STUDIES ON CHLOROPLAST DNA RESTRICTION FRAGMENT LENGTH POLYMORPHISMS OF THREE *SPECIES OF GUIZOTIA CASS. (G. abyssinica, G. scabra ssp. scabra and G. scabra ssp. schimperi)*

By

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ABSTRACT

Chloroplast DNA from three *Guizotia* species (*G. abyssinica, G. scabra ssp. scabra* and *G. scabra ssp. schimperi*) were isolated using sucrose gradient centrifugation method and Dynabeads DNA DIRECT™ System I. Each of the DNA extracted by sucrose gradient method was separetely digested with BamHI, EcoRI and HindIII restriction endonuclease enzymes, recognizing 6 base pairs at 37°C for 4hr. The digestion products were then loaded on 0.8% agarose gel and electrophoresed at 30-50 volts for 3-4 hours. After visualizing with 0.5µg/ml of ethidium bromide solution and photographed using polaroid films, the fragment bands were compared. In addition, the DNAs extracted by Dynabeads DNA DIRECT™ System I method were used for PCR amplification of chloroplast DNA non-coding region (*trn*L (UAA) intron) using Primer C and D of Taberlet *et al.* (1991). The amplified chloroplast DNA non-coding region was digested with Sau3AI restriction endonuclease enzyme recognizing 4 base pairs at 37°C for 2 hr. and electrophoresed in 2% agarose gel at 100 volts for 2 hr. After being visualized with 0.5µg/ml ethidium bromide solution and photographed using polaroid films, the fragment bands were compared. In both cases, detectable variation was not observed among the *Guizotia* species compared, indicating that these species are either closely related and recently diversified or their chloroplast DNAs are evolving at a relatively slower rates in comparison to the rates of cpDNA evolution in other angiosperm species.

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1. Introduction

1. 1. Taxonomy of the genus *Guizotia*

The genus belongs to the family Compositae, tribe Heliantheae, subtribe Corepsidinae. The taxonomy of the genus was revised by baagoe (1974) and it comprises three annual and four perennial species/subspecies . The annual species are *G. abyssinica* (L. f.) CASS., *G. scabra* (V. S.) CHIOV. *ssp. schimperi* (SCH. BIP.) Baagoe, and *G. villosa* SCH. BIP. in WALP, while the perennial species include *G. scabra* (VIS.) CHIOV. *ssp. scabra, G. arborescens* I. FRIIS, *G. zavattari* LANZA in CHIOV, and *G. reptans* HUTCH (Baagoe, 1974). According to Kifle Dagne (1994) there are more *Guizotia* taxa/accessions than recognized so far (e. g. Ketcha and Chelelu populations).

Baagoe (1974) grouped *G. scabra* and *G. schimperi* as subspecies of *G. scabra*, and described them as *G. scabra* (VIS.) CHIOV. *ssp. schimperi* SCH. BIP. in WALP Baag. and *G. scabra* (VIS.) CHIOV. *ssp. scabra* respectively. But, on the basis of their differences in habit, distribution, karyotype and cytology of the hybrids, the two subspecies could have been treated as two independent species (Hiremath *et al.*, 1992; Murthy *et al.*, 1993; Kifle Dagne, 1994).

The result obtained from the hybridization study indicated that *G. scabra ssp. scabra* is more similar to *G. villosa* than to *G. scabra ssp. schimperi* (Kifle Dagne, 1994). On the other hand, *G. scabra ssp. schimperi* is morphologically (e.g. annual habit, ovate outer phyllaries and lower number of florets) more similar to *G. abyssinica* than to *G. scabra*

ssp. scabra which is characterized by it's perennial habit, stiff leaves, lanceolate outer phyllaries, and higher number of florets (Baagoe, 1974; Kifle Dagne, 1994; Getinet and Sharma, 1996). From the chromosome pairing data in the hybrids, Hiremath *et al.* (1992) and Murthy *et al.* (1993) concluded that *G. abyssinica* and *G. scabra ssp. schimperi* are almost equivalent in their relationships to *G. scabra ssp. scabra*. Murthy *et al.* (1993) further suggested that *G. abyssinica* and *G. scabra ssp. schimperi* are conspecific members of the same species for they cross easily, their F_1 meiosis is nearly complete and the F_1 are highly fertile. *Guizotia abyssinica* is more crossable with *G. scabra ssp. schimperi* (41%) than with both *G. scabra ssp. scabra* and *G. villosa* (20%). On the other hand, *G. scabra ssp. schimperi* is more crossable with both wild taxa *G. scabra ssp. scabra* (62%) and *G. villosa* (46%) than to the cultivated *G. abyssinica* (41%), and *G. scabra ssp. scabra* is more crossable with *G. villosa* (82%) than with *G. scabra ssp. schimperi* (62%) (Kifle Dagne, 1994). In general, there is an indication of uncertainity with the taxonomic position of *G. scabra ssp. schimperi* (whether to treat it as an independent species, as member of the same species together with *G. abyssinica* or remain as suspecies of *G. scabra*).

1. 2. Geographic distribution of the genus Guizotia

The genus *Guizotia* occurs mainly in the tropical Eastern Africa with the highest species concentration in Ethiopia (Baagoe, 1974). *G. abyssinica* is cultivated in Ethiopia, some other East African countries and Indian subcontinent; *G. scabra ssp. scabra* is widely distributed in Eastern part of Africa and extends to West Africa; and *G. scabra ssp. schimperi* occurs in the Ethiopian highlands and few collections from Yemen were

suspected to be alien (Baagoe, 1974). *G. villosa* is endemic to the Northern part of the Ethiopian highlands. *G. arborescens* is endemic in South West Ethiopia and the Imantong Mountains on the border between Uganda and Sudan. *G. zavattarii* is endemic around Mount Mega in southern Ethiopia and the Huri Hills in northern Kenya. *G. reptans* has been known from Kenya and Uganda. In general, All *Guizotia* taxa are found in Ethiopia with the exception of *G. reptans,* indicating that this genus probably has originated or genetically diversified in this country (Baagoe, 1974; Kifle Dagne, 1994).

1. 3. Economic importance of the genus Guizotia

The genus *Guizotia* is of economic valve since it contains *G. abyssinica* (noug) (niger) which is an edible oil seed crop cultivated predominantly in Ethiopia and India (Hiremath and Murthy, 1988; Getinet and Sharma, 1996). The domestication of *G. abyssinica* (niger) was assumed to begin in Northern Ethiopia as early as 3000 B. C and the crop probably was introduced into India through trade routes (Hiremath and Murthy, 1988).

Niger is a cool season crop (Abebe *et al.*, 1978) with a very low yield of 200-400 kg/ha. on farmers fields and 1000-1500 kg/ha. under experimental conditions (Riley and Belayneh, 1989). This low yield is probably due to preharvest seed loss resulted from uneven ripening of heads on individual plants and the whole field (Seegeler, 1983). More over, the self incompatibility of the species hinders the development of inbred lines to produce synthetic varieties that yield more (Riley and Belayneh, 1989).

Niger seeds contain 30-50% oil (Seegeler, 1983), and it provides about 50-60% of edible oil in Ethiopia and accounts for 2% of the total oil seed production in India (Riley and Belayneh, 1989). Studies indicate that there is significant difference between *G. abyssinica* and the wild species in their seed oil content, being 42-44% in the former and 21-33% in the later, indicating that domestication has accounted for increased oil content of *G. abyssinica* (Kifle Dagne). On the other hand, strong similarity was observed in terms of fatty acid composition of *Guizotia* taxa/accesions (Kifle Dagne, 1994).

Niger oil is mainly used for cooking purposes, and also used in the manufacture of soap, paints, and as illuminant or lubricant (Vaughan, 1970). In addition, the press cake which contains 24-30% protein is used as animal feed, manure or fuel (Riley and Belayneh, 1989).

From fatty acid composition point of view, the oil of wild *Guizotia* seems safe for human consumption. But, for the wild *Guizotia* taxa have low seed oil content and have no new oil quality in terms of fatty acid composotion, other than what is contained in the *G. abyssinica*, the utilization of the wild *Guizotia* taxa as sources of oil is not feasible at present (Kifle Dagne, 1994).

1. 4. Cytology and karyotype of the genus Guizotia

Cytological studies showed that all *Guizotia* taxa have 2n= 30, indicating that

speciation within the genus did not involve changes in the chromosome number (Hiremath and Murthy, 1992; Kifle Dagne, 1994). This similarity of chromosome number among *Guizotia* taxa/accessions makes it difficult to determine the basic chromosome number of the genus, which still remains unknown (Murhty *et al.*, 1993; Kifle Dagne, 1994). On the basis of centromeric position and number of satellite chromosomes, *G. abyssinica and G. scabra ssp. schimperi* could be placed together for they resemble each other by having predominantly median type centromeric position and eight satellite chromosomes, whereas *G. scabra ssp. scabra* is more related to *G. villosa*, whose chromosomes are predominantly submedian and subterminal types and have four satellite chromosomes (Kifle Dagne, 1974).

1. 5. Evolutionary trends and phylogenetic relationships within the genus Guizotia

According to Baagoe (1974), the study on evolutionary trends and phylogenetic relationships within the genus *Guizotia* indicate that *G. scabra ssp. scabra* has the highest correlation of primitive characters (e.g. caudex perennial habit, leaves sessile, leaves isophyllous, heads solitary and florets numerous), and *G. reptans* and *G. arborescens* have the highest correlation of advanced characters (e.g. annual habit, leaves petiolate, leaves anisophylous, synflorescences and florets few), while the other species are approximately intermidiate.

