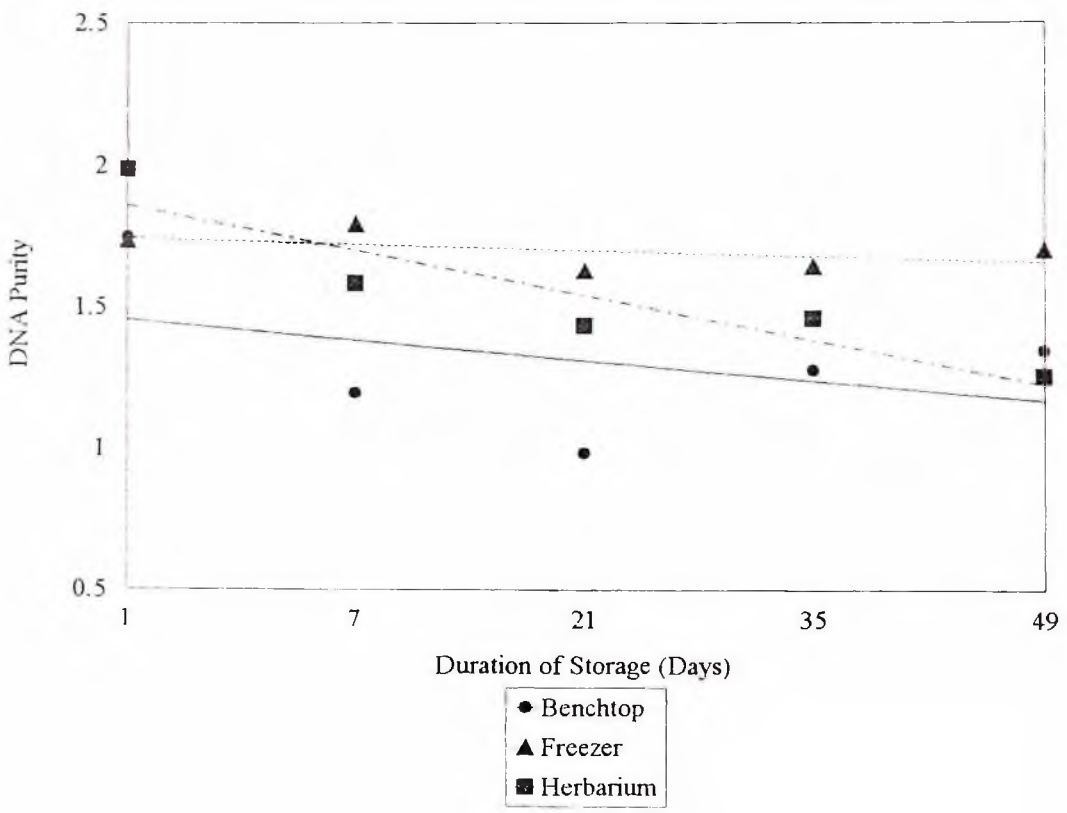


fig. 4: Effect of Different Storage Conditions on DNA Purity



3.5 DISCUSSION

The present study has clearly shown that under benchtop conditions, significantly higher yield of DNA was obtained from day 1 samples of either flush or mature leaves stored for longer periods. This may be the reason behind extracting plant DNA from fresh samples since higher yields of DNA are usually obtained from fresh samples (Hoelzel *et al.*, 1992).

It was observed that differences in variety did not significantly account for the differences in the DNA yield from samples kept in the freezer (both flush and mature leaves) and those kept under herbarium conditions. However, there was no clear pattern on the effect of differences in variety from samples kept on benchtop. Significant difference in DNA yield was found between the flush leaves of Amantin and Ejura Red, Amantin and Soronko, Asontem and Ejura Red, and Asontem and Soronko, and between the mature leaves of Amantin and Asontem, Amantin and Ejura Red, and Amantin and Soronko. This latter observation could have been a consequence of the low DNA yields recorded for mature leaves from Amantin in this study.

However, no differences were found in the purity of extracts from flush leaves stored at room temperature. Similarly, no significant differences in purity were found in DNA extracted from flush leaf samples, but significant differences were found when the mature leaves were used. Similar conclusions can be drawn for the material kept under simulated herbarium conditions.

It is known that the condition of the original tissue affects the purity and yield of DNA (Hoelzel *et al.,* 1992). At room temperatures, the data obtained (pooled data) showed significant difference in DNA yield between leaf types. However, no significant difference in DNA yield was found between frozen samples of different leaf types. These observations, contrary to expectation, could be attributed to the definition of a 'mature' leaf. 'Mature' leaves were considered as leaves which were more than two days old. This group included the very few old leaves found on the plants and many leaves which retained level of freshness, that is, majority of leaves classified as 'mature' leaves were still fresh and green but they were considerably larger in size than the rest. In addition, harvesting of 'mature' leaves was done without considering the duration between sowing and harvesting and because very few leaves had fully developed within this period (that is 14 days), it was possible that many of the 'mature' leaves had not produced secondary compounds such as complex carbohydrates in enough quantities to significantly affect DNA yield and purity from the matured leaves used (Hoelzel *et al.*, 1992). Thus in order to obtain a clearer picture of the effect of leaf age on DNA yield and purity, criteria for determining the ages of samples must be well-defined than was applied in this study. In that instance, harvesting of leaves could commence at the start of flowering, that is about 28 days after sowing.

Storage at room temperature at any time significantly produced the least amount of DNA when compared with the yield from the two other conditions of storage. Similarly, though higher DNA yields were obtained from herbarium samples there was a significantly sharp decline in yield during the period of the study. On the contrary, frozen samples yielded a fairly constant amount of DNA of relatively higher purity than the other samples. These

observations could be a result of loss of moisture from the samples stored on benchtop and in the herbarium. Moisture loss results in the drying of leaves. Grinding dried samples was difficult than grinding frozen ones. Moreover, freezing enhanced cell fracture and this might explain why more DNA was isolated from frozen samples than from fresh samples (see Table 12). Freezing as a preservation method also lowers the activity of enzymes unlike at the elevated temperatures of herbarium and benchtop which are ideal for nucleases and other degradative enzymes activities. Since herbarium temperatures were maintained constant during the study whereas benchtop temperatures varied between 22°C and 32°C, it was possible that samples kept on benchtop were drier than the herbarium samples and this might have accounted for the differences in DNA yields of their samples.

3.6 CONCLUSION

To conclude, the yield and quality (purity) of the DNA extracted is affected by the duration of storage and the preservation condition (temperature) and in some cases by the type of leaf. Time was found to correlate inversely to the purity and yield of DNA in almost all cases. More DNA was isolated from flush than from mature leaves.

Of the three conditions, leaves kept at room temperature yielded relatively the least amount of DNA. Though leaves preserved under simulated herbarium conditions yielded more DNA, this was not significantly different from the yield of DNA obtained from samples stored at -20°C (that is in a freezer). Furthermore, storage at -20°C yielded relatively constant amount of DNA and maintained high purity during the period of study. Therefore, of the three non chemical conditions of storage, freezing could be the best method for the preservation of cowpea leaves for further molecular analysis with particular reference to yield and purity (quality).

CHAPTER FOUR

DETECTION OF DNA POLYMORPHISM IN COWPEA VARIETIES BY MOLECULAR METHODS

4.1 INTRODUCTION

Molecular analysis of genomes of organisms do provide valuable supplementary information about their degree of relatedness. This is because the genetic material, DNA, is itself, very rarely influenced by the environment. Thus, the sequences of nucleotides (as molecular structures) which constitute heritable genes of organisms remain conserved over a long period of time. Moreover, because it is a simple sequence of different combinations of four bases, the position of each base is a potential data point. However, some DNA sequences can undergo changes because a segment can be translocated or the exact base sequences can be altered as point mutations originating from nucleotide deletions, insertions, or substitutions. These unit base changes and translocations can be detected either as the absence or presence of a segment of DNA or as differences in the size(s) of the DNA fragments (polymorphisms) when genomes of similar individuals are analyzed. For example, deletions or insertions can result in a gain or a loss of a site at which a particular restriction enzyme acts and this can alter the length of fragments between two restriction sites. Similarly, the absence of an amplified product could be the consequence of a modified site at which a DNA oligonucleotide primer binds to the template DNA. These polymorphisms once detected can then be utilized as genetic markers for the identification and characterization of families, species or individuals.

