Table of contents

List of Figures III

List of Tables III

Acknowledgements IV

Abstract V

1. Introduction 1

2. Literature review 3

2.1 The plant materials used in the study 3

2.2 The afroalpine climate and plant adaptations 5

2.2.1 The afroalpine and its climate 5

2.2.2 Adaptation of plants to the afroalpine climate 6

2.3 Variation in C-value 8

2.3.1 Interspecific plant C-value variation 8

2.3.2 Intraspecific C-value variation in plants 9

2.3.3 Factors leading to DNA amount variation 10

2.3.3.1 Polyploidy 10

2.3.3.1.1 Factors promoting polyploidy 11

2.3.3.1.2 DNA amount changes following polyploid formation 12

2.3.3.2 Aneuploidy 13

2.3.3.3 Molecular factors as causes of DNA amount variation 14

2.3.3.4 Other factors which cause DNA amount variation 15

2.3.4 Correlation of DNA amount with phenotype 16

2.4 Methods of genome quantification 17

2.4.1 Flow cytometry 17

2.4.1.1 Methods of flow cytometry for plant DNA study 19

2.4.1.2 Applications of flow cytometry in plant studies 22

2.4.1.3 Artefactual data of flow cytometry.....24
2.4.1.4 Best practice for avoiding artefactual data..... 25
2.4.1.5 Correction on C-values using best practice..... 26
2.4.2 Static cytometry.....27
2.4.3 Advantages and disadvantages of flow cytometry and static cytometry.....27
2.5 Objectives of the study..... 29
3. Materials and methods..... 29
3.1 Plant materials and sites of collection..... 29
3.2 Methods..... 33
3.2.1 Chromosome counting.....33
3.2.2 DNA-ploidy and C-value estimation.....33
4. Results..... 36
4.1 Chromosome count and Ploidy levels..... 36
4.2 Intraspecific and interspecific 2C DNA value.....38
5. Discussion.....47
5.1 Chromosome count and Ploidy levels..... 47
5.2 DNA-ploidy and ploidy levels.....48
5.3 2C DNA value..... 49
6. Summary and Recommendations..... 52
7. References.....54

List of Figures

Figure 1. The cell cycle and the components of the DNA histogram.....18
Figure 2. A histogram..... 19
Figure 3. Four of the plant species.....31
Figure 4. Map of Ethiopia.....32
Figure 5. Chromosomes of the plant materials studied..... 37
Figure 6. Histogram of *Cotula cryptocephala* accession CcBa6a.....38
Figure 7. Histogram of *Cotula cryptocephala*, accession CcBa6b.....39
Figure 8. Histogram of *Cotula cryptocephala*, accession CcBa7.....39
Figure 9. Histogram of *Cotula cryptocephala*, accession CcBa13.....40
Figure 10. Histogram of *Cotula cryptocephala*, accession CcSe9..... 40
Figure 11. Histogram of *Deschampsia caespitosa*, accession DcBa4.....41
Figure 12. Histogram of *Deschampsia caespitosa*, accession DcBa8.....41
Figure 13. Histogram of *Ranunculus stagnalis*, accession RsSe3.....42
Figure 14. Histogram of *Ranunculus stagnalis*, accession RsSe4.....42
Figure 15. Histogram of *Ranunculus oreophytus*, accession RoBa2.....43
Figure 16. Histogram of *Ranunculus oreophytus*, accession RoBa4.....43
Figure 17. Histogram of *Ranunculus oreophytus*, accession RoBa6.....44
Figure 18. Histogram of *Ranunculus oreophytus*, accession RoSe..... 44
Figure 19. Histogram of *Ranunculus oreophytus*, accession RoSe6..... 45
Figure 20. Histogram of *Ranunculus oreophytus*, accession RoBa8.....45

List of Tables

Table 1. Internal reference standards recommended 20
Table 2. Plant materials studied.....30
Table 3. Chromosome number, accession number and altitude..... 36
Table 4. 2C DNA values.....46
Table 5. Mean 2C-value..... 46