As of the phylogenetic relationships within the genus *Guizotia*, Baagoe (1974) proposed

that *G. abyssinica* (niger) was derived from *G. scabra ssp. schimperi* and acquired it's specific characters through selection under cultivation. From the results of hybridization study, the chromosomes of *G. abyssinica* and *G. scabra ssp. schimperi* were found to be basically similar, and this supports the assumption that *G. abyssinica* was originated from *G. scabra ssp. schimperi* through selection and further cultivation of large achene mutant (Murthy *et al.*, 1993). Baagoe (1974) also suggested that *G. villosa* was probably derived from *G. scabra* (ssp. not specified), and Hiremath *et al.* (1992) speculated that *G. scabra ssp. schimperi* was a possible progenitor of *G. villosa*. But, on the basis of karyotype and chromosome pairing in hybrids, *G. villosa* shows more affinity to *G. scabra ssp. scabra* than to *G. scabra ssp. schimperi* (Kifle Dagne, 1994) and this is in line with the sugestion of Baagoe (1974) that *G.villosa* was possibly derived from *G. scabra ssp. scabra* or from an unknown ancestor like this taxon.

Strictly speaking, the phylogenetic relationships as well as the taxonomic positions of some *Guizotia* species have not been resolved. For example, the need to reconsider the taxonomic status of *G. scabra ssp. scabra* and *G. scabra ssp. schimperi* as subspecies, the possibility of other forms of Guizotia (e. g. Chelelu population) to be a possible progenitor of *G. abyssinica*, and the question of considering *G. scabra ssp. scabra* or *G. scabra ssp. schimperi* as a progenitor of *G. villosa* have not been yet resolved.

Kifle Dagne (1994) summarized the genomic relationships and phylogenetic affinities within the genus *Guizotia* on the basis of karyotype and crossability. But, to construct a final phylogeny of all taxa/accessions of the genus *Guizotia,* additional data from morphological, anatomical, biochemical and molecular studies are needed (Kifle Dagne, 1994). Molecular techniques could provide solution to the phylogenetic problems of the species of *Guizotia* such as investigation of the proginator of *G. abyssinica* (Getinet and Sharma, 1996). One of the data obtained from molecular genetic analysis is generated by restriction fragment length polymorphisms (RFLPs) through the use of type two restriction endonucleases to fragment DNA molecules at specific recognition sites (Clegg *et al.*, 1984).

Restriction fragment length polymorphisms (RFLPs) of the entire cpDNA and cpDNA non-coding regions are often used to describe phylogenetic relationships between related genera and species and the method has been applied to many taxa (e.g. Palmer *et al.*, 1985; Sytsma and Gottlieb, 1986). In case of the genus *Guizotia*, inspite of its economic importance and limited species number, data from molecular studies are not well documented and in particular restriction fragment length polymorphisms of chloroplast DNA have not been worked out. Thus, the present study was conducted with the following objectives in mind.

General objectives

To document molecular information of taxonomic and phylogenetic importance for the genus *Guizotia* Cass.

To enrich information on the role of chloroplast DNA to resolve interspecific phylogenetic questions.

Specific objectives

To determine the patterns of cpDNA restriction fragment length polymorphisms for *G. abyssinica* and the two subspecies of *G. scabra* using three restriction endonuclease enzymes (BamHI, EcoRI and HindIII).

To investigate the comparative rate of differentiation among these species using the present data and previously studied characters.

To make phylogenetic and taxonomic inferences using the result obtained in this study in relation with previously studied morphological and cytological data.

2. Literature Review

2. 1. Molecular techniques

The study of systematic relationships and phylogenies among plant taxa traditionally has depended on comparative analysis of phenotypic characters. Problems can potentially arise when these phenotypes are utilized to infer phylogenetic taxonomies, and these problems stem from apparent plasticity of many characters and our lack of knowledge of the genetic bases or developmental pathways responsible for the plasticity of characters (Sytsma and Schaal, 1985). In contrast, recombinant DNA technology is providing evolutionary biologists with another tool for making phylogenetic inference through contrasts of molecular genetic analysis (Templeton, 1983). Phylogenetic analysis of plants at the molecular level is increasingly providing detailed and often unexpected evidence of species relationships (Sytsma and Gottlieb, 1986). Molecular genetic analysis is providing two types of data. The first class of data derives from comparative analysis of complete DNA sequences (Aqudro and Greenberg, 1983; Zurawski *et al.*, 1984). The second class of data comes from restriction fragment length polymorphism (RFLP) through the use of type two restriction endonucleases to fragment DNA molecules at specific recognition sites (Clegg *et al.*, 1984). The power of these molecular techniques for systematic and evolutionary studies lies in their direct analysis of the genotype rather than of drived phenotypes and in their ability to detect numerous mutation events that can be used to infer phylogenies (Sytsma and Schaal, 1985).

2. 1. 1 Sequences of homologous DNA segments

Definitive studies of genetic variation can only be made at the primary sequence level in regions of known genetic function (Holwerda *et al.*, 1986). DNA sequences contain useful information about evolutionary history (Palmer *et al.*, 1988; Fangan *et al.*, 1994). The main limitations for a more extensive use of DNA sequence data until the mid of the past decade was its high cost and time consuming method (Clegg, 1993). But, the situation changed in the mid 1980s when the polymerase chain reaction (PCR) method appeared. The appearance of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) has been a major step forward in making a rapid path from plant tissue to DNA sequencing. PCR, coupled with the direct dideoxynucleotide sequencing of amplified products has suddenly produced a great wealth of sequence data (Clegg, 1993). Two methods are avialable for DNA sequencing : the chemical degradation method of Maxam and Gilbert (1977), and dideoxy chain termination method of Sanger *et al.* (1977). DNA sequencing by the Sanger dideoxy chain termination method has undergone significant refinement in recent years, including the development of additional enzymes (Tabor and Richardson, 1987), an instrument for partial automation of DNA sequence analysis (Smith *et al.*, 1986). The basic procedure of DNA sequencing by Sanger dideoxy chain termination method involves: hybridization of an oligonucleotide primer to a suitable single or denatured double stranded DNA template, extention of the primer with DNA polymerase in four separate reaction mixtures (each containing one alpha- labeled dNTP, a mixture of unlabeled dNTPs and one chain terminating ddNTP), resolution of the four sets of reaction products on a high reaction polyacrylamide/urea gel, production of an autoradiographic image of the gel, which can

be examined to infer the DNA squence (Innis *et al.*, 1988).

2. 1. 2. Restriction fragment length Polymorphisms (RFLPs)

Several studies have demonstrated variability in DNA fragments after digestion by one or more sequence specific restriction enzymes (Beckmann and Soller, 1986). The demonstration of RFLP involves extraction and purification of DNA from an individual and digestion of the DNA with a restriction endonuclease to form a mixture of restriction fragments, differing in length according to the specific distribution of cleavage sites along the DNA molecules that can be separated by gel electophoresis (Engels, 1981). A major advantage of this technique is that it can be used for any segment of the genome, regardless of whether it codes for a soluble protein.

The main properties that make RFLP a convenient genetic marker, useful at all levels of biological organizations, are its detectability in all tissues at all ages, thus enabling early detection, long shelf life of the DNA samples, its being informative about the nature of the variation, virtually unlimited number of probe and enzyme combinations available, and probes not being restricted to coding sequences (Beckman and Soller, 1986).

2. 1. 3. Application of molecular techniques

Molecular markers reveal variability at the primary DNA sequences of both coding and non-coding regions of the genome, which is the most basic material to address issues of evolutionary descent (Crawford, *et al.*, 1990; Engels, 1981; Song *et al.*, 1988; Sytsma and Schaal, 1985). Isozyme markers have proved useful to this end, however, they are restricted to a limited number of coding regions of the genome and they can detect only those mutations that can cause changes in protein mobility (Aldrich and Doebley, 1992). Estimates of over all levels of genetic diversity may be more reliable when measured with molecular markers such as restriction site analysis than by isozymes, because of the greater number of loci avilable and the ability to survey both coding and non-coding regions of the genome (Doebley and Wendel, 1989 cited in Aldrich and Doebley, 1992). In addition, RFLP markers are potential land marks for the physical mapping of chromosomal regions that can not be further resolved by the classical genetic methods (Graner *et al.*, 1990).

Molecular markers are also valuable tool for studying the evolution of crops and their wild relatives. Wild relatives of crop plants have often been considered to be important sources of genes of special interest in plant breeding programs. Thus, comparative molecular assessments are necessary in order to obtain knowledge concerning genetic variability in crop and their wild relatives (Saghi-Maroof *et al.*, 1995).

Genetic variability data from molecular markers at the interspecific, intraspecific and intrapopulation levels are used in understanding evolutionary mechanisms for the

development of plant and animal breeding programs and conservation programs aimed at conserving the gene pools of endangered species (Banks and Birky, 1985). For all these purposes it is important to know the variability of genes in mitochondria, chloroplasts as well as in of the nucleus. The application of molecular markers such as RFLPs to the phylogenetic problems in plants has been almost exclusively confined to the relatively small genome of chloroplasts (Bowman *et al.*, 1983; Clegg *et al.*, 1984; Gordon *et al.*, 1982; Palmer and Zamir, 1982; Palmer *et al.*, 1983). More over, analysis of organelle genome such as chloroplast DNA (cpDNA) has several advantages over the analysis of nuclear genes and their products, because the former is less prone to DNA alterations through mutations, recombinations and introgression (Bowman *et al.*, 1983). In addition, nuclear DNA is larger than organellar DNA by many orders of magnitude and its restriction analysis is almost impossible without cloned probes that can isolate specific DNA segment (Sytsma and Schaal, 1985). The mitochondrial DNA (mtDNA) of plants is also both more difficult to isolate and more complex than is DNA from chloroplasts (Stern and Lonsdale, 1982; Stern and Palmer, 1984).