Detection of these genetic sequences can therefore generate immense evidence to analyze relationships and refine taxonomy and classification of organisms. This molecular phylogeny derived from sequences of nucleotides has been reported to be consistent with, but in many cases more precise than, classical phylogeny based on macroscopic structures. Moreover, molecular structures have been conserved in evolution even though organisms have continuously diverged at the level of gross anatomy (Lehninger *et al.*, 1993). It is possible that minute differences in anatomy and morphology could be revealed at the molecular level.

To this end, a number of modern techniques are now available for detecting genetic polymorphism among individuals, species and families at the molecular level. These include RFLP (Young, 1992), pulse field electrophoresis, SDS-PAGE and Western Blotting and Ribosequencing (Davis *et al.*, 1994). The principles and practices of these relatively new techniques have been described elsewhere (see sections 1.5 to 1.6).

4.1.1 Objective

The objective of this study was to employ the techniques of PCR, RAPDs and PCR-RFLP to detect DNA polymorphism for the identification of the six varieties of cowpea, *Vigna unguiculata* (L.) Walp.

4.2 MATERIALS AND METHODS

All the six varieties of cowpea, Amantin, Asonten, Ayiyi, Bengpla, Ejura Red and Soronko were used in this study.

Genomic DNA of the cowpea leaves were isolated using the method described under section 2.3.2 of Materials and General Molecular Methods

The primers and their respective sequences used in this study are listed in Table 13.

Table 11: List of Random Primers used and their Sequences

Primer	Length	Sequence
AR	20	AAGTGTCCTCTCCATTCCTA
GA	20	CTGGTTTGGTCGCGACGTTT
UN	20	GTGTGCCCCTTCCTCGATGT
QD	20	CAGACCAAGATGGTTAGTAT
ME	20	TGACCAACCCACTCCCTTGA
BD1	20	GTCGTAACAAGGTTTCCGTA
BD1F	20	TCCTGCAGTTCACATTCTGAC
HISP	20	TAGGTATAGATGTGGACAGC
ITS	20	TACATGGCGGGGTCAGTTTG
ITS4S	20	TAGTGACGCGCATGAATGGA
MT1	20	TCAAACGGCGGGAGTAACTA
MT2	20	CCCCTTCGTATGTGTTGTTT

PR2	20	CCTGTGCGATTTCACGCAGG
VI4	20	CCTGTGCGATTTCACGGACC
V15	20	CTGGACGTGGAAAAGGTGGT

4.2.1 Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR)

Initial trials following eight published RAPD-PCR protocols (Welsh & McClelland, 1990, 1991; Williams *et al.*, 1990; Love *et al.*, 1990; Ballinger-Crabtree *et al.*, 1992; Innis *et al.*, 1990 & Saghai-Maroof *et al.*, 1994) were carried out and modified in order to optimize conditions for the amplification of the random target. This was done by either varying the concentrations of the solutions of the reaction mix or by varying the thermocycling conditions.

Each PCR reaction mix in 25 µl contained 1X PCR reaction buffer,2 mM $MgCl_2$, 0.4% w-1 (Detergent), 25 µM each of Deoxyribonucleotides (dATP, dCTP, dGTP & dTTP), 1 U/µl Taq DNA Polymerase (1U), 800 µM Primer, 20 ng Template DNA. The reactions were thoroughly mixed and overlaid with 25 µl of mineral oil to minimize evaporation and refluxing during thermocycling. Low stringency amplification condition of 35°C was used. The thermal cycler was programmed for an initial denaturation at 94°C for 2 minutes which was succeeded by 3 cycles of low stringency annealing at 94°C for 1 minute (denaturation), 35°C for 1 minute (annealing) and 72°C for 2 minutes (extension). This temperature profile was followed by another 32 cycles at low stringency at 94°C for 10 seconds (denaturation), 35°C for 30 seconds (annealing) and 72°C for 1 minute

(extension). The reaction was concluded with a final extension step of 72°C for 5 minutes to enhance completion of all synthesized strands. In all, 15 random oligonucleotide primers were used singly or in combination in various assays that amounted to 38 trials. Details of primer sequences are presented in the Table 13

4.2.1.1 Analysis of Amplified DNA

Agarose Gel Electrophoresis

Following PCR, 10 µl of the PCR reaction was electrophoresed in ethidium bromide-stained 2% agarose gel at 80V. To estimate the sizes of the DNA products λ/*Hae*III digest was used as molecular weight marker. In cases where the PCR products were to be sliced from gels, 15 µl of the products were electrophoresed in 1% agarose gels. The bands were visualized on the UV transilluminator (UVP, U.S.A.) and photographed.

Restriction Analysis of RAPD-PCR Products

The eight restriction endonucleases (*Alu*1, *Dra*1, *Eco*R1, *Eco*RV, *Hin*f1, *Hae*111, *Kpn*1, *Pvu*1) were used to digest and the details of the protocol followed are described in section 2.5.3. The digested products were separated on either 2% agarose gels or on SDS-PAGE gels. 5 µl of each digested product was electrophoresed using a 5-12% gradient SDS PAGE gels for 2.5 hours (see section 2.5.3.2), and silver stained to visualise the DNA fragments, and the gels were photographed.

Estimation of Sizes of RAPD-PCR Products

The sizes of the RAPD-PCR products and the restriction fragments were estimated from 2% agarose gel by comparing with molecular weight markers as described in section 2.5.3.1 under Materials and General Molecular Methods.

4.3 RESULTS

4.3.1 RAPD-PCR

4.3.1.1 Estimation of the Size of fragments of the Marker

In this study, the products in a λ/*Hae*III digest were employed as the standard against which the sizes of the PCR products, both digested and undigested, were estimated. This was done by comparison with the mobility of fragments of the λ/*Hae*III digest. The sizes of the fragments in the latter marker were initially estimated by interpolating from a calibration curve of the logarithm of molecular weight of a 100 bp DNA ladder against their mobility (Fig. 5). The fragments sizes of these markers are listed in section 2.2.1.

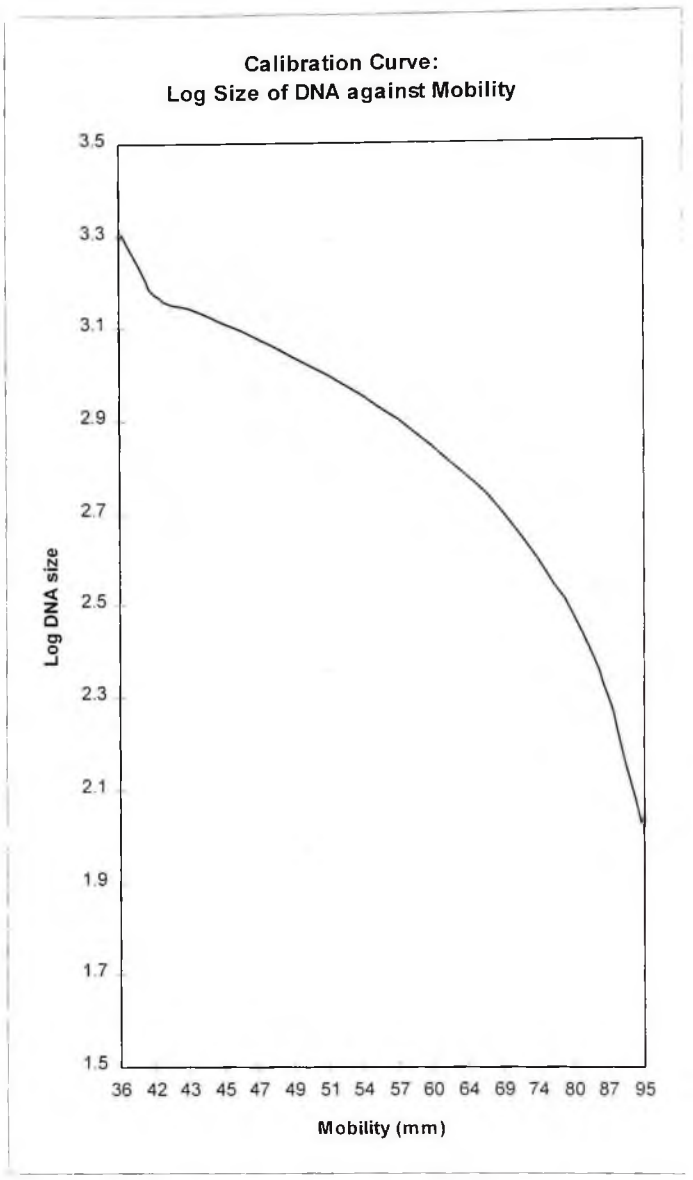


Fig. 5

Calibration curve showing the logarithm of the size of DNA of a 100 bp ladder (100 bp to 2072 bp) against its mobility on an agarose gel. This curve was used to estimate the sizes of fragments in the λ/*Hae*III digest employed as the marker DNA in this study. The sizes of PCR products, digested or undigested, were then estimated by comparison with the mobility of fragments in a λ/*Hae*III digest.