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Abstract

This study includes five species of the afroalpine flora- *Cardamine obliqua* (Brassicaceae), *Cotula cryptocephala* (Asteraceae), *Deschampsia caespitosa* (Poaceae), *Ranunculus oreophytus* and *Ranunculus stagnalis* (Ranunculaceae) collected from Bale and Simen Mountains. Estimates of 2C-values and DNA-ploidy analysis were done for *C. cryptocephala, D. caespitosa*, *R. oreophytus* and *R. stagnalis* from leaf tissue using flow cytometry. Chromosome counting from mitotic root tips was done for *C. cryptocephala, D. caespitosa*, *R. oreophytus*, *R. stagnalis* and *C. obliqua*. Mean 2C-values were found to be 10.2 pg, 9.2 pg, 27.5 pg and 14.2 pg for *C. cryptocephala, D. caespitosa*, *R. oreophytus* and *R. stagnalis*, respectively. Chromosome numbers found were the same as earlier reports, 2n=62, 26, 32, 28 for *C. obliqua*, *D. caespitosa*, *R. oreophytus* and *R. stagnalis*, respectively but for *C. cryptocephala* 2n= 64 is a new report. For all species analyzed by flow cytometry, no intraspecific DNA-ploidy level difference was found whereas slight intraspecific 2C-value variation up to 1.047-fold was found, which could be either artefactual or real intraspecific variation correlated with molecular, morphological or environmental factors. These results indicate that further morphological, cytological, flow cytometric and molecular investigations of the afroalpine flora will provide data that answer many important biological and evolutionary questions.

Key words: Nuclear DNA amount, DNA ploidy, chromosome counts, afroalpine

1. Introduction

Of the plant materials used in the present study, *Cardamine obliqua* (Brassicaceae), *Cotula cryptocephala* (Asteraceae), *Ranunculus oreophytus* (Ranunculaceae) and *Ranunculus stagnalis* (Ranunculaceae) are dicot perennial herbs distributed in Ethiopia and other African countries (Edwards *et al*., 2000; Mesfin, 2004). *Deschampsia caespitosa* is a tall tussock forming perennial grass distributed in Ethiopia, other African countries and in temperate regions of both hemispheres (Phillips, 1995). From the afroalpine environment, *C. obliqua* has 5 cytotypes known so far with 2n=36, 56, 62, 64 and 72. *D. caespitosa, C. cryptocephala, R. oreophytus* and *R. stagnalis* each have one cytotype known so far from the afroalpine environment with 2n= 26, 80, 32 and 28, respectively (Hedberg and Hedberg, 1977). There is a nuclear DNA amount report of 2C= 18 pg for the tetraploid cytotype of *D. caespitosa* (2n= 52) in the angiosperm C-value database (Bennett and Leitch, 2005b) and 2C= 10.43 pg for the diploid cytotype (2n= 26) from New Zealand (Murray et al., 2005).

The afroalpine flora comprises the floras of the upper parts of the high mountains of Tropical East Africa (Hedberg, 1957), which provide unique opportunities for the study of speciation in connection with geographical isolation and adaptation to extreme environmental conditions (Hedberg, 1970). The afroalpine plants are divided into five distinct life forms based on the different types of morphological adaptations to afroalpine climate (Hedberg, 1995). The representatives of acaulescent rosette plants (*R. oreophytus* and *R. stagnalis*) and tussock grasses (*D. caespitosa*) are included in this study.

Nuclear DNA amount varies over several orders of magnitude among eukaryotes (Gregory, 2005). In plants, it varies 1000 fold (Gregory, 2005) and in angiosperms 800 fold, ranging from 2C-value= 0.32 pg in *Arabidopsis thaliana* (Brassicaceae) to 2C-value= 254.8 pg in *Fritillaria assyriaca* (Liliaceae) (Schneeweiss *et al*., 2006). Polyploidy (Cavalier Smith, 2005), aneuploidy (Greilhuber, 1998), B-chromosomes (Rayburn *et al*., 1985; Bures *et al*., 2004), transposable elements (Kidwell, 2002), different repetitive sequence families (Ohmido *et al*., 2000; Rees and Narayan, 1981; Flavell, 1986) and deletion (Bennetzen *et al*., 2005) are some of the factors that cause DNA amount variation. Although intraspecific variation has been reported in many plants (Ohmido *et al*., 2000; Rayburn *et al*., 1985; Greilhuber, 1998), there are also species with constant DNA content (Bennett *et al*., 2000b; Johnston *et al.*, 2005; Suda, 2002).