2. 2. Chloroplasts and chloropplast DNAs

Chloroplasts are plant cellular organelles having an important role in photosynthesis. The endosymbiont hypothesis postulates that modern chloroplasts are descendants of endosymbiotic prokaryotes, particularly cyanobacteria which have entered eukaryotic cells during evolution (Bonen *et al.*, 1975).

Chloroplasts contain their own autonomously replicating DNA. The majority of the proteins present in the chloroplasts are encoded by nuclear DNA and imported post translationally into the chloroplast. But, the rest of the proteins in the chloroplasts are encoded by (cpDNA) and synthesized by the chloroplast transcription-translation machinery (Gantt, 1991; Sugiura, 1992). The translation process within a chloroplast is mediated by its own ribosomes of the 70 S type, which are different from the 80 S ribosomes of the cytoplasm (Schwarz and Kossel, 1979).

One important feature of organelle genes is that they are often inherited from the female parent. This is often caused by the paternal gametes transmitting few or no organelle genomes to the egg, which is a large cell with many copies of each organelle gene (Birky *et al.*, 1983), or by the action of DNA modification enzymes leading to methylation of the maternal DNA and destruction of the homologous paternal DNA (Burton *et al.*, 1979).

2. 2. 1. Structural organization of chloroplast DNA

Plant chloroplasts contain 20-200 copies of closed circular DNA molecules ranging from 120-180 kbp, which are organized into a large (78-100 kbp) single copy region and a small (12-30 kbp) single copy region, separated by two sequences (20-24 kbp) arranged in an inverted orientations (Close *et al.*, 1989; Kolodner and Tewari, 1979; Morton and Clegg, 1993; Palmer and Thompson, 1981). The arrangement of the genome is conserved throughout of the angiosperms with the exception of several species of the Viceae tribe of the legume family in which the inverted repeat is lacking

(Bonnard *et al.*, 1985; Palmer and Thompson, 1981, 1982).

2. 2. 2. Evolution of chloroplast DNA

In higher plants (angiosperms) the chloroplast genomes appear to be very stable in evolutionary terms (Coates and Cullis, 1987). Studies suggest that the inverted repeats evolve more slowly than the unique sequence regions and different portions of the large unique sequence region have dissimilar rates of sequence evolution (Ogihara and Tsunewaki, 1988; Tassopulu and Kung, 1984). The low frequency of structural changes in angiosperm chloroplast genomes is complemented by a conservative rate of sequence evolution. By some estimates, the synonymous substitution rate is 0.1% per million years (Zurawski *et al.*, 1984). An important contributing factor to this conservation arises from the DNA sequences coding for the chloroplast ribosomal RNA (cprRNA), which are highly conserved in evolution (Duvall and Doebley, 1990). Although it is accepted that evolution of cpDNA is conservative, restriction fragment analysis and nucleotide sequencing revealed a number of mutations of this genome (Ogihara and Tsunewaki, 1988; Gordon *et al.*, 1982; Zurawski and Clegg, 1987). These mutations are structural changes such as inversions, rearrangements of gene order, and insertions/deletions as well as base substitutions. Most of the cpDNA variation is due to nucleotide substitutions or small deletions and additions of 1-10 bp in the non-coding regions (Palmer 1987; Zurawski *et al.*, 1984).

2. 2. 3. Chloroplast DNA as a marker molecule

The high sequence conservation combined with the cytoplasmic inheritance of chloroplast DNA makes the molecular analysis of cpDNA a particularly useful tool for understanding evolutionary relationships between closely related species (Curtis and Clegg, 1984). The popularity of cpDNA as marker molecule is based on generalizations about its structure and evolution (Palmer *et al.*, 1988). The chloroplast DNA is small, with a relatively low rate of structural and sequence evolution (low intra specific cpDNA variation, recombination is rare or absent), and the genome is inherited predominantly uniparentally maternal (Harris and Ingram, 1991). On the other hand, the low evolutionary rate of this molecule seriously limits its role as a biosystematically useful marker to the higher taxonomic level (Taberlet *et al.*, 1991).

Chloroplast DNA has been widely employed in phylogenetic studies at higher taxonomic levels using both restriction fragment length polymorphism (RFLP) and nucleotide sequence comparison of homologous DNA segments in different groups (Palmer *et al.*, 1988; Clegg, 1993).

2. 2. 4. Analysis of chloroplast DNA restriction fragment length polymorphisms

The analysis of restriction fragment length polymorphisms (RFPLs) in chloroplast DNA has been used since the early 1980s to study species relationships (Dowling *et al.*, 1996

cited in Yonemori *et al.*, 1998). A number of studies have demonesrated the potential of chloroplast DNA restriction site mutations for resolving phylogenetic relationships among closely related species (Palmer, 1986, 1987; Wang *et al.*, 1992). Restriction fragment variation in the chloroplast genome may also be utilized to study a crop gene pool (Aldrich and Doebley, 1992).

In this paper, we report the results of chloroplast DNA restriction fragment length polymorphisms of three *Guizotia* species (*G. abyssinica*, *G. scabra ssp. scabra*, and *G. scabra ssp. schimperi*) by restricting the entire chloroplast DNA with three restriction endonuclease enzymes (BamHI, EcoRI and HindIII) recognizing six base pairs, and the chloroplast DNA non-coding region (*trn*L (UAA) intron) with one restriction endonuclease enzyme recognizing four base pairs (Sau3AI).

3. Materials and methods

3. 1. Plant materials

A total of 9 accessions representing three species of the genus *Guizotia*, were investigated in the present study (Table 1). Seeds of the wild species (*G. scabra ssp. scabra* and *G. scabra ssp. schimperi*) were collected from the field. The seed samples of cultivated species (G. abyssinica) were kindly provided by Dr. Sileshi Nemomsa (Department of Biology Science Faculty Addis Ababa University). The natural sites from where the seed samples of *G. abyssinica* used in this study were collected are recorded. Representative seeds of each accession were grown under green house conditions at Science Faculty, Addis Ababa University. Leaves were harvested from 5-6 weeks old young plants of each accession and used for extraction of chloroplast DNA (cpDNA).

Table 1. Localities of Guizotia species and accessions used as sources of chloroplast DNAs in the present study.

Species name	Acc.no.	Place of collection
G. abyssinica	1	Region 1, Ma'ikelawi Admimistrative Zone (Lay Maychew, Aksum).
	2	Region 4, West Shoa Administrative Zone (Addis Alem, Kimoye).
	3	Region 4, North Shoa Administrative Zone (Jira Jarso Fiche).
G. scabra ssp. scabra	1	Region 4, Jimma Administrative Zone (9 km. east of Jimma town on A/A road).
	2	Region 4, East Wellega Administrative Zone (7 km. south west of Arjo town on Bedele road).
	3	Region 4, East Wellega Administrative Zone (20 km. west of Bako town on Nekemte road).
G. scabra ssp. schimperi	1	Region 4, Jimma Administrative Zone (5 km. west of Jimma town on Bedele road).
	2	Region 4, East Wellega Administrative zone (12 km. north of Arjo town on Nekemte road).
	3	Region 4, West Shoa administrative Zone (12 km. west of Ijaji town on Bako road).

3. 2. Extraction of chloroplasts and chloroplast DNAs

3. 2. 1. Isolation of chloroplasts

Chloroplasts were isolated from fresh leaves of all accessions of *Guizotia* species considered in this study by sucrose gradient centrifugation method of Palmer (1986). Prior to extraction, plants were kept in the dark for 1-4 days to reduce chloroplast starch levels. Young, healthy, destarched leaves were washed in distilled water and cut into small pieces. To every 25 grams of cut leaves, about 100 ml of ice-cold extraction buffer containing 0.35M sorbitol, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, o.1% (w/v) BSA, and 0.1% (v/v) 2-mercaptoethanol was added (2-mercaptoethanol was added immediately prior to use). The leaf and buffer mixture was homogenized in a prechilled 0.2 litre waring blender for 5 sec. brusts at high speed. The homogenate was filtered through four layers of cheese cloth (with squeezing) and then through two layers of mira cloth (without squeezing) and centrifuged at 5000 rpm at 4°C for 15 minutes to pellet the chloroplasts. The chloroplast pellet was resuspended in 8 ml washing buffer containing o.35 M sorbitol, 50 mM Tris-HCl pH 8.0 and 25 mM EDTA. The resuspended chloroplast pellet was loaded onto a step gradient consisting of 10 ml of 60% sucrose in 50 mM Tris-HCl pH 8.0 and 25 mM EDTA, and over layered with 5ml of 30% sucrose in 50 mM Tris-HCl pH 8.0 and 25 mM EDTA. The sucrose step gradient containing the chloroplast suspension was then placed in SW-(Beckman) ultracentrifuge and centrifuged at 26,000 rpm at 4°C for 45 minutes. The chloroplast band at 30-60% sucrose interface was removed with a micropipette and diluted with 3-10 volumes of washing buffer. The chloroplasts were then pelleted by centrifugation at

5000 rpm for 15 minutes at 4°C. Depending on its size, the chloroplast pellet was resuspended in a washing buffer to a final volume of 2 or 15 ml. 0.5 μl of chloroplast suspension was transferred to each of several eppendorf tubes and 1/5 volume of lysis buffer (2% Sodium Lauryl Sarcosinate solution or 5% triton x-100 in 50 mM Tris-HCl pH 8.0 and 25 mM EDTA) was gently added and mixed by slowly inverting the tube several times over a period of 10-15 minutes, and 0.1 volume of a 10 mg/ml proteinase k solution was added and incubated overnight at 25°C to digest the protein content of the suspension.