4.3.1.2 Random Amplified Polymorphic DNA-Polymerase Chain Reaction

Of the 15 random oligonucleotide primers used (either singly or in combination) only two (AR and GA) were found to anneal to segments of the genomic DNA in all the varieties. Each of these primers produced two sizes of DNA fragments in all the six varieties. Amplifications which employed the primer AR yielded two products of sizes, 369 bp and 545 bp in all the varieties whilst amplifications using primer GA resulted in the synthesis of two products of about 1200 bp and 1065 bp (Fig 6). However, no differences in the sizes of the amplified products of the varieties were observed. Primers BDIF, ITS4S, MT1 and MT2 also resulted in successful amplifications but the results were not reproducible.

4.3.2 Restriction Enzyme Digestion of RAPD-PCR Products

The fragments generated by digesting the GA-primed PCR products of all the varieties using the nine restriction enzymes were resolved on both agarose and SDS PAGE gel systems. In both gel systems, no differences in the banding patterns and sizes of fragments were observed. Of the nine restriction endonucleases used in the digestion reactions, only four (*Alu*I, *Hae*III, *Hinf*I and *Kpn*I) digested the GA-primed PCR product of 1200 bp. The digestion with *Alu*I generated three fragments, a faint fragment of about 200 bp and two fragments which co-migrated around 460 bp (Fig 7). *Hae*III digestion yielded three fragments of sizes of about 240 bp, 400 bp and 490 bp (Fig 7). The *Hinf*I digestion also produced three relatively smaller DNA fragments of approximate sizes; 290 bp and 330 bp and 470 bp (Fig 7). The *Kpn*I digestion yielded two smaller fragments of 400 bp and 800 bp in size (Fig 8).

4.4 DISCUSSION AND CONCLUSION

Of the four restriction enzymes which digested the GA-primed product of 1200 bp only the two fragments generated by KpnI digestion added up to 1200 bp. The sizes of the restriction fragments obtained using *Alu*I, *Hae*III, and *Hinf*I added up to 1120 bp, 1130 bp, and 1090 bp respectively. The shortfall observed could be attributed to very small fragments which ran off the gel or were so faint that they could not be observed on both agarose and SDS polyacrylamide gels.

However, the results of RAPD-PCR have clearly shown that the six varieties of cowpea were identical at the loci amplified. Restriction analysis of the GA-primed PCR product also failed to reveal any differences among them. These observations suggest that the varieties of cowpea examined might be very closely related since their banding patterns were identical. However, it is possible that if more random primers had been used some polymorphism among the six varieties could have been detected. This is because RAPD polymorphisms are noted by the presence or absence of an amplification product from a single locus arising from either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region (Tingey *et al.,* 1994). In addition, since the sequence of nucleotides amplified in RAPDs is not known and one random primer on the average is capable of directing the amplification of DNA at more than one loci within a genome, the greater the number of random DNA sequences amplified by a single primer the greater the chances of detecting some nucleotide sequence polymorphisms between individuals. Therefore, it is possible that the number of amplified

random bands observed for each primer in this study were so small to facilitate the utility of the RAPD assay for discriminating between closely related individuals.

Another explanation for the failure to detect polymorphism among the six varieties could be attributed to the region(s) within the cowpea genome (that is the loci) which were amplified by the two primers (AR and GA). It is possible that these regions amplified were not located in the hypervariable region of the cowpea genome and therefore were unsuitable for population studies within cowpeas or that the regions amplified evolve slowly and they might be suitable for systematic studies at the higher taxonomic levels of species, genus and the family. On the other hand, further analysis of the amplified random bands using techniques such as DNA sequencing, could probably have revealed some differences in the sequence of nucleotides of the products. However, this technnique could not be employed because facilities for DNA sequencing were not available.

These observations notwithstanding, several groups have reported on the usefulness of RAPD markers, as sources of phylogenetic information (Arnold *et al.,* 1991); for DNA fingerprints of individuals and of taxonomic relationships in eukaryotes and prokaryotes (Caetano-Anollés *et al.*, 1991; Welsch *et al.,* 1991; Hedrick, 1992). For instance, Hu and Quiros (1991) were able to demonstrate that the amplification products from only 4 random primers were significant to discriminate between 14 different broccoli and 12 different cauliflower cultivars (*Brassica oleracea* L.). Similarly, using only 25 different decamer oligonucleotide primers, Kresovich *et al.,* (1992) were able to collect information on 140 different polymorphic characters in an array of individuals representing *Brassica oleracea* L. and *B. Rapa.* Thus, from the foregoing, it is likely that the part of the cowpea genome amplified by the two primers were not suitable for identification of varieties. At best, it revealed how closely related the six varieties are.

Another approach to the present study could have been to conduct a search of the DNA databases such as EMBL and Genebank, for published hypervariable regions of plant genome in general, from close relatives of cowpea such as soy bean, or from the cowpea genome. From the information obtained, oligonucleotide primers could be designed from sequences flanking conserved regions of genes coding for single or repetitive sequences. These primers would then be used to screen for variation among the six cowpea varieties. Similarly, a search for published DNA sequences from cowpeas or any closely related species such as soybeans could be done from Genebanks for the design of primers. This was the approach used by Akkaya *et al.,* (1992).

Alternatively, microsatellite primers could have been used. Microsatellites are highly repetitive DNA sequences of tandemly repeated motifs of 6 bp or less. They vary in both the size of the motif and the length of the repeat and also abound in high proportion within the genome of both plants and animals. Normally, the sequences flanking microsatellites are generally conserved in individuals of the same species and this facilitates selection of PCR primers that will amplify the intervening microsatellite in all genotypes. Variation in the number of tandem repeats at a locus generate PCR products of varying sizes which are highly informative DNA markers (Weber and May, 1989; Litt and Luty, 1989; Tautz, 1989). Thus, the detection and exploitation of microsatellites have revolutionized both human and mammalian research and their utility in the study of plant genetics have been demonstrated (Akkaya *et al.*, 1992). To date, they are being developed in a number of plant species including maize (*Zea mays* L.) (Senior and Heun, 1993) and soybean (Akkaya *et al.*, 1992). Therefore, it is possible that by searching the DNA data banks of cowpea sequences, microsatellites could be identified from which PCR primers could be designed for identification purposes.

Similarly, further restriction analysis using more restriction enzymes, or more approriately using rare cutting enzymes, could be exploited to enhance the chances of detecting some polymorphism among the varieties within 1200 bp PCR product. Such digestion products could be run on longer gel plates so that the minute differences in migratory rates between any two closely migrating bands between different varieties could be detected. However, the lack of these logistics during the study made it impossible to carry out further restriction analysis of this PCR product and on the other products as well.

CHAPTER FIVE

SINGLE STRANDED CONFORMATION POLYMORPHISM ANALYSIS (SSCP) OF COWPEA VARIETIES

5.1 INTRODUCTION

Single stranded conformation polymorphism analysis (SSCP) is a technique first described by Orita *et al.* (1989) and it is used for the rapid detection of small DNA sequence differences such as point mutations in PCR amplified products. It is based on the relationship between the electrophoretic mobility of a single stranded DNA (ssDNA) and its folded conformation, which in turn, reflects its internal nucleotide sequence. Any change in the sequence theoretically causes a shift in the mobility of the analyzed conformers. Double stranded DNA (dsDNA) is first denatured (by heat and/or a chemical) to generate single stranded DNA and the ssDNA are electrophoresed on either denaturing or non-denaturing polyacrylamide gels. Single stranded DNA which have undergone point mutations migrate at different rates from their wild type. These individual SSCP bands can then be visualized by autoradiography or directly by silver or ethidium bromide staining. The combined application of PCR amplification of DNA segments of suitable length followed by screening using SSCP thus facilitates rapid identification of genetic polymorphisms, whether resulting from germ-line heterozygosity or somatic cell mutations, at virtually any site within a given moderately long segment of DNA.

So far, this technique has enabled the efficient detection of mutations if the PCR fragment is not more than 400 bp in size (Hayashi, 1992). However, Hongyo *et al.*, (1993) have

demonstrated that using denatured molecular weight marker DNA, sharp resolution of single strands up 1350 bp in length is possible.