Although nuclear DNA amount data are important, there are many gaps in terms of geographical and life form representation (Bennett and Leitch 2005b; Bennett *et al*., 2000a). The only C-value data in the angiosperm C-value database from Ethiopia is that of *Eragrostis tef* (Bennett *et al*., 2000a).

Flow cytometry is the most popular method of genome quantification (Dolezel and Bartos, 2005) in addition to its application in large-scale ploidy level screening (Suda, 2004). It gives the relative value of DNA amount of the target plant expressed either in picogram or megabase pairs by including an internal standard of known DNA amount. Chromosome count is used to relate the flow cytometry estimated DNA amount to the actual ploidy level (Bennett and Leitch 2005a).

This study provides estimates of 2C-value, DNA ploidy and chromosome count in four afroalpine species: *C. cryptocephala, D. caespitosa*, *R. oreophytus* and *R. stagnalis* and chromosome count for *C. obliqua*.

2. Literature review

2.1 The plant materials used in the study

Five species of the afroalpine flora- *Cardamine obliqua* (Brassicaceae), *Cotula cryptocephala* (Asteraceae), *Deschampsia caespitosa* (Poaceae), *Ranunculus oreophytus* (Ranunculaceae) and *Ranunculus stagnalis* (Ranunculaceae) have been used in this study.

C. obliqua is a dicot perennial herb distributed in Gondar, Gojjam, Welo, Shewa, Arsi, Bale and Harar regions in Ethiopia. It is also found in other East African mountains outside Ethiopia. It grows along montane stream banks and wet meadows, in altitude ranging from 2200 to 4000 m.a.s.l. (Edwards *et al*., 2000). It exhibits intraspecific ploidy differences with 5 polyploid cytotypes known so far from the afroalpine environment. Chromosome number of 2n= 36, 56 and 72 were counted from East African mountains outside Ethiopia. 2n= 62 was counted from Simen and 2n=64 was counted from Bale in Ethiopia (Hedberg and Hedberg, 1977).

R. oreophytus is a dicot perennial herb distributed in Arsi, Sidamo, Bale, Shewa, Gondar, Gojjam and Welo regions in Ethiopia; and other African countries of Kenya, Sudan, Tanzanya, Uganda and Zaire. It is found in moist peaty places in streambeds, in gravel and grassy meadows and afroalpine associations including *Alchemilla* and other herbs, in altitude ranging from 2500 to 4000 m.a.s.l. (Edwards *et al*., 2000). *R.*

oreophytus is a morphologically variable species, but the variation in most features is too continuous to allow the separation of distinct varieties. The reason of this extensive morphological variation is controversial. Both caulescent and acaulescent plants of this species are found. The caulescent habit may perhaps be partly environmentally conditioned, since there is a widespread tendency in the afroalpine flora towards a reduction of the stem at high altitudes. Many species become more or less completely sessile in the alpine belt (Hedberg, 1957). But, normal acaulescent specimens of this species may also occur at low altitude level. The caulescent specimens display considerable resemblance to an assumed hybrid between *R. oreophytus* and *R. multifidus*. Since *R. multifidus* is common on the lower parts of the mountains, the caulescent specimens may possibly be due to hybrid introgression from that species (Hedberg, 1957). From the afroalpine environment, the species has one cytotype with 2n= 32 chromosome number counted from East African Mountains (Hedberg and Hedberg, 1977).

R. stagnalis is a dicot perennial herb found in *Erica-Philippia* groves, by streams and swampy places, in altitude ranging from 2250 to 4100 m.a.s.l. It is distributed in Gondar, Gojjam, Welo, Arsi, Sidamo, Bale and Shewa regions in Ethiopia; and other African countries of Kenya, Tanzanya, Uganda and Zaire (Edwards *et al*., 2000). From the afroalpine environment, the species has one cytotype with 2n= 28 chromosome number counted from East African mountains (Hedberg and Hedberg, 1977). *R. stagnalis* varies morphologically and it was suggested that these plants of different morphology are of hybrid origin (Edwards *et al*., 2000).