3. 2. 2. Chloroplast DNA isolation from lysed chloroplasts

The chloroplast DNA was isolated from lysed chloroplasts according to phenol/chloroform extraction method of Zimmer and Newton (1982) cited in Doebley *et al.* (1987). Equal volume of phenol/chloroform (1:1) was added to each eppendorf tube containing lysed chloroplasts and gently mixed for 10 minutes or vortexed for few seconds and centrifuged at 7500 rpm at 4°C for 10 minutes. The supernatant (phenol phase) was transferred to a new eppendorf tube (1.5 ml), washed and centrifuged at 7500 rpm for 10 minutes at 4°C twice with equal volume of chloroform/isoamyl alcohol (24:1) taking the upper phase each time to remove the phenol.

The final supernatant (upper phase) was transferred to a new eppendorf tube (1.5 ml), and mixed with 2/3 volume of ice-cold 99% ethanol and 30 μl NaAc and placed overnight at -20°C to allow DNA (cpDNA) precipitation. The chloroplast DNA was then pelleted by centrifugation at 7500 rpm for 15 minutes at 4°C. Ethanol was removed (poured off) and the cpDNA pellet was washed with 70% ethanol and repelleted through centrifugation for 10 minutes at 7500 rpm at 4°C. The chloroplast DNA pellet was then air dried to remove the alcohol and dissolved in 100 μl of sterile distilled water and stored at -20°C until used.

3. 3. Digestion of extracted cpDNA samples

The extracted chloroplast DNA of *Guizotia* species in the present study were digested with three restriction enzymes (EcoRI, BamHI and HindIII) recognizing six base pairs (Table 2).

Restriction enzymes were purchased from Promega Corporation (Madison, WI, U. S. A.). The restriction enzymes were used according to manufacturers' recommendations. 2-3 μl of cpDNA aliquot containing 1 to 1.5 μg cpDNA from each sample for each enzyme was added to separate sterile eppendorf tube (0.5 ml) containing 2 μl restriction enzyme 10x buffer E (restriction enzyme 1x buffer E is composed of 6 mM Tris-HCl pH 7.5, 100 mM NaCl, 6 mM $MgCl_2$ and 1mM DTT at 37°C), 0.2 μl of Acetylated 10 μg/μl BSA, 13-14 μl of sterile deionized water and 1 μl of one of the restriction enzymes recognizing 6 bp (Table 2). After mixing gently by pipetting, the tubes were closed, centrifuged for a few seconds in a microcentrifuge and incubated at the optimum temperature (37°C) for 4hrs.

Table 2. Restriction enzymes used in the present study

Enzyme	No of base pairs in the recognition sites	Sequences of the recognition sites
BamHI	6	5'..G□ G A T C C...3' 3'..C C T A G□ G...5'
EcoRI	6	5'...G□ A A T T C...3' 3'...C T T A A□ G...5'
HindIII	6	5'...A□ A G C T T...3' 3'...T T C G A□ A...5'
Sau3AI	4	5'..□G A T C...3' 3'...C T A G□...5'

3. 4. Electrophoresis of digested cpDNA samples

Chloroplast DNA fragments obtained from retriction enzyme digests were separated by electrophoresis at 30-50 voltage using 0.8% agarose gels with TAE running buffer composed of 40 mM Tris acetate pH 8.0 and 1 mM EDTA. The DNA fragments were made visible by staining the gel with ethidium bromide (0.5 μg/ml) and were photographed under UV light using polariod films. From the photographs, the restriction fragment patterns of cpDNA with each enzyme was drawn by measuring the distance of each fragment from the origin (Tsunewaki and Ogihara, 1983). The molecular sizes of each restriction fragment was estimated from its mobility in comparison to HindIII/EcoRI restricted Lamda DNA fragments of known molecular sizes.

3. 5. Isolation of PCR-ready chloroplast DNA using Dynabeads DNA DIRECT™ system I

Besides the use of the entire cpDNA, extracts for PCR amplification of cpDNA non-coding regions, PCR-ready chloroplast DNA was isolated from representative samlpes of *Guizotia* species under consideration using Dynabeads DNA DIRECT™ system I. Dynabeads DNA DIRECT™ System I was purchased from Dynal A.S, Oslo, Norway and used according to the suppliers recommendations. Approximately 30-100 mg fresh plant leaf from 10 days old seedlings of representative samples were used for each isolation. The plant leaf was

homogenized in 1.5 ml eppendorf tube for two minutes in liquid nitrogen with a plastic pestle to mechanically break open the hard cell walls and increase DNA yield and 200 μl (1 unit) fully resuspended Dynabeads DNA DIRECT™ (uniform, superparamagnetic, polymer particles suspended in a lysis buffer) (bottle 1) was added in a single rapid pipetting action. After keeping at room temperature for 5 minutes, the tube was placed in a Dynal MPC (Dynal Magnetic Particle Concentrator) to allow the DNA/Dynabeads DNA DIRECT™ complex to move to the side of the tube facing the magnet. After 1-2 minutes the supernatant was discarded by carefully pipetting off. The tube was then removed from the Dynal MPC and 200 μl washing buffer (Proprietary mix of salts in a colourless aqueous solution) (bottle 2) was added in a single pipetting action so that the complex is flushed off the side wall. The tube was then replaced back into the Dynal MPC for 30 seconds or until the supernatant was cleared, and the washing step was repeated after discarding the supernatant by carefully pipetting off. Finally the DNA/Dynabeads complex was resuspended in 20-40 μl of resuspension buffer composed of 10mM Tris HCl pH 8.0 (bottle 3), pipetted several times until the suspension was homogeneous, eluted by incubation at 65°C for 5 minutes to prevent degradation and stored at -20°C.

3. 6. PCR amplification of the chloroplast DNA non-coding region (*trnL* (UAA) intron)

Up to 20% of the DNA/Dynabeads suspension or 1-3 μl of phenol/chloroform extracted

cpDNA were subjected to 28 cycles of amplification (1 min. at 94°C of denaturation, 1 min. at 49°C of annealing and 2 min. at 72°C of extension) in a total volume of 50 μl PCR reaction mix. consisting of 5 μl of 10x PCR buffer without $MgCl_2$ (100 mM Tris-HCl pH 8.3, 500 mM KCl), 5μl of $MgCl_2$, 5 μl of 200 μM DNTPs, 1 μl of 1 μM of each primer and 0.5 μl of Taq DNA polymerase. The pair of universal primers C and D (Table 3) were used for the amplification of chloroplast DNA non-coding region (*trnL* (UAA) intron) (Fig. 1). 5-10 μl of each PCR products of each representative sample was electrophoresed in 1.6% agarose gel to check for the successful application of the primers.

Table 3. Sequences of a pair of universal primers (primers C and D) used for the amplification of the chloroplast DNA non-coding region (*trnL* (UAA) intron). The code denotes the 3' most base pair in the published tobacco chloroplast DNA sequence. The B and A in the code refer to each strand of the DNA (Taberlet *et al.*, 1991).

Primer	Code	Sequence 5'-3'
C	B49317	CGAAATCGGTAGACGCTACG
D	A49855	GGGGATAGAGGGACTTGAAC

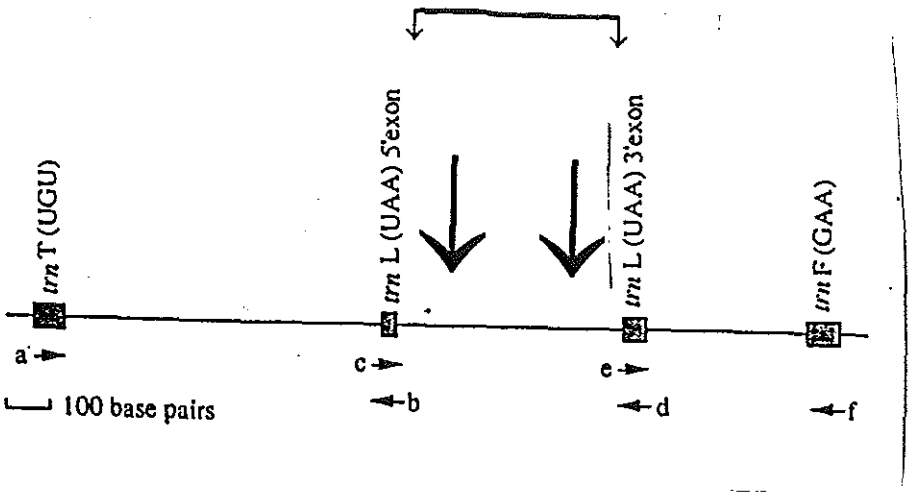

Figure 1. Positions and directions of a pair of universal primers used to amplify the non-coding region (*trnL* (UAA) intron) of the chloroplast DNA. Tips of arrows indicate the 3' ends of the primers (Taberlet *et al.*, 1991).