Two basic methods of SSCP analysis have been described. The first described by Orita *et al.* (1989), is radioisotope based and uses radiolabelled PCR primers or oligonucleotides to generate radioactive PCR products. The amplified DNA is then highly diluted, denatured by heating, and electrophoresed in a large-formatted (40x20 cm) non-denaturing gel. The SSCP bands are then visualized by autoradiography. Although sensitive, several limitations observed in the use of radioisotopes have made this approach unpopular. Also, the gel must be run at low voltage (usually overnight) to avoid heat-induced conformational changes; precise control of the temperature which has been shown to be important in obtaining consistent results is often difficult to achieve with large-formatted gels (Sarkar *et al.,* 1992). Autoradiography, usually with multiple exposures, requires additional time to produce a clear image of the resulting SSCP pattern; besides, it is difficult to cut out SSCP bands from these gels for sequence characterization since they cannot be visualized directly. These disadvantages hampered the extensive use of SSCP as a routine screening procedure in clinical laboratories.

Another approach which is non-isotopic has several advantages over the former in that the lack of radioisotpes makes this procedure less dangerous. It is simple and very rapid since it is amenable to automated commercial minigel electrophoresis. The SSCP band image developing time also is greatly reduced because rapid means of detecting individual SSCP bands such as ethidium bromide and silver staining techniques can be applied. In addition, the individual bands can be easily sliced from the gel for subsequent

reamplification or sequencing. The fact that the temperature at which the gel is run can be regulated enhances the efficiency of the procedure.

Several protocols purported to be improvement of non-isotopic SSCP analysis based on non-denaturing gels have been published. But more recently, the use of denaturing gels in discontinuous phase non-isotopic SSCP has also been reported (Yap and McGee, 1993) and is increasingly becoming popular.

SSCP has become one of the most commonly used techniques for screening and detecting minute genetic alterations as single base mutations. It is being widely applied in biomedical research especially in the diagnosis of certain human diseases caused by gene defects. Ainsworth *et al.*, (1991) have successfully adapted it to diagnose a B1 variant of Tay-Sachs disease (TSD) whiles Spinardi *et al.*, (1991) have applied it to study five variants of a 60 bp fragment of human papilloma virus (HPV) differing from each other by a single point mutation. Similarly, Dockhorn-Dworniczak *et al.*, (1991) applied this technique in the diagnosis of phenylketonuria caused by well-defined mutations in the gene coding for phenylalanine hydroxylase (PAH). SSCP has also been used to detect mutated *ras* genes and in the diagnosis of at least two of the exon 11 mutations responsible for cystic fibrosis. SSCP has been applied to the genetic profiling of individuals using the highly polymorphic D-loop region of human mitochondrial DNA, with bands staining with much greater intensity as well as better resolution, using conventional UV transillumination and Polaroid photography. These results altogether have indicated that PCR-SSCP might expedite clinical molecular diagnosis of diseases because it bypasses the time-consuming and tedious techniques of cloning and sequencing (Yap and McGee, 1993). However, certain changes cannot be detected by

SSCP analysis (White *et al.*, 1992). Cai and Touitou (1993) also failed to discriminate between two 144 bp cathepsin D sequences which only differ by a C/T base substitution at position 224, using the original or modified SSCP protocols on PCR products obtained from homozygous (T/T), heterozygous (C/T) and homozygous (C/C) breast cancer cell lines(MCF7, MDA-MB-231 and T47D respectively). These setbacks notwithstanding the use of SSCP is gaining much popularity and the technique would be of tremendous importance in molecular systematics and population biology studies.

5.1.1 Aims and Objectives

For the present study, SSCP was employed with view of detecting slight differences that might be present in the PCR products of the six cowpea varieties. The two *AR*-primed PCR products of about 369 bp and 545 bp in size were used for analysis. These were chosen because the bands were conspicuous on agarose gels and were also relatively smaller than the products generated by amplifying genomic DNA with primer GA.

5.2 RESULTS

The re-PCR of the DNA fragments extracted from the agarose gels were all successful. A single band was obtained in all cases when the PCR fragments of 369 bp was reamplified (Fig 9). The 545 bp product also produced a single band (Fig 10), but smears denoting non-specific amplification were observed. These resulted in the faint bands observed on the SDS PAGE gels (Fig 11). To establish that the DNA fragments were actually denatured, all the gels ran included the undenatured PCR product.

5.2.1 SSCP Analysis

Using non-denatured polyacrylamide gels, the denatured DNA were observed as the fainter bands and moved relatively slower than their corresponding undenatured products. For some unexplained reasons, all attempts at denaturing the 369 bp DNA fragment were unsuccessful. However, the bigger DNA fragment of 545 bp were successfully denatured, but no differences in mobility were observed between all the cowpea varieties that were studied (Fig 11). In addition, other denatured bands were observed on the gel for the 545 bp product.

5.3 DISCUSSION

The results obtained has shown that there are no detectable polymorphism among the six varieties with the DNA fragment that was analyzed by SSCP. To obtain unambiguous results, it was important that a single, clear band of PCR product (double stranded) is found on the electrophoretic gel after PCR. Since two main bands (369 bp and 545 bp) were observed on the agarose gel (Figs 9 & 10), by cutting them out separately, re-extracting and reamplifying increased the possibility of obtaining only single products for SSCP analysis. This practice in essence eliminated possibility introducing artefacts into the study.

The size of the band 545 bp fragment is beyond the range proposed as optimum for SSCP analysis (Hongyo *et al.*, 1993). This might have accounted for the inability to detect different migration patterns among the denatured PCR products of the six cowpea varieties. Strand separation in SSCP analysis is affected also by the temperature of the running buffer, buffer type and its concentration, the nature of denaturant, concentration of DNA in the PCR product, and the gel polyacrylamide concentration (Hongyo *et al.*, 1993). Among these parameters, the running buffer temperature and the gel concentration are considered the most important in SSCP analysis. The ideal temperature for SSCP analysis however varies among individual PCR products and this requires empirical study to obtain optimal results for each product (Hongyo *et al.*, 1993). For instance, an optimum temperature of 10°C was established as the optimum when analyzing PCR products of 117 bp in size (Hongyo *et al.*, 1993). In the present study, regulating the running buffer chamber temperature was impossible because the kit used lacked a buffer

circulator for this purpose. Replacing the running buffer with cold buffer every sixty minutes to keep the temperature below 12°C did not improve strand separation. Also when gels were run at room temperature, the bands were blurred and not sharp. However, Yap and McGee (1993) were able to detect differences in migration patterns of the highly polymorphic D-loop region of human mitochondrial DNA when electrophoresis was performed at 4-20°C. Also, Spinardi *et al.,* (1991) have observed that gels run at room temperature were even more discriminative, and that for certain sequences a second run at 4°C was more informative. Optimizing the buffer temperature is therefore a crucial step in determining the success of a separation.

Different authors have successfully detected differences in band migration patterns using different concentrations of gels. For the present study, three gel concentrations (30, 40 and 50% acrylamide gel mixtures) were tested. The 30% polyacrylamide gels gave relatively sharper and consistent bands whereas the 40% and 50% gels resulted in diffuse and blurred bands (Figs 18 & 19).

Of the two 545 bp bands observed, the leading band was blurred whereas the lagging band was clear and sharp. This difference in band intensity could be attributed to insufficient ssDNA generated by alkali denaturation as reported by Yap and McGee (1993) or excessive reannealing owing to the choice of denaturant, the concentration of DNA present in the PCR product or the loading volume of the product used (Hongyo *et al.,* 1993). For this study, sodium hydroxide was used as the denaturant and denaturation mix was heated for 15 minutes to ensure that denaturation had been effected. It is possible that the 369 bp product separated into two single strands when boiled for a period of 15

minutes. However, the denaturant, sodium hydroxide, might not have been sufficiently strong to prevent reassociation so that the complimentary strands reannealed and then migrated as a single band prior to loading of the gel. The use of a stronger denaturant could have resolved this anomaly. For example, Hongyo *et al.*, (1993) used methyl mercury hydroxide, a very strong denaturant. This denaturant permitted sufficiently large amounts of DNA to be loaded so that weakly staining bands were readily visualized. Alternative denaturant such as urea has proved less effective than methyl mercury hydroxide. Blurred bands could also be a result of high concentrations of the DNA used for denaturation. High DNA concentrations facilitate rapid kinetics of complete and partial reannealing that occurs at high DNA concentrations (Hongyo *et al.*, 1993). However, the assumption that the DNA concentration in the products were high is not plausible, because most single stranded and double stranded bands were weakly stained on the polyacrylamide gel. Nonetheless, it is possible that the size of DNA product (that is, 545 bp) did not facilitate complete strand separation since the ability of SSCP to detect mutations declines as PCR fragments approach 400 bp in size (Hayashi, 1992) though sharp resolution of single strands up to 1.35 kbp in length has been reported when denatured molecular weight marker was used (Hayashi 1992). Moreover, certain changes have been reported to remain undetected by SSCP analysis. Cai and Touitou (1993) failed to discriminate between two 144 bp cathepsin D sequences which only differ by a C/T base substitution at position 224. It might be possible that there are some unit base changes within the DNA fragments studied but these could not be detected by SSCP analysis. Such minute differences therefore might be detected through sequencing of the fragments.