C. cryptocephala is a dicot perennial herb found on moist ground, along paths, on roadside margins and riverbanks, at altitude range of 2800-3750 m.a.s.l. It is distributed

in Gondar, Bale, Shewa regions in Ethiopia and also in Uganda and Kenya (Mesfin, 2004). From the afroalpine environment, the species has one cytotype with 2n= 80 chromosome number counted from East African mountains (Hedberg and Hedberg, 1977).

D. caespitosa is a tall, tussock forming perennial grass that is found in marshy, waterlogged ground alongside streams, in altitude ranging from 3100 to 4300 m.a.s.l. In Ethiopia, it is found in Gondar, Gojjam, Arsi and Bale. It is widespread in temperate regions of the hemispheres, Cameroon Mountain and the East African Mountains (Phillips, 1995). From the afroalpine environment, the species has one cytotype with 2n chromosome number of 26 counted from East African Mountains (Hedberg and Hedberg, 1977).

2.2 The afroalpine climate and plant adaptations

2.2.1 The afroalpine and its climate

Afroalpine Mountains refers to the group of isolated mountain peaks in East Africa. These mountains are of volcanic origin and they protrude above the surrounding plains like islands out of the sea (Hedberg, 1957).

By definition, the 'Afroalpine flora' comprises the floras of the upper parts of the high mountains of Tropical East Africa (Hedberg, 1957). These mountains harbor a flora and fauna entirely different from those of the lower country. Their vegetation has a marked zonation, which makes it possible to distinguish three major vegetation belts, which from below are: the Montane Forest belt, the Ericaceous belt and the Alpine belt

(Hedberg, 1957). The vegetation of the afroalpine belt in these mountains has its lower limit at 3500-4000 m.a.s.l (Hedberg, 1970).

The climate of these mountains is very different from that of the surrounding plains. The afroalpine climate is diurnal, i.e., frost occurs on many or most nights of the year, whereas day temperatures may be fairly high. Hedberg called this climate 'summer every day and winter every night' (Hedberg, 1957). The afroalpine thus provides unique opportunities for the study of speciation in connection with geographical isolation and adaptation to extreme environmental conditions (Hedberg, 1970).

2.2.2 Adaptation of plants to the afroalpine climate

A climate of the afroalpine type must evidently exert a rigorous selection on the flora; the plants must be able to withstand frequent night frosts and rapid heating by intense radiation during the day. Detailed studies of these life forms and their relation to the afroalpine climate are essential for a proper understanding of the evolution of the afroalpine flora (Hedberg, 1957). Few environments populated by plants on this planet could have greater exposure to extremeness of climate than the afroalpine (Hedberg, 1995).

The afroalpine plants are divided into five distinct life forms based on the different types of morphological adaptations to afroalpine conditions of 'summer every day and winter every night'. These are giant rosette plants, tussock grasses, acaulescent rosette plants, cushion plants and sclerophyllous shrubs (Hedberg, 1995). The representatives of acaulescent rosette plants (*Ranunculus oreophytus* and *R. stagnalis*) and tussock grasses (*Deschampsia caespitosa*) are included in this study.

Thick tussocks form grassland on afroalpine, which is separated by a network of lower vegetation or open soil (Hedberg, 1995). The dense and firm tussocks of the grasses consist not only of living shoots but also large amounts of dead and decaying culms and leaves (Hedberg, 1995). These grass tussocks dominate the grassland and the large amount of dead and decaying leaves and culms provide good temperature insulation for the living inner parts of the tussock (Hedberg, 1970). Tussock formation is an adaptation of the plant to temperature insulation. *D. caespitosa* is one example of tussock grass (Hedberg, 1995).

Acaulescent rosette plants are characterized by the more or less complete absence of an above ground stem, the leaf rosette arising at or below the general level of the soil surface (Hedberg, 1995). These plants utilize the favorable microclimate near the soil surface in daytime. These life forms also protect the water conducting tissues of the stem by burying it in the ground or among a mass of living leaves (Hedberg, 1970).