3. 7. Digestion of PCR amplified chloroplast DNA non-coding region (*trnL* (UAA) intron)

The enzyme Sau3AI was purchased from New England Bio Labs and used following manufacturers'recommendations. 8 μl of PCR amplified cpDNA non-coding region (*trnL* (UAA) intron) from the representative samples of the three *Guizotia* species (*G. abyssinica, G. scabra ssp. scabra* and *G. scabra ssp. schimperi*) were digested for 2 hours at 37°C with Sau3aI restriction enzyme recognizing 4 bp (Table 2) in 2 μl of 10x NEB restriction buffer (100 mM NaCl, 10 mM Bis Trispropane-HCl, 10 mM $MgCl_2$, 1mM dithiothretiol, pH 7.0 at 25°C), 2 μl of BSA, 1 μl of 10 μg/μl of Sau3AI restriction enzyme and sterile deionized distilled water to a final reaction volume of 20 μl.

3. 8. Electrophoresis of PCR amplified and digested cpDNA non-coding region (*trnL* (UAA) intron)

Sau3AI enzyme restricted cpDNA non-coding region (*trnL* (UAA) intron) fragments were separated by electrophoresis at 100 voltage using 2% agarose gels with 1x TBE running buffer containing 50 mM Tris-HCl pH 8.3, 50 mM H_3BO_3 and 10 mM EDTA). The cpDNA non-coding region (*trnL* (UAA) intron) fragments were made visible by staining the gel with ethidium bromide (0.5 μg/ml and photographed under UV light using polariod

films. From photographs, the restriction fragment patterns of cpDNA non-coding region (*trnL* (UAA) intron) generated by Sau3AI enzyme was drawn by measuring the distance of each fragment from the origin (Tsunewaki and Ogihara, 1983). The molecular sizes of each restriction fragment was estimated from its mobility in comparison to the molecular sizes of 123 bp DNA ladder.

3. 9. Data analysis

The molecular sizes estimated by Bowman *et al.* (1981) was used as references for estimating the molecular sizes of cpDNA fragment bands. The molecular size of each fragment was estimated in relation to the molecular size of the molecular wt. marker band closest to it following the equation given below.

$$D_1 x Wt_1 = D_2 x Wt_2$$

$$Wt_2 = \frac{D_1 x Wt_1}{D_2}$$

in which D_1 is the distance migrated by the closest fragment of the molecular wt. marker, D_2 is the distance migrated by the DNA fragment in question, wt_1 is the estimated molecular size of the molecular wt. marker fragment closest to the DNA fragment in

question and wt_2 is the estimated molecular wt. of the DNA fragment in question. Suspected homologies were confirmed by side-by-side comparison of electrophoretic mobilities of cpDNA fragments (Carr *et al.*, 1987). All bands with an identical electrophoretic mobility were treated as homologous.

Fragment patterns generated by restriction endonuclease digestions were used to calculate the extent of sequence divergence between each pair of samples. The degree of genetic divergence between two species is expected to be correlated with the proportion of DNA fragments shared by them. The proportion of DNA fragments shared by two species (F) is given by the following formula of Nei and Li (1979) (equation 21)

$$F = \frac{2n_{xy}}{n_x + n_y}$$

in which n_x and n_y are the number of fragments in species x and y respectively, whereas n_{xy} is the number of fragments shared by the two species.

In addition, restriction sites of the different taxa were aligned and presence/absence of restriction sites were scored as 1/0 data matrix from which similarity or disimilarity indexes and dendrograms were generated from the similarity matrix using a soft ware program NTSYS-PC (Rholf, 1993).

4. Results

Extractions of chloroplast DNAs following the sucrose gradient method of Palmer (1986) was successfully optized for all the *Guizotia* species considered in the present study (Fig. 2). The isolation of PCR-ready DNA using Daynabeads DNA DIRECT™ System I and subsequent amplification of chloroplast DNA non-coding region (*trnL* (UAA) intron) (Fig. 3) using primers C and D of Taberlet *et al.* (1991) was also done.

The digestion of the total chloroplast DNA extracts from reperesentative samples of *Guizotia* species in the present study using the three type two restriction endonuclease enzymes recognizing six base pairs (BamHI, EcoRI and HindIII) produced a total of 20 fragments (Fig. 3). This means that, 20 recognition sites were detected and about 120 base pairs were surveyed in each of the chloroplast DNA studied and this represents 0.086% of the chloroplast genome, based on estimated average molecular size of the chloroplast genome ranging from 120 to 180 kb (Close *et al.*, 1989). The number of restriction fragments per restriction enzyme recognizing six bp used ranges from 6-8, while the sizes of the restriction fragments ranges from 1.33-19.5 kb.

The digestion of PCR amplified chloroplast DNA non-coding region (*trnL* (UAA) intron) from representative samples of *Guizotia* species considered in the present study with type two restriction endonuclease enzyme (Sau3AI) (Table 2), recognizing 4 base pairs produced 3 restriction fragments (Fig. 4). This means that, two recognition sites were detected and 8

base pairs were surveyed in each of this PCR amplified chloroplast DNA non-coding region (*trnL* (UAA) intron), and this represents 1.63% of this particular chloroplast DNA non-coding region based on the estimated molecular size of this non-coding region to be 490 bp in the present study.

An attempt to digest the PCR amplified cpDNA non-coding region (*trnL* (UAA) intron) with the three restriction endonuclease enzymes recognizing six base pairs (Table 2) did not generate any restriction fragment indicating that these restriction enzymes lack recognition sites in this particular cpDNA non-coding region.

Because the resolution of the smaller fragments produced by the restriction endonuclease enzymes (BamHI, EcoRI and HindIII) were inadequate on 0.8% agarose gels, most deductions were made by comparing only the larger fragments. Thus, we have probably underestimated the variation existing among these species as some differences may be evident among the smaller fragments. However, the digestion of the cpDNA non-coding region (*trnL* (UAA) intron) with Sau3AI restriction enzyme producing smaller fragments ranging in size from 0.27-0.4 kbp partially confirms the absence of variation even among the smaller restriction fragments expected to be produced.

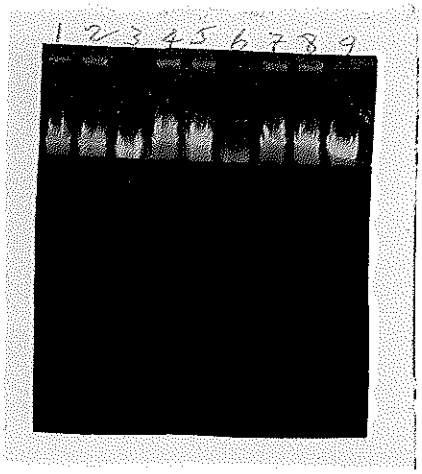

Figure 2. Electrophoresis in 0.8% agarose gel showing successful extraction of chloroplast DNA from *Guizotia* species in the present study. Lanes 1-3, 4-6 and 7-9 are *G. abyssinica, G. scabra ssp. scabra* and *G. scabra ssp. schimperi*, respectively.

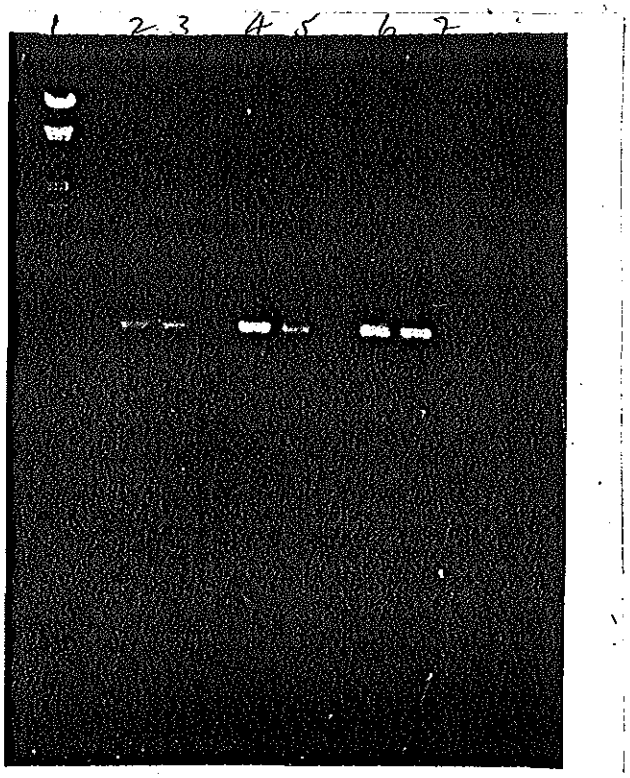

Figure 3. Electrophoresis of PCR amplified chloroplast DNA non-coding region (trnL (UAA) intron) in a 1.6% agarose gel showing the successful application of primers C and D (Tab. 3). Lane 1 is molecular wt. marker (EcoRI/HindIII digested Lamda DNA), lanes 2 and 3, 4 and 5, 6 and 7 are *G. abyssinica, G. scabra ssp. scabra* and *G. scabra ssp schimperi,* respectively.