These notwithstanding, the similarities observed in the banding patterns of the denatured 545 bp and the undenatured 369 bp products on the polyacrylamide gels at least indicated that the six varieties of cowpea might be closely related. On the other hand, it is also possible that the portion of the genome amplified is not suitable for separating closely related individuals and varieties within *Vigna unguiculata* (L.) Walp.

5.4 CONCLUSION

Discontinuous phase single stranded conformation polymorphism analysis (DP-SSCP) was used to detect possible differences in the internal sequence of nucleotides present in two PCR products, 369 bp and 545 bp, amplified from the genomic DNA of six cowpea varieties. The two fragments were obtained from an amplification reaction that used a random primer AR, originally designed for the identification of the mosquito, *Anopheles arabiensis*. The products were denatured by boiling for 15 minutes in a denaturation mix which included also sodium hydroxide and ETDA (500 mM:50 mM) as the denaturants, The denatured products were electrophoresed on a 30% discontinuous phase polyacrylamide gel at 120V for five hours. Only the 545 bp DNA fragments obtained from the six cowpea varieties were successfully denatured but the single stranded DNA were not separated on gels to enable their differentiation be made. Moreover, there were no differences observed in the banding patterns in all six varieties. It was therefore concluded that DP-SSCP could not be used to identify any of the six varieties of cowpea studied using this DNA fragment.

SUMMARY

1 The yield and quality (purity) of the extracted DNA is affected by the duration of storage, the preservation condition (temperature) and in some cases by the type of leaf (that is the age of the leaf). Time was found to correlate inversely to the purity and yield of DNA in almost all cases. More DNA was isolated from flush leaves than from mature leaves. Of the three conditions studied, leaves kept at room temperature yielded relatively the least amount of DNA. Though leaves preserved under simulated herbarium conditions yielded more DNA, this was not significantly different from the yield of DNA obtained from samples stored at -20°C (that is in a freezer). Furthermore, storage at -20°C yielded relatively constant amount of DNA and maintained high purity during the period of the study. Therefore, of the three conditions of storage studied, storage in freezer is the best non-chemical method for the preservation of cowpea leaves.

2 Fifteen oligonucleotide primers were randomly assayed with the aim of amplifying DNA sequences which would be analyzed for polymorphism and for identification of 6 varieties of cowpea (*Vigna unguiculata* L. Walp.) found in Ghana. Using RAPD-PCR only 2, GA and AR out of the 15 random primers, successfully amplified segments of the genomic DNA isolated from the 6 varieties of cowpea - Amantin, Asontem, Ayiyi, Bengpla, Ejura Red and Soronko. Amplification using primer AR yielded two products of sizes, 369 bp and 545 whilst primer GA yielded one product of 1200 bp in size. When these PCR products were electrophoresed on both 2% agarose and 5-12% SDS

polyacrylamide gels no differences were observed in their profiles for all the 6 varieties.

Subsequently, Restriction Fragment Length Polymorphism (RFLP) studies were carried out on the GA-primed product (1200 bp) using eight restriction endonucleases, *Alu*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hinf*I, *Kpn*I and *Pvu*I. Of these eight, only 4 enzymes, *Alu*I, *Hae*III, *Hinf*I and *Kpn*I digested the product. Digestion with *Alu*I gave rise to 3 fragments of sizes, 240 bp, and two fragments which co-migrated around 400 bp. Digestion with *Hae*III yielded three fragments of sizes, 240 bp, 400 bp and 490 bp. *Hinf*I restricted the product into three fragments of sizes 290 bp, 330 bp and 470 bp whilst *Kpn*I digested the product into two fragments, 400 bp and 800 bp in size respectively. However, the restriction profiles observed using each endonuclease was the same in all 6 varieties, thus showing that the 6 varieties did not differ in their DNA sequence at the regions of the genomic DNA that were amplified.

The two AR-primed products were further analyzed using the technique single stranded conformation polymorphism (SSCP). This exploits the detail of DNA sequences due to base substitution, deletion to and differential mobility of secondary structures of single stranded DNA on SDS PAGE electrophoresis to demonstrate polymorphism. The profiles of the denatured bands, in all 6 varieties, and therefore single-stranded 545 bp products of the six varieties were identical when electrophoresed on 30% discontinuous phase polyacrylamide gel. It was concluded that all the 6 varieties were identical in their DNA sequence for the

region of the genomic DNA of coupes. Therefore, they were not suitable for the identification of the 6 local varieties of cowpea, Amantin, Asontem, Ayiyi, Bengpla, Ejura Red and Soronko. It was suggested that further analysis could be done by firstly conducting a search of the DNA databases such as EMBL and Genbank, for published hypervariable regions of the plant genome in general, for close relatives of cowpea such as soy bean and for the cowpea genome. From the information obtained, oligonucleotide primers could be designed from sequences of conserved regions flanking the hypervariable regions for PCR. These primers would then be used to screen for variation among the 6 cowpea varieties. Similarly, the use of microsatellite primers for DNA fingerprinting could also be exploited.

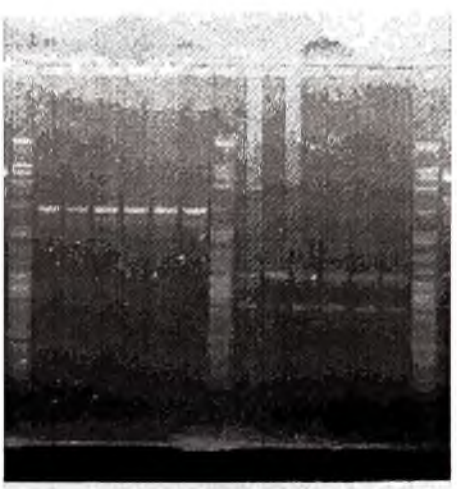


Fig. 6 Agarose gel electrophoresis of PCR products amplified using primers GA and AR on the genomic DNA extracted from six cowpea varieties. Lanes M, DNA/*Hae*III digest (molecular marker); lanes 2 to 7, products amplified using primer GA and lanes 9 to 14, products amplified using primer AR. The templates in lanes 2 & 9, are Amantin; 3 & 10, Asontem; 4 & 11, Ayiyi, 5 & 12, Bengpla; 6 & 13, Ejura Red; 7 & 14, Soronko.

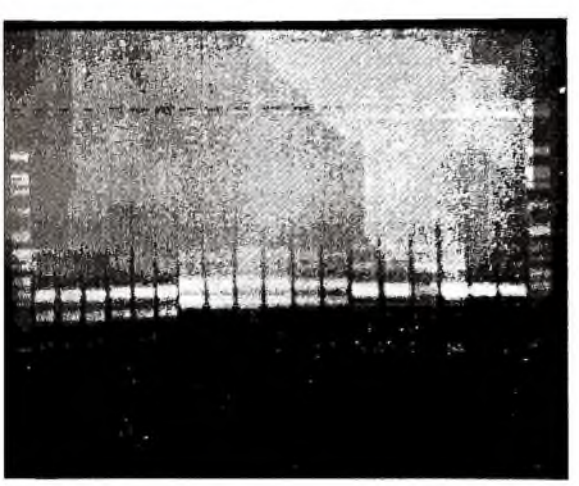


Fig. 7 Restriction fragment length polymorphism studies (RFLP). Agarose gel electrophoresis of the digested PCR product (1200 bp) amplified using primer GA on genomic DNA extracted from the six varieties. Lanes M, λDNA/*Hae*III digest (molecular marker); Lanes 2 to 7, products digested by HinfI, lanes 8 to 13, products digested by *Hae*III and lanes 14 to 19, products digested by *Alu*I: The templates in lanes 2, 8 & 14 are Soronko; 3, 9, & 15, Ejura Red; 4, 10, 16, Bengpla; 5, 11 & 17, Ayiyi; 6, 12 & 18, Asontem; 7, 13 & 19, Amantin.