Other life forms of the afroalpine environment include giant rosette plants, which are the Giant *Senecios* and Giant *Lobelias*. They are characterized by thick and unbranched or sparsely branched stems, which are usually covered either by a dense mantle of marcescent dry leaves or by a thick and cork-like bark (Hedberg, 1995). Each stem is terminated by a huge dense leaf rosette, which folds together at night providing good temperature insulation for its interior, and unfolds again in the morning (Hedberg, 1970). The inflorescence is terminal and thus both shoot apex and inflorescence are protected against extreme temperatures (Hedberg, 1995).

Cushion plants are few in the afroalpine flora and their adaptation is similar to that of acaulescent rosette plants. They utilize microclimate of the soil during day and protect

water-conducting tissues by burying it in the ground or among a mass of living leaves (Hedberg, 1995; Hedberg, 1970).

2.3 Variation in C-value

C-Value is the nuclear DNA content of the unreplicated haploid chromosome complement, i.e. the whole chromosome complement with chromosome number *n* irrespective of the ploidy level. It is expressed either in picogram (10^{-12} gram) or megabase pairs (1pg= 978 Mbp, where 1 Mbp= 10^6 bp). On the other hand, genome size is the nuclear DNA amount in the monoploid chromosome set i.e. one chromosome set of an organism and its DNA having the chromosome base number *x*. It is calculated by dividing the 2C-value by the ploidy level (Suda, 2004).

C-value varies over several orders of magnitude among eukaryotes and it bears no connection to organismal complexity. The size of the eukaryotic genome need not imply anything at all about the number of genes it contains because it is mostly non-coding DNA (Gregory, 2005).

Variation in C-value can be considered at the interspecific or intraspecific level.

2.3.1 Interspecific plant C-value variation

Bennetzen and Kellog, (1997) raised the question 'Does C-value continue to increase from ancestral plants to the most recently originated plants?' This led to the investigation of C-value variations across plants in a phylogenetic context.

The first large-scale analysis of C-value evolution in angiosperms, based on analysis of C-value data and phylogenetic tree, revealed that the C-value of ancestral angiosperm was small (Leitch *et al*., 1998). When Leitch *et al*., (2005) repeated this investigation on a

wider scale across land plants and using the most recently available phylogenetic tree, they found similar results. Their investigation revealed that C-value evolution across land plants has been dynamic with several independent increases and decreases. Although angiosperms, bryophytes and some clades of monilophytes have very small ancestral C-value, gymnosperms were found to have intermediate ancestral C-value (Leitch *et al*., 2005).

Several studies, focused on angiosperm families and genera, were also done following the question raised by Bennetzen and Kellog (1997), which show that C-value both increases and decreases within a phylogenetic context of families and there exists wide range of C-value variation within families and genera. For example, Brassicaceae showed an evolutionary decrease in C-value in three branches and increase in one branch of their phylogenetic tree (Johnston *et al*., 2005). C-value was found to vary 57-fold within the family Orchidaceae (Cox *et al*., 1998). An 8.1 fold variation exists among the 21 *Sorghum* species, with 3.6 fold variation among the 2n= 10 and 5.8 among the 2n= 20 species (Price *et al*., 2005).

In plants in general, the largest variation in C-value occurs in angiosperms (Gregory, 2005). It varies 800 fold ranging from 2C= 0.32 pg in *Arabidopsis thaliana* (Brassicaceae) to 254.8 pg in *Fritillaria assyriaca* (Liliaceae) (Schneeweiss *et al*., 2006). This is followed by monilophytes, lycophytes and gymnosperms. The least variation occurs in bryophytes (Gregory, 2005).

2.3.2 Intraspecific C-value variation in plants

In some cases, DNA content is constant within a species. For example, DNA content was found to be constant among *Allium cepa* cultivars compared across four continents

(Bennett *et al*., 2000b). There is constancy of C-value among ten geographically diverse ecotypes of *Arabidopsis thaliana* (Johnston *et al.*, 2005). Limited variation has also been reported within species of *Empetrum* (Suda, 2002).