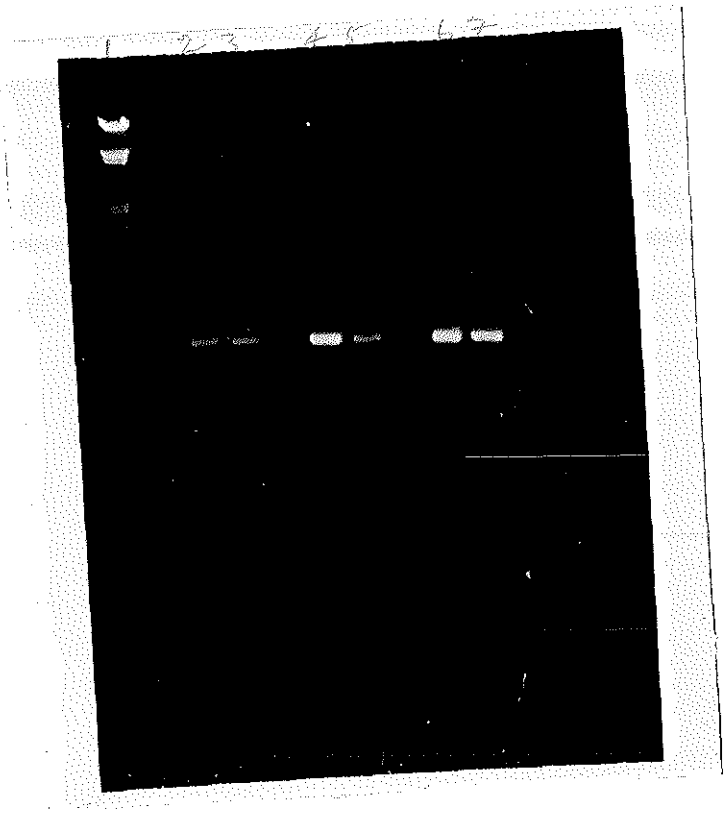

Figure 3. Electrophoresis of PCR amplified chloroplast DNA non-coding region (trnL (UAA) intron) in a 1.6% agarose gel showing the successful application of primers C and D (Tab. 3). Lane 1 is molecular wt. marker (EcoRI/HindIII digested Lamda DNA), lanes 2 and 3, 4 and 5, 6 and 7 are *G. abyssinica, G. scabra ssp. scabra* and *G. scabra ssp schimperi,* respectively.

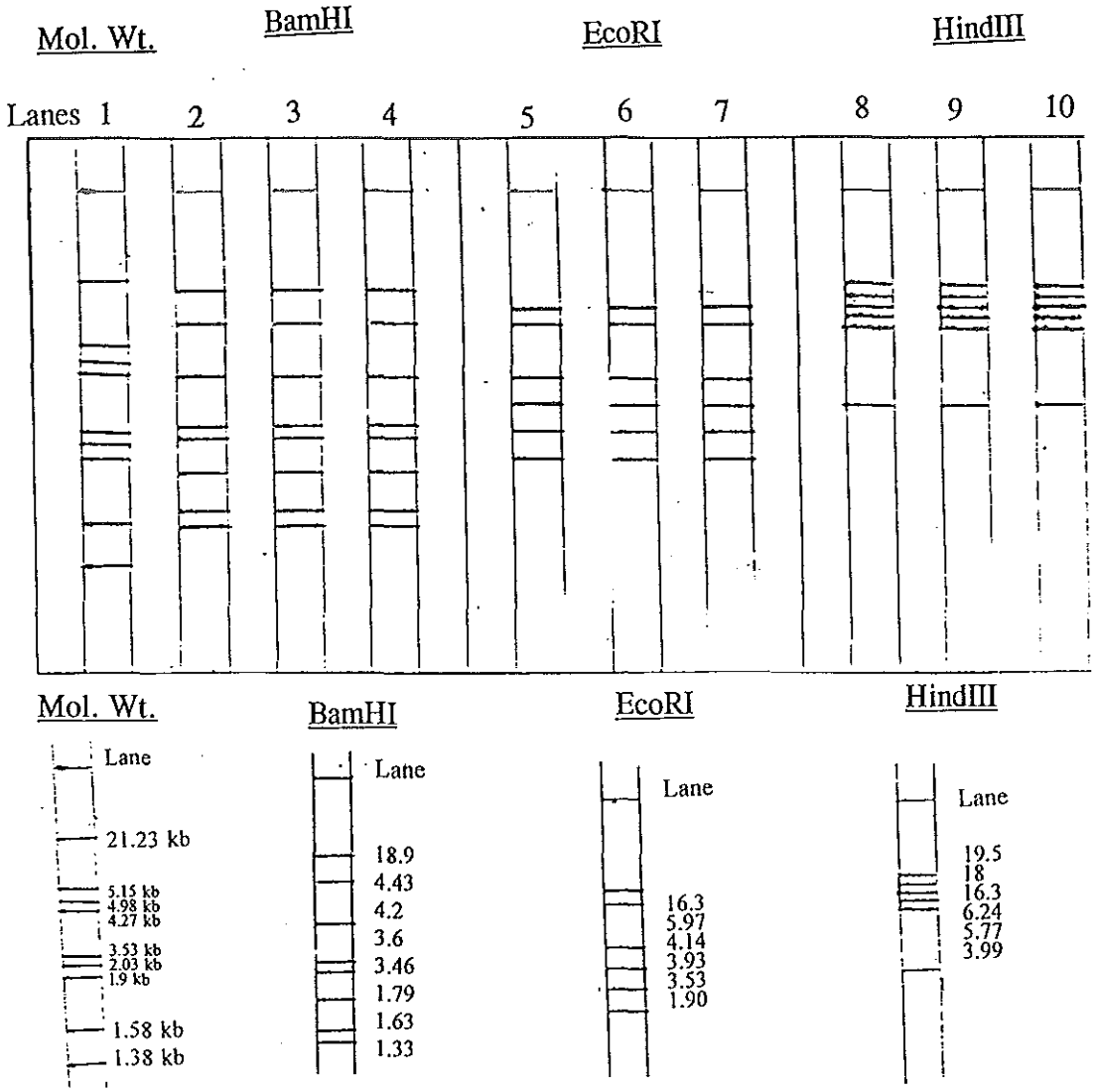

Figure 4. Agarose gel electrophoretic patterns and the estimated molecular sizes of restriction endonuclease digests of chloroplast DNAs isolated from the three *Guizotia* species. Lane 1 is molecular wt. marker (EcoRI/HindIII digested Lamda DNA) and lanes 2, 3 and 4 are BamHI; 5, 6 and 7 are EcoRI and 8, 9 and 10 are and HindIII restriction fragment patterns, repectively. Lanes 2, 5 and 8 are *G. abyssinica*; 3, 6 and 9 are *G. scabra ssp. scabra* and lanes 4, 7 and 10 are *G. scabra ssp. schimperi* chloroplast DNA restriction fragments, respectively.

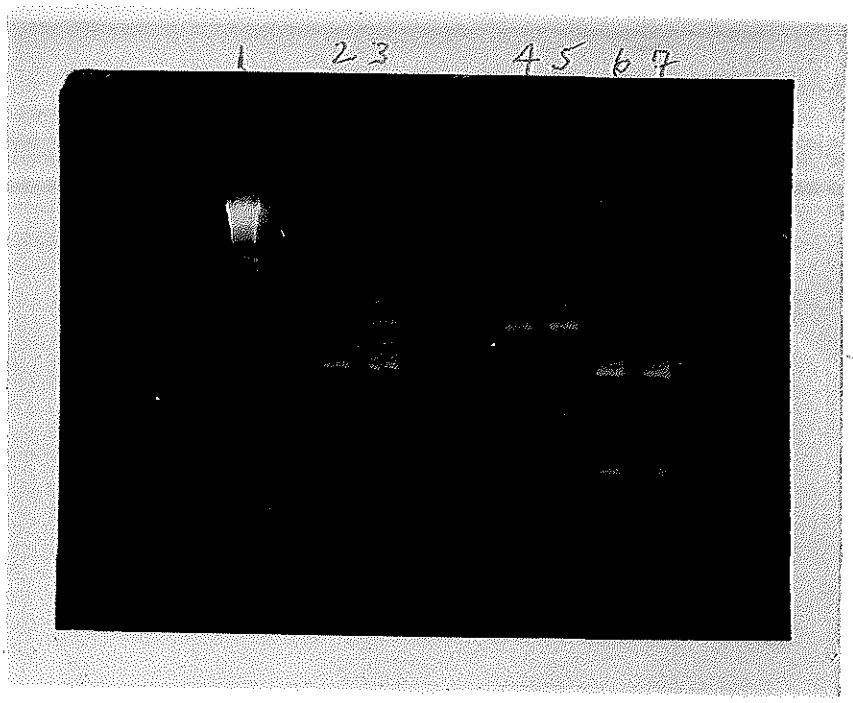

Figure 5. Agarose gel electrophoresis of the three Guizotia species chloroplast DNA non-coding region (*trn*L (UAA) intron) fragments produced by digestion with restriction endonuclease enzyme (Sau3AI) recognizing 4 base pairs. lane 1 is molecular wt. marker (EcoRI/HindIII digested Lamda DNA or 123 base pair ladder DNA), while lanes 2-3, 4-5, 6-7 are *G. abyssinica, G. scabra subsp. scabra* and *G. scabra ssp. schimperi* respectively.

Table 4. Number of bands scored for each *Guizotia* species with each restriction enzyme used.

Enzyme	*G. abyssinica*	*G. scabra subsp. scabra*	*G. scabra subsp. schimperi*
BamHI	8	8	8
EcoRI	6	6	6
HindIII	6	6	6
Sau3AI	3	3	3

Table 5. Proportion of restriction fragments shared by each pair of *Guizotia* species compared.