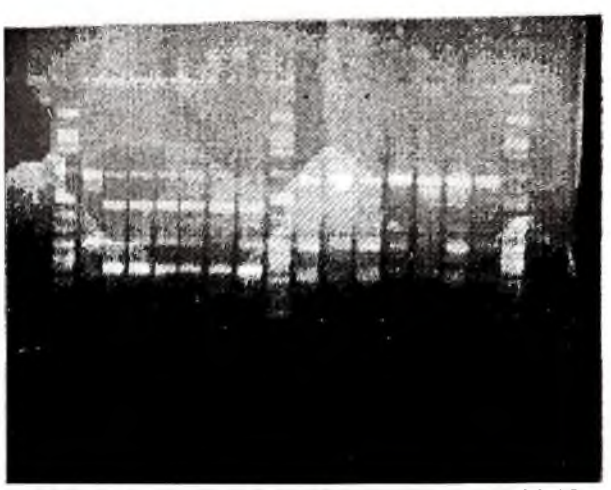


Fig. 8 Restriction fragment length polymorphism studies (RFLP). Agarose gel electrophoresis of the the digestd PCR product (1200 bp) amplified using primer GA on genomic DNA extracted from all six varieties. Lanes M, DNA/*Hae*III digest (molecular marker); lanes 2 & 16, undigested PCR product from genomic DNA of Soronko; 3 to 8, products of *Kpn*I digestion; 10 to 15, products of *Pvu*I digestion; the templates in lanes 3 & 10 are Soronko.; 4 & 11, Ejura Red ; 5 & 12, Bengpla; 6 & 13, Ayiyi; 7 & 14, Asontem; 8 & 15, Amantin; 18, Negative Control

Fig. 9 Single stranded conformation polymorphism analysis (SSCP) Agarose gel electrophoresis of re-amplified products (369 bp and 545 bp) from AR amplication of the genomic DNA extracted from agarose gel. Lanes M, DNA/HaeIII digest (molecular marker); 2, Soronko ; 3, Ejura Red; 4, Bengpla; 5, Ayiyi; 6, Asontem; 7, Amantin; 8, Negative Control.

Fig. 10 Single stranded conformation polymorphism analysis (SSCP). Agarose gel electrophoresis of the re-PCR products amplified using primer AR on the 545 bp product re-extracted from the agarose gel from all six cowpea varieties. The templates in Lane 1 is Negative control; 2, Soronko; 3, Ejura Red ; 4, original PCR products obtained from primer AR on genomic DNA of Amantin ; 5, Bengpla; 6, Ayiyi; 7, Asontem; 8, Amantin.

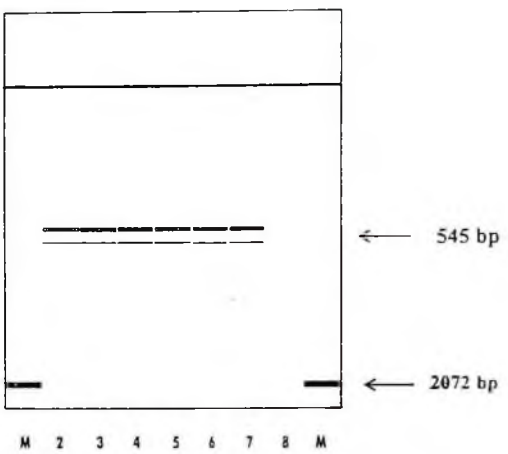


Fig.11 Single stranded conformation polymorphism analysis (SSCP). Discontinuous phase Sodium dodecyI Sulphate polyacrylamide gel elctrophoresis of the denatured re-PCR 545 bp product amplified using primer AR on the original 545 bp product extracted from 1% agarose gel from all six varieties. Lanes M, DNA/HaeIII digest (molecular marker); the denatured products in lane 2 is Soronko; 3. Ejura Red; 4.

APPENDIX

Appendix A

Table A1:
Data for the determination of the best nonchemical preservation method for cowpea leaves for molecular analysis

V	L	S	TIME (Days)	WEIGHT(g)[1]	D_{OD260}	P_{OD280}	E_{OD340}	DNA YIELD	PURITY[2]
1	1	1	1	1.005	0.334	0.160	0.014	6.368	2.000
1	1	1	1	1.005	0.228	0.112	0.012	4.299	1.930
1	1	1	7	1.269	0.149	0.083	0.020	1.654	1.550
1	1	1	7	1.274	0.132	0.072	0.017	1.474	1.600
1	1	1	21	1.255	0.247	0.130	0.360	2.740	1.620
1	1	1	21	1.255	0.114	0.065	0.021	1.200	1.490
1	1	1	35	1.256	0.082	0.047	0.020	0.700	1.130
1	1	1	35	1.256	0.063	0.038	0.020	0.850	1.320
1	1	1	49	1.238	0.082	0.047	0.020	0.816	1.320
1	1	1	49	1.238	0.063	0.038	0.020	0.566	1.130
1	2	1	1	1.030	0.016	0.009	0.001	0.291	1.670
1	2	1	1	1.030	0.023	0.013	0.000	0.446	1.770
1	2	1	7	2.023	0.118	0.058	0.010	0.585	1.860
1	2	1	7	2.023	0.154	0.083	0.021	0.721	1.600
1	2	1	21	2.031	0.137	0.088	0.041	0.520	1.090
1	2	1	21	2.031	0.118	0.860	0.024	0.509	1.340
1	2	1	35	2.105	0.176	0.102	0.034	0.744	1.390
1	2	1	35	1.980	0.158	0.096	0.032	0.711	1.390
1	2	1	49	2.013	0.073	0.045	0.019	0.296	1.200
1	2	1	49	2.006	0.061	0.039	0.019	0.231	1.070
2	1	1	1	1.020	0.152	0.089	0.026	4.844	1.420
2	1	1	1	1.020	0.328	0.186	0.041	7.529	1.510
2	1	1	7	1.528	0.049	0.027	0.016	2.546	1.220
2	1	1	7	1.520	0.081	0.037	0.038	3.252	1.770
2	1	1	21	0.997	0.028	0.018	0.009	0.862	1.060
2	1	1	21	0.997	0.019	0.013	0.005	0.782	1.390
2	1	1	35	1.294	0.129	0.066	0.914	2.876	1.740
2	1	1	35	1.294	0.086	0.066	0.140	1.422	1.650
2	1	1	49	1.298	0.100	0.067	0.036	0.988	.960
2	1	1	49	1.298	0.066	0.041	0.018	0.741	1.170
2	2	1	1	1.020	0.138	0.074	0.020	2.902	1.730
2	2	1	1	1.020	0.057	0.030	0.009	1.275	1.530
2	2	1	7	2.104	0.050	0.033	0.023	3.888	1.000
2	2	1	7	2.170	0.055	0.033	0.028	1.677	0.820
2	2	1	21	2.057	0.056	0.037	0.022	0.837	0.920
2	2	1	21	2.057	0.039	0.027	0.016	0.671	0.860
2	2	1	35	2.140	0.070	0.045	0.010	0.804	1.330
2	2	1	35	2.089	0.063	0.044	0.011	0.718	1.180
2	2	1	49	2.004	0.035	0.016	0.009	0.260	1.310
2	2	1	49	1.997	0.041	0.026	0.012	0.290	1.120
3	2	2	1	1.045	0.061	0.028	0.007	6.560	1.990
3	2	2	1	1.045	0.062	0.031	0.006	5.872	1.980
3	2	2	7	1.058	0.134	0.078	0.025	2.060	1.400
3	2	2	7	1.058	0.119	0.063	0.018	1.906	1.600
3	2	2	21	1.074	0.086	0.044	0.013	1.360	1.660
3	2	2	21	1.074	0.089	0.060	0.016	1.360	1.220
3	2	2	35	1.068	0.187	0.096	0.025	3.035	1.690

3	2	2	35	1.068	0.166	0.102	0.032	2.511	1.310
3	2	2	49	1.0775	0.221	0.138	0.063	1.150	1.200
3	2	2	49	1.0775	0.225	0.137	0.061	1.200	1.250
4	2	2	1	1.005	0.334	0.160	0.014	5.980	1.960
4	2	2	1	1.005	0.228	0.112	0.012	5.423	2.000
4	2	2	7	1.0960	0.152	0.082	0.013	2.537	1.700
4	2	2	7	1.0960	0.141	0.082	0.016	2.281	1.620
4	2	2	21	1.2215	0.109	0.061	0.017	1.507	1.510
4	2	2	21	1.2215	0.128	0.075	0.028	1.637	1.330
4	2	2	35	1.2695	0.137	0.084	0.026	1.749	1.320
4	2	2	35	1.2695	0.158	0.086	0.028	2.048	1.510
4	2	2	49	1.6200	0.173	0.105	0.038	1.667	1.290
4	2	2	49	1.6200	0.152	0.097	0.033	1.469	1.270
5	1	1	1	1.035	0.122	0.058	0.017	2.230	1.810
5	1	1	1	1.035	0.036	0.014	0.006	1.588	2.140
5	1	1	7	2.506	0.036	0.018	0.010	0.711	1.390
5	1	1	7	2.520	0.021	0.006	0.006	0.674	2.500
5	1	1	21	0.543	0.039	0.027	0.016	0.600	1.260
5	1	1	21	0.543	0.035	0.027	0.016	0.586	1.250
5	1	1	35	1.953	0.344	0.253	0.164	0.664	0.710
5	1	1	35	1.933	0.243	0.163	0.087	0.748	0.960
5	1	1	49	1.950	0.145	0.083	0.035	0.746	1.330
5	1	1	49	1.950	0.097	0.055	0.019	0.529	1.420
5	1	3	1	1.035	0.122	0.058	0.017	2.230	1.810
5	1	3	1	1.035	0.036	0.014	0.006	1.588	2.140
5	1	3	7	2.500	0.117	0.055	0.013	2.080	1.890
5	1	3	7	2.500	0.118	0.055	0.012	2.180	1.900
5	1	3	21	2.500	0.542	0.278	0.048	3.757	1.780
5	1	3	21	2.500	0.465	0.245	0.051	3.162	1.690
5	1	3	35	2.500	0.214	0.108	0.024	0.760	1.760
5	1	3	35	2.500	0.188	0.581	0.131	3.828	1.650
5	2	1	1	1.030	0.145	0.072	0.010	2.609	1.780
5	2	1	1	1.030	0.020	0.010	0.010	1.689	1.930
5	2	1	7	2.318	0.020	0.010	0.010	0.083	1.000
5	2	1	7	2.318	0.018	0.009	0.009	0.068	1.000
5	2	1	21	0.616	0.002	0.011	0.011	0.670	0.830
5	2	1	21	0.616	0.011	0.006	0.006	0.720	0.830
5	2	1	35	0.951	0.099	0.057	0.022	0.730	1.350
5	2	1	35	0.939	0.116	0.070	0.029	0.994	1.240
5	2	1	49	0.632	0.042	0.023	0.009	0.234	1.430
5	2	1	49	0.654	0.114	0.064	0.025	0.610	1.390
5	2	3	1	1.030	0.145	0.072	0.017	2.609	1.780
5	2	3	1	1.030	0.082	0.037	0.009	1.689	1.930
5	2	3	7	5.000	0.063	0.028	0.014	0.980	1.750
5	2	3	7	5.000	0.085	0.035	0.014	1.420	2.030
5	2	3	21	1.250	0.371	0.198	0.047	2.459	1.640
5	2	3	21	1.250	0.248	0.126	0.022	1.700	1.720
5	2	3	35	2.532	0.888	0.499	0.109	3.116	1.560
5	2	3	35	2.532	0.567	0.315	0.080	1.948	1.550
6	1	1	1	1.045	0.061	0.028	0.007	1.550	1.930
6	1	1	1	1.045	0.062	0.031	0.006	1.872	1.870
6	1	1	7	1.000	0.145	0.072	0.017	0.720	1.400
6	1	1	7	1.000	0.063	0.028	0.014	0.700	1.200
6	1	1	21	1.610	0.122	0.058	0.017	0.680	1.230
6	1	1	21	1.610	0.122	0.058	0.017	0.600	1.250
6	1	1	35	1.195	0.256	0.168	0.082	1.535	1.040
6	1	1	35	1.195	0.302	0.211	0.141	1.912	0.600
6	1	1	49	1.195	0.310	0.180	0.066	1.016	1.360

6	1	1	49	1.195	0.289	0.155	0.048	0.956	1.560
6	1	3	1	1.045	0.061	0.028	0.007	2.230	1.930
6	1	3	1	1.045	0.062	0.031	0.006	1.588	1.870
6	1	3	7	2.500	0.075	0.040	0.012	2.185	1.800
6	1	3	7	2.500	0.087	0.049	0.014	1.885	1.170
6	1	3	21	2.500	0.328	0.176	0.038	3.192	1.970
6	1	3	21	2.500	0.259	0.120	0.027	2.668	1.500
6	1	3	35	2.500	0.331	0.202	0.059	3.828	1.350
6	1	3	35	2.500	0.092	0.060	0.020	0.760	1.200
6	2	1	1	1.035	0.069	0.034	0.008	2.214	2.100
6	2	1	1	1.035	0.020	0.088	0.002	0.348	1.290
6	2	1	7	1.000				0.840	1.200
6	2	1	7	1.000				0.870	1.100
6	2	1	21	1.616				0.650	0.980
6	2	1	21	1.616				0.710	1.000
6	2	1	35	1.221				0.750	1.150
6	2	1	35	1.221				0.760	1.160
6	2	1	49	1.163	0.346	0.179	0.059	1.148	1.600
6	2	1	49	1.163	0.540	0.282	0.085	1.820	1.610
6	2	3	1	1.035	0.069	0.034	0.008	2.214	2.100
6	2	3	1	1.035	0.020	0.008	0.002	0.348	1.290
6	2	3	7	5.000	0.066	0.032	0.015	1.320	1.590
6	2	3	7	5.000	0.075	0.039	0.014	1.220	1.560
6	2	3	21	2.500	0.566	0.289	0.059	4.056	1.750
6	2	3	21	2.500	0.615	0.317	0.068	4.376	1.730
6	2	3	35	2.517	0.079	0.052	0.020	0.469	0.880
6	2	3	35	2.517	0.044	0.029	0.012	0.254	0.710

Variety
1- Amantin
2- Asontem
3- Ayiyi
4- Bengpla
5- Ejura Red
6- Soronko

Leafage (Type of Leaf)
1- Flush leaves
2- Mature Leaves

Storage (Storage condition)
1- Benchtop (Room Temperature, >25°C)
2- Herbarium
3- Freezer (-20°C)

TIME (Days)- Duration of storage of leaves prior to extraction of DNA.
WEIGHT(g)[1] - Fresh weight of harvested leaves on day 1 (on harvesting).
DNA YIELD calculated in µg/mg of leaf
D_{OD260}- Absorbance measured at 260 nm to estimate the quantity of nucleic acids present in the extract.
P_{OD280}- Absorbance measured at 280 nm to estimate the quantity of proteins present in the extract.
E_{OD300}- Absorbance measured at 300 nm to estimate the quantity of proteins present in the extract which absorb at 260 nm.
PURITY- Ratio of DNA to Protein calculated as outlined in Appendix B

Appendix B

Table B.1: Weights of leaf varieties taken, spectrophotometric measurements, DNA yield, the ratio of DNA to Protein, and the ratio of DNA to polysaccharides

Variety	Weight (g)	D_{OD260}	P_{OD280}	E_{OD300}	DNA Yield	Purity (Protein)
Amantin	6.651	0.716	0.349	0.024	3460	2.12
Asontem	9.990	0.889	0.437	0.030	4345	2.11
Ayiyi	9.920	0.720	0.354	0.034	4343	2.02
Bengpla	3.675	0.708	0.348	0.029	3395	1.94
Ejura Red	21.330	0.048	0.029	0.007	205	0.91
Soronko	11.055	0.804	0.386	0.026	3887	2.22

D_{OD260}- Absorbance measured at 260 nm to estimate the quantity of nucleic acids present in the extract.

P_{OD280}- Absorbance measured at 280 nm to estimate the quantity of proteins present in the extract.

E_{OD300}- Absorbance measured at 300 nm to estimate the quantity of proteins present in the extract which absorb at 260 nm.