Species	*G. abyssinica*	*G. scabra subsp. scabra*	*G. scabra subsp. schimperi*
G. abyssinica	1	1	1
G. scabra subsp. scabra	1	1	1
G. scabra subsp. schimperi	1	1	1

5. Discussion

Variability of cpDNA from three closely related species of the genus *Guizotia* was examined by digesting with restriction endonuclease enzymes (Table 2). The observation of the electrophoretic fragment bands indicated that there was no variation among these species. The absence of variation found here is in line with the conservative nature of the chloroplast genome that has been reported at both the intraspecific and interspecific levels in other angiosperm species (Bowman *et al.*, 1983; Palmer *et al.*, 1985). Higher plant chloroplast genomes are highly conserved across a broad array of taxa (Plamer, 1985). Birky (1988) cited in Doyle *et al.* (1990) has also pointed out that low levels of cpDNA variation are commonly observed in interspecific comparison (e.g Koch *et al.* 1996) found only 4 restriction site mutations in section Cochleria (Brassicaceae) with 91 accession representing 11 species analysed by 25 restriction enzymes). One possible explanation for low genetic variability of cpDNA is its low mutation rate (the rate of base substitutions is significantly lower for chloroplast DNA than for a single copy nuclear DNA from the same species (Belford and Thompson, 1981). Another possiblity is the uniparental transmission of chloroplast genome resulting in rarity of heteroplasmic individuals (Thomson, 1977).

A general criticism of absence of cpDNA variation is inadequate sampling, either as a result of too few restriction enzymes used (e.g. Hantula *et al.*, 1989) or considering too few individuals per taxon (e.g.Coates and Cullis, 1987). Therefore, adequate sampling is also

essential in studies using cpDNA variation for phylogeny reconstruction among closely related species. Selection of 1-3 accessions to repesent each of the three species or digestion with few restriction enzymes could generate any variation. Thus, the observed affinities of species represented by a single or few accessions or limited number of restriction enzymes used should be considered preliminary. But, if very little or no interspecific cpDNA variation is encountered, like in the case of *Guizotia* species in the present study, increasing intraspecific samples would probably be a waste of resources.

The level of nucleotide change among genera in different tribes and subtribes of the family Asteraceae is found to be surprisingly low in comparison to intrageneric divergence value in several other angiosperm families (Jansen and Palmer, 1988). These low levels of intergeneric cpDNA nucleotide change in the Asteraceae (the family to which the genus *Guizotia* belongs) suggests that the Asteraceae is either a relatively young family or the rate of chloroplast DNA divergence is slower within the Asteraceae (Jansen and Palmer, 1988). Similarly, the absence of cpDNA restriction fragment variation among the three species of the genus *Guizotia* in the present study could be explaned in terms of their close relationship and recent diversification or having lower rate of cpDNA nucleotide divergence.

In contrast to the remarkable morphological variability of the *Guizotia* species as judged by Baagoe (1974), the cpDNA restriction fragment patterns of these species were found to be identical. The same phenomena was found by Clegg *et al.* (1984) in the genus *Hordeum* where strong morphological variability does not correlate with detectable cpDNA variability. Morphological data are not always in line with molecular data, a situation which

is also seen in other taxa too (Koch *et al.*, 1996). Introgression has been suggested to account for incongruence between cpDNA and morphological variation patterns in some plant species (e.g. *Brassica napus*, Palmer *et al.*, 1983). Introgression requires at least a possible limited gene flow among the taxa involved.

The present cpDNA restriction fragment variation analysis revealed that the three *Guizotia* species are equally related to one another. This is in line with the similarity of *Guizotia* species in their chromosome number and their fatty acid composition

6. Conclusion

The present results of cpDNA restriction fragment analysis among the three species of the genus *Guizotia* showed absence of variation indicating a close relationship among these species due to their recent diversification or their chloroplast DNA evolving relatively at a slower rate. Our findings for the homologous cpDNAs in these species of the genus *Guizotia* support the assumption of Palmer and Zammir (1982) who emphasized the conservative nature of the chloroplast DNA.

Although we found no variation in cpDNA restriction fragment analysis among the presently studied species, it is evident that cpDNA can still provide substantial phylogenetic insights at both intergeneric and interspecific level in other angiosperms, because a number of studies have demonstrated the potential of cpDNA restriction site mutations for resolving phylogenetic relationships among closely related species (Palmer, 1986b cited in Jansen and Palmer, 1988). Finally, inorder to get more reliable RFLP information, the use of more restriction enzymes and cloned probes for the future restriction fragment variation analysis of all *Guizotia* species and other angiosperm species are recommended.

References

Abebe, M., Yermanos, D. M. and Bingham, F. T. 1978. The ecophysiology of noug ('*Guizotia abyssinica*, Cass.') I. Effects of photoperiod and temperature on growth. *Afr. J. Agr. Sci.* **5** (1): 55-66.

Aldrich, P. R. and Doebley, J. F. 1992. Restriction fragment variation in the nuclear and chloroplast genomes of cultivated and wild *Sorgum bicolor*. *Theor. Appl. Genet.* **85**: 293-302.

Aquadro, C. F. and Greenberg, B. D. 1983. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics* **103**: 287- 312.

Baagoe, J. 1974. The genus *Guizotia* (Compositae): A taxonomic revision. *Bot. Tidsk.* **69**: 1-39.

Banks, J. A. and Birky, C. W. 1985. Chloroplast DNA diversity is low in a wild plant , *Lupinus texensis*. *Proc. Natl. Acad. Sci. U. S. A.* **82**: 6950-6954.

Beckmann, J. S. and Soller, M. 1986. Restriction fragment length polymorphisms and genetic improvement of agricultural species. *Euphytica* **35**: 111-124.

Belford, H. S. and Thompson W. F. 1981. Single copy DNA homologies in Atriplex II. Hybrid thermal stabilities and molecular phylogeny. *Heredity* **46**: 102-122.

Birky, C. W., Maruyama, T. and Fuerst, P. 1983. An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. *Genetics* **103**: 513-527.

Bonnard, G., Weil, J. H. and Strinmetz, A. 1985. The intergenic region between the *Vicia faba* chloroplast tRNA Leu/CAA and tRNA Leu/UAA genes contains a partial copy of the split tRNA Leu/UAA gene. *Current Genetics* **9**: 417-422.

Bonen, L. and Dolittle, W. F. 1975. On the prokaryotic nature of red algal chloroplasts. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 2310-2314

Bowman, C. M., Koller, B., Delius, H. and Dyer, T. A. 1981. A physical map of wheat chloroplast DNA showing the location of the structural genes for the ribosomal RNAs and the large subunit of ribulose 1, 5-bisphosphate carboxylase. *Mol. Gen. Genet.* **183**: 93-101.

Bowman, C. M., Bonnard, G. and Dyer, T. A. 1983. Chloroplast DNA variation between species of *Triticum* and *Aegilops*. Location of the variation on the chloroplast genome and its relevances to the inheritance and classification of the cytoplasm. *Theor. Appl. Genet.* **65**: 247-262.

Burton, W. G., GraBowy, C. T. and Sager, R. 1979. Role of methylation in the modification and restriction of chloroplast DNA in Chlamydomonas. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 1390-1394.

Carr, S. M., Brothers, A. J. and Wilson, A. C. 1987. Evolutionary inferences from restriction maps of mitochondrial DNA from nine taxa of Xenopus frogs. *Evol.* **41**(1): 176-188.

Clegg, M. 1993. Chloroplast gene sequences and the study of plant evolution. *Proc. Natl. Acad. Sci. U. S. A.* **90**: 363-367.

Clegg, M. T., Rawson, J. R. and Thomas, K. 1984. Chloroplast DNA variation in Pearl millet and related species. *Genetics* **106**: 449-461.

Close, P. S., Shoemaker, R. C. and Keim, P. 1989. Distribution of restriction site polymorphism within the genus *Glycine* subgenus *Soja*. *Theor. Appl. Genet.* **77**: 768-776.

Coates, D. and Cullis, C. A. 1987. Chloroplast DNA variability among *Linum* species. *Amer. J. Bot.* **74**: 260-268.

Crawford, D. J., Palmer, J. D. and Kobayashi, M. 1990. Chloroplast DNA restriction site variation and the phylogeny of *Coreopsis* section *Coreopsis* (Asteraceae). *Amer. J. Bot.* **77**: 552-559.

Curtis, S. E. and Clegg, M. T. 1984. Molecular evolution of chloroplast DNA sequences. *J. Biol. Evol.* **1**: 291-301.

Doyle, J. J., Doyle, J. L. and Brown, A. H. D. 1990. Chloroplast DNA polymorphism and phylogeny in the B genome of *Glycine* subgenus *Glycine* (Leguminase). *Amer. J. Bot.* 77(6): 772-782.

Doebley, T., Renfroe, W. and Blanton, A. 1987. Retriction site variation in the *Zea* chloroplast genome. *Genetics* **117**: 139-147

Duvall, M. R. and Doebley, J. F. 1990. Restriction site variation in the chloroplast genome of *Sorghum* (Poceae). *Syst. Bot.* **15**: 472-480.

Engels, W. R. 1981. Estimating genetic divergence and genetic variability with retriction endonucleases. *Proc. Natl. Acad. Sci. U. S. A.* **78**: 6329-6333.

Fangan, B. M., Stedje, B., Stabbetrop, O. E., Jensen, E. S. and Jakobsen, K. S. 1994. A general approach for PCR amplification and sequencing of chloroplast DNA from crude vascular plant and algal tissue. *Biotechniques* **16**: 484- 494.

Gantt, J. S., Baldauf, S. L., Calie, P. J., Weeden, N. F. and Palmer, J. D. 1991. Transfer of rpL22 to the nucleus greatly preceded it's loss from the chloroplast and involved in the gain of an intron. *EMBO Journal* **10**(10): 3073-3078.

Getinet, A. and Sharma, S. M. 1996. Niger (*G. abyssinica* (L. f.) Cass.): *Promoting the conservation and use of underutilized and neglected crops*. 5. Intrnatioal Plant Genetic Resources Institute.