The yield of DNA in the original sample was estimated as:

$$\text{DNA yield } (\mu g/ml) = 50\mu g(D_{OD260} - E_{OD300}) \times A \times B^{-1}$$

where A: Total volume (µl) of sample prepared, 500 µl
B: Aliquot (µl) of sample taken, 50 µl

The purity of the DNA extract was estimated as follows:

$$\text{Ratio of DNA:Protein, Purity (protein)} = (D_{OD260} - E_{OD300}) \times D_{OD280}^{-1}$$

Table B2: Summary of data on Storage Conditions on DNA Concentration and Purity of the Pooled Flush leaf Samples

	Mean DNA Yield[1] (µg/mg)		Mean DNA Purity[2] of Extract	
Time (Days)	Benchtop	Freezer	Benchtop	Freezer
1	3.785	1.810	1.826	1.938
7	1.467	2.083	1.440	1.690
21	1.006	3.210	1.319	1.735
35	1.331	2.350	1.165	1.490
49	0.795	2.399	1.303	1.713

Table B3: Summary of data on Storage Conditions on DNA Concentration and Purity of the Pooled Mature Leaf Samples

	Mean DNA Yield[1] (µg/mg)			Mean Purity[2] of Extract		
Time (Days)	Benchtop	Freezer	Herbarium	Benchtop	Freezer	Herbarium
1	1.704	2.182	5.959	1.730	1.778	1.983
7	1.256	1.235	2.191	1.213	1.733	1.580
21	0.661	3.374	2.333	0.961	1.710	1.506
35	0.777	1.675	1.460	1.374	1.550	1.480
49	0.361	2.026	1.372	1.216	1.708	1.253

Appendix C Reagents

The following reagents were used:

Ethylene-diaminetetraacetate, disodium salt, (EDTA) from M&B Chemicals, England; Tris(hydroxymethyl)aminomethane, Sodium hydroxide, Sodium chloride, Isopropanol, Phenol, Hydrochloric acid (concentrated), Sodium dodecylsulphate (SDS), Ethanol (96%), Acetic acid, Potassium acetate, Sodium acetate, Isoamyl alcohol, Silver nitrate and Ethidium bromide were obtained from Fluka AG Garantie Chemika, Switzerland.

Polyvinylpyrrolidone (PVP MW 10,000), Agarose, Sucrose, Mineral oil (Light white oil) and Bromophenol blue were also obtained from Sigma Chemical Company, USA.

Chloroform, 2-mercaptoethanol, 8-Hydroxyquinoline and Boric acid, Formamide were obtained from BDH Chemicals, England.

Acrylamide (electrophoresis grade) and Bisacrylamide (electrophoresis grade) were obtained from Bio-Rad Laboratories, U.S.A.

Ammonium persulphate and Orange C were obtained from Kanto Chemical Company Industries Ltd., Japan; Formaldehyde was obtained from Riedel-de-Haën AG D-3016, Seelze, Germany

Agarose (Molecular Biology grade) was obtained from Promega Corporation, U.S.A.

Enzymes

The following enzymes were used in the present work:

Ribonuclease 1, Deoxyribonuclease 1 were obtained from Sigma Chemical Company, U.S.A., and Taq DNA Polymerase (5U/µl). The Taq polymerase was supplied as a set with its

accompanying buffer, $MgCl_2$ (50 mM) and W-1 (1.0%) were supplied by GIBCOBRL (Life Technologies), U.S.A.

The restriction enzymes *Alu*I, *Dra*I, *Eco*RI, *Hae*III, and *Hinf*I and their accompanying buffers, were obtained from Sigma Chemical Co., U.S.A.

Eco RV, *Kpn*I, *PVu*I were obtained from Nortumbria Biologicals Limited,UK.

The following reagents were supplied by Pharmacia, Netherlands

Primers

Primer	Length	Sequence
AR	20	AAGTGTCCTCTCCATTCCTA
GA	20	CTGGTTTGGTCGCGACGTTT
UN	20	GTGTGCCCCTTCCTCGATGT
QD	20	CAGACCAAGATGGTTAGTAT
ME	20	TGACCAACCCACTCCCTTGA
BD1	20	GTCGTAACAAGGTTTCCGTA
BD1F	20	TCCTGCAGTTCACATTCTGAC
HISP	20	TAGGTATAGATGTGGACAGC
ITS	20	TACATGGCGGGGTCAGTTTG
ITS4S	20	TAGTGACGCGCATGAATGGA
MT1	20	TCAAACGGCGGGAGTAACTA
MT2	20	CCCCTTCGTATGTGTTGTTT
PR2	20	CCTGTGCGATTTCACGCAGG
VI4	20	CCTGTGCGATTTCACGGACC
V15	20	CTGGACGTGGAAAAGGTGGT

Deoxyribonucleotide triphosphate (dNTPs)

The four deoxyribonucleotides, deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythiamine triphosphate (dTTP).

Bacteriophage Lambda (λ)DNA was obtained from GIBCOBRL (Life Technologies), U.S.A.

Appendix D

Standard Solutions

The following standard solutions were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121lbs for 15 minutes in an Eyela Autoclave (Rikikkaki, Tokyo).

DNA Extraction Buffer

50 mM EDTA, pH 8.4, 100 mM Tris.HCl, pH 8.0, 0.1% 2-mercaptoethanol, 10% SDS, 1.5% PVP (MW 11,000), 500 mM NaCl

Tris.HCl-EDTA (TE) pH 8.0

10 mM Tris.HCl (pH 8.0), 1 mM EDTA(pH 8.0). Stored at room temperature

1 M Tris.HCl (pH 8.0)

121.1 g Tris base was dissolved in 800 ml of sddw. The pH was adjusted to 8.0 by the addition of concentrated HCl. The solution was allowed to cool to room temperature and the volume made up to 1000 ml with sddw. It was then autoclaved and stored at room temperature

0.5 M EDTA (pH 8.4)

186.1 g/l was dissolved in 800 ml of sddw while stirring. The pH was adjusted to 8.4 with NaOH pellets, autoclaved and stored at room temperature

5.0 M NaCl

292.2 g of NaCl was dissolved in 800 ml of sddw. The volume was adjusted to 1000 ml with sddw, autoclaved and stored at room temperature.

5.0 M KAc (pH 4.8)

Autoclaved and stored at room temperature

5.0 M NaCl

146.1g of NaCl crystals dissolved completely while stirring. Autoclaved and stored at room temperature.

3.0 M NaAc (pH 7.6)

102 g NaAc was dissolved in 200 ml of sddw. The pH was adjusted to 7.6 with glacial acetic acid and the volume made up to 250 ml with sddw. Autoclaved and stored at room temperature

10% SDS

50 g of electrophoresis-grade SDS was added to 225 ml of sddw, heated to 68°C in a waterbath to dissolve and the pH adjusted to 7.2 by the addition of drops of concentrated HCl. The volume was made up to 500 ml with sddw.

70% EtOH

72.9 ml of 96% EtOH was adjusted to 100 ml with sddw and stored at -20°C

EtBr (10 mg/ml)

1 g EtBr was completely dissolved in 100 ml ddw and stored in the dark 4°C.

Ribonuclease

10 mg/ml of ribonuclease (powder) was dissolved in water, heated to 65°C to inactivate any DNAse present, sterilized by filtration and stored at -20°C.

Solutions for Electrophoresis

Agarose Gels

10x TAE buffer

242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with ddw

SDS Polyacrylamide Gels

10x TBE

15.1g/l Tris base, 72.0g/l glycine in ddw. stored at room temperature. pH 8.3 is reached when diluted to 1x working solution.

29% Acrylamide: 1% Bisacrylamide Solution

29g Acrylamide, 1 g bisacrylamide in 100 ml ddw. Stored in the dark at 4°C.

5% Stacking gel solution (50 ml)

8 ml 29% Acrylamide:1.0% Bisacrylamide solution, 6.25 ml Tris.HCl pH 6.8, 0.5 ml 10% SDS in ddw. Stored in the dark at 4°C.

12.5 % Separating (Resolving) gel solution (30 ml)

15.0 ml 29%Acrylamide:1.0% Bisacrylamide solution, 3.75 ml Tris. HCl (pH 8.8), 0.3 ml 10% SDS.

Gel Loading

Buffers

6x Bromophenol blue 0.25% bromophenol blue, 40% sucrose in water. Stored at 4°C.

5x Orange G

20% (w/v) Ficoll, 25 mM EDTA, 2.5% (w/v) orange G. Stored at room temperature.

Bromophenol blue and Xylene cyanol in Formamide

0.5% bromophenol blue, in 75% formamide. Stored at 4°C

Silver Staining Solutions

Fixative	10% EtOH:0.5% glacial acetic acid. Stored at room temperature
Staining solution	0.984 g $AgNO_3$ in 500 ml ddw (11 mM). Stored in the dark at room temperature
Developing solution	15 ml 5.0 M NaOH, 0.8 ml Formalin made up to 100 ml with sddw. (Prepared when needed).
Quenching solution	10% glacial acetic acid. Stored at room temperature.

All solutions and buffers were prepared from their standard solutions using sddw.

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