Gordon, K. H., Crouse, E. J., Bohnert, H. J. and Hermann, R. G. 1982. Physical mapping of differences in chloroplast DNA of the wild type plastomes in *Oenothera* subsection *Euoenothera*. *Theor. Appl. Genet*. **61**: 373-384.

Graner, A., Siedler, H., Jahoor, A., Herrmann, R. G. and Wenzel, G. 1990. Assessment of the degree and the type of restriction fragment length polymorphism in barley (*Hordeum vulgare*). *Theor. Appl. Genet*. **80**: 826-832.

Hantula, J., Uotila, P., Saura, A. and Lokki, J. 1989. Chloroplast DNA variation in *Anemone senu lato*. *Plant Syst. Evol.* **163**: 81-85.

Harris, S. A. and Ingram, R. 1991. Chloroplast DNA and biosystematics: The effect of intraspecific diversity and plastid transmission. *Taxon* **40**: 393-412.

Hiremath, S. C. and Murthy, H. N. 1988. Domestication of Niger (*Guizotia abyssinica*). *Euphytica* **37**: 225-228.

Hiremath, S. C. and Murthy, H. N. 1992. Cytogenetical studies in *Guizotia* (Asteraceae). *Caryologica* **45**: 69-82.

Hiremath, S. C., Murthy, H. N. and Salimath, S. S. 1992. Quantitative nuclear DNA differences associated with genome evolution in *Guizotia* (Compositae). *Genetica* **85**: 241-247.

Holwerda, B. C., Jana, S. and Crosby, W. L. 1986. Chloropast and mitochondrial DNA variation in *Hordeum spontaneum*. *Genetics* **114**: 1271-1291.

Innis, M. A., Myambo, K. B., Gelfand, D. H. and Brow, M. A. D. 1988. DNA sequencing with Thermus aquaticus DNA and direct sequencing of polymerase chain reaction amplified DNA. *Proc. Natl. Acad. Sci.* **85**: 9436- 9440.

Jansen, R. K. and Palmer, J. D. 1988. Phylogenetic implications of chloroplast DNA restriction site variation in the *Mulisiieae* (Asteraceae). *Amer. J. Bot.* **75**: 753-766.

Kifle Dagne 1994. Cytology, phylogeny and oil quality of *Guizotia* Cass. (Compositae). Ph.D. Dissertation Addis Ababa University

Kolodner, R. and Tewari, K. K. 1979. Inverted repeats in chloroplast DNA from higher plants. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 41-45.

Koch, M., Hurka, H. and Mummenhoff, K. 1996. Chloroplast DNA restriction site variation and RAPD analysis in *Cochlearia* (Brassiceae): Biosystematics and speciation. *Nordic J. Bot.* **16**(6): 585-603.

Maxam, A. M. and Gilbert, W. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 560- 564.

Morton, B. R. and Clegg, M. T. 1993. A chloroplast DNA mutational hot spots and gene conversion in non-coding region near rbcL in the grass family (Poaceae). *Current Genetics* **24**: 357-365.

Murthy, H. N., Hiremath, H. N. and Salimath, S. S. 1993. Origin, evolution and genome differentiation in *Guizotia abyssinica* and it's wild species. *Theor. Appl. Genet.* **87**: 587-592.

Nei, M. and Li, W. 1979. Mathematical model for studying variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 5268-5273.

Ogihara, Y. and Tsunewaki, K. 1988. Diversity and evolution of chloroplast DNA in Triticum and Aegilops as revealed by restriction fragment analysis. *Theor. Appl. genet.* **76**: 321-332.

Palmer, J. D. 1985. Comparative organization of chloroplast genomes. *Ann. Rev. Genet.* **19**: 325-354.

Palmer, J. D. 1986. Isolation and structural analysis of chloroplast DNA. Methods *Enzymology* **118**: 167-186.

Palmer, J. D. 1987. Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *The American Naturalist* **130** Supplement: 6-29.

Palmer, J. D. and Thompson, W. F. 1981. Rearrangements in the chloroplast genomes of Mung bean and pea. *Proc. Natl. Acad. Sci. U. S. A.* **78**: 5533-5537.

Plamer, J. D. and Thompson, W. F. 1982. Chloroplast DNA rearrangements are more frequent when a large inverted repeated sequence is lost. *Cell* **29**: 537-550.

Palmer, J. D. and Zamir, D. 1982. Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon. Proc. Natl. Acad. Sci. U. S. A.* **79**: 5006-5010.

Palmer, J. D., Shields, C. R., Cohen, D. B. and Orton, T. J. 1983. Chloroplast DNA evolution and the origin of amphidiploid *Brassica species.* Theor. Appl. Genet. **65**: 181-189.

Palmer, J. D., Jorgensen, R. A. and Thompson, W. F. 1985. Chloroplast DNA variation and evolution in *Pisium*: Patterns of change and phylogenetic analysis. *Genetics* **109**: 195-213.

Palmer, J. D., Jansen, R. K., Michaels, H. J., Chase, M. W. and Manhart, J. R. 1988. chloroplast DNA variation and plant phylogeny. *Ann. Missouri. Bot. Gard.* **75**:1180-1206.

Riley, K. M. and Belayneh, H. 1989. Niger. In: Robbelen, G., Downey, R. k., Ashir, A. (eds) *Oil Crops of the World: Their Breeding and Utilization*. McGraw Hill, New York. pp. 394-403.

Rohlf, F. J. 1993. *NTSYS-PC Numerical taxonomy and multivariate analysis system*, version 1.80, Exter Publishing, Ltd., Setauket, New York.

Saghai-Maroof, M. A., Zhang, Q. and Biyashev, R. 1995. Comparison of restriction fragment length polymorphisms in wild and cultivated barley. *Genome* **38**: 298-306.

Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487- 491.

Sanger, F., Nicklen, S. and Coulson, R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* 74: 5463- 5467.

Schwarz, Z. and Kossel, H. 1979. Sequencing of the 3'-terminal region of a 16S rRNA gene from Zea mays chloroplast reveals homology with E. coli 16S rRNA. *Nature* **279**: 520-522.

Seegeler, C. J. P. 1983. *Oil plants in Ethiopia, their Taxonomy and Agricultural Significance*. Center for Agricultural Publishing and Documentation, Wageningen, pp. 122-146.

Smith, L. M., Sanders, J. Z., Kaiser, R. J., Hughes, P., Dodd, C., Connell, C. R., Heiner, C., Kent, S. B. H. and Hood, L. E. 1986. Fluorescence detection in automated DNA sequence analysis. *Nature* **321**: 674- 679.

Song, K. M., Osborn, C. and Williams, P. H. 1988. Brassica taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). *Theor. Appl. Genet.* **75**: 784-789.

Stern, D. B. and Lonsdale, D. M. 1982. Mitochondrial and chloroplast genes of maize have a 12 kilobase DNA sequence in common. *Nature* **299**: 698-702.

Stern, D. B. and Palmer, J. D. 1984. Extensive and wide spread homologies between mitochondrial DNA and chloroplast DNA in plants. *Proc. Natl. Acad. Sci. U. S. A.* **81**: 1946-1950.

Sugiura, M. 1992. The chloroplast genome. *Plant Molecular Biology* **19**: 149-168.

Sytsma, K. J. and Gottielb, L. D. 1986. Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* sec. *Peripetasma* (Onagraceae). *Evolution* **40**: 1248-1261.

Sytsma, K. J. and Schaal, B. A. 1985. Phylogenetics of the *Lisianthius skinneri* (Gentianaceae) species complex in Panama utilizing DNA retriction fragment analysis. *Evolution* **39**: 594-608.

Taberlet, P., Gielly, L., Pautou, G. and Pouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant molecular Biology* **17**: 1105-1109.

Tabor, S. and Richardson, C. C. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **84**: 4767-4771.

Tassopulu, D. and Kung, S. D. 1984. *Nicotiana* chloroplast genome. 6. Deletion and hot-spot a proposed origin of the inverted repeats. *Theor. Appl. genet.* **67**: 185-193.

Templeton, A. R. 1983. Phylogenetic inference from restriction endonuclease site maps with particular reference to the evolution of humans and apes. *Evolution* **37**: 221-244.

Thomson, G. 1977. The effect of selected locus on linked neutral loci. *Genetics* **85**: 753-788.

Tsunewaki, K. and Ogihara, Y. 1983. The molecular basis of genetic diversity among cytoplasms of *Triticum* and *Aegilops* species. II. On the origin of polyploid wheat cytoplasms as suggested by chloroplast DNA restriction fragment patterns. *Genetics* **104**: 155-171.

Vaughan, J. G. 1970. *The structure and utilization of oil seeds*. Chapman and hall, London, pp. 41-43.

Wang, Z. Y., Second, G. and Tanksley, S. D. 1992. Polymorphism and phylogenetic relationships among species in the genus *Oryza* as determined by analysis of nuclear RFLPs. *Theor. Appl. Genet.* **83**: 565-581.

Yonemori, K., Kanzaki, S., Parfitt, D. E. and Sugiura, A. 1998. Phylogenetic relationships of *Diospyros kaki* (persimmon) to *Diospyros* spp. (Ebenaceae) of Thailand and four temperate zone *Diospyros* spp. from analysis of RFLP variation in amplified cpDNA. *Genome* **41**: 173-182.

Zurawski, G., Clegg, M. T. and Brown, A. H. D. 1984. The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. *Genetics* **106**: 735-749.

Zurawski, G. and Clegg, M. T. 1987. Evolution of higer plant chloroplast DNA- encoded genes: implications for structure function and phylogenetic studies. *Ann. Rev. Plant Pysiol.* **38**: 398- 418.