On the other hand, some plant species exhibit intraspecific C-value variation. For example, *indica* rice variety has 9.7% higher DNA content than *japonica* rice variety (Ohmido *et al*., 2000). DNA content varies up to 23% in different lines of maize (Rayburn *et al*., 1985).

2.3.3 Factors leading to DNA amount variation

2.3.3.1 Polyploidy

Polyploidy is the presence of three or more chromosome sets in an organism. On the basis of the source of the chromosome sets, polyploids are divided into autopolyploids and allopolyploids. An autopolyploid is a polyploid derived by doubling or adding the chromosome sets of a structural homozygote. Hence, an autopolyploid is an organism containing three or more sets of homologous chromosomes. An allopolyploid is the product of doubling in a species hybrid; it is therefore a polyploid containing different sets of non-homologous chromosomes derived from two or more parental species (Grant, 1981).

Polyploidy is a major force of evolution, particularly in plants (Grant, 1981). Other authors from their recent studies also agree that it plays a major role in the evolution and diversification of the plant kingdom (Bretagnolle and Thompson, 1995; Soltis *et al*., 2003). The abundance of polyploidy in evolutionary history of plants further suggests its importance in their evolution. An estimate of the frequency of polyploid angiosperm species has reached as high as 80%, and 95% in pteridophytes (Liu and Wendel, 2002).

Stebbins (1971) presents many examples of plant taxa with possible secondary and multiple polyploid origins. Recent evidences also suggest that lineages may undergo repeated cycles of polyploidization followed by extensive diploidization. The advancement of molecular techniques have revealed that flowering plants and perhaps all eukaryotes possess genomes with considerable gene redundancy, much of which is likely the result of polyploidy or whole genome duplication (Soltis *et al*., 2003).

2.3.3.1.1 Factors promoting polyploidy

A number of investigations consider the following factors to be important in promoting polyploidy.

Allopolyploids recover the lost fertility due to hybrid formation. They have advantages of hybridity like increased vigor and heterosis combined with true breeding. They can reproduce sexually and yet breed true for a highly heterozygous genotype. They have greater buffering in their genotypes because of numerous duplications (Grant, 1981). Allopolyploids and autopolyploids maintain higher levels of heterozygosity than diploids (Soltis and Rieseberg, 1986).

The increased size of certain organs particularly seeds, which accompanies polyploidy, may also help in the process of stabilization and establishment of polyploids since it increases seedling vigor (Stebbins, 1971).

These advantages that polyploids have over diploid, i.e. increased heterozygosity, increased vigor and heterosis, increased size of certain organs and tolerance to a wide range of environmental conditions might contribute to the occurrence of polyploids in harsh conditions like cold environments and in high altitudes (Grant, 1981).

The frequency of polyploid species tends to be relatively high in higher mountains, sometimes higher than the neighboring lowlands (Grant, 1981). There are cases where frequency of polyploids increases with altitude on a mountain (Hedberg and Hedberg, 1977).

2.3.3.1.2 DNA amount changes following polyploid formation

Formation of polyploids results in hybridity and polyploidy (Finnegan, 2001). Newly formed auto- and allopolyploids exhibit considerable meiotic complexity, including multivalent pairing, multisomic inheritance and the production of unbalanced gametes which lead to sterility (Ramsey and Schemske, 2002).

One mechanism that increases fertility of newly formed polyploids is diploidization, which leads to bivalent inheritance in polyploids (Singh, 1993) and reduction of the expressed gene set to an effective diploid level (Finnegan, 2001).

In newly formed allopolyploids, pairing between homeologous parental genomes can be frequent, but are rare in established polyploids (Comai *et al*., 2000). In case of the genus *Aegilops*, allopolyploidy-induced sequence elimination occurred in a sizable fraction of the genome and in sequences that were apparently non-coding. This finding suggests a role of sequence elimination in augmenting the differentiation of homoeologous chromosomes at the polyploid level, thereby providing the physical basis for the diploid-like meiotic behavior of allopolyploids (Ozkan *et al*., 2001). This can also be seen from other data in wheat (Shaked *et al*., 2001), *Brassica* species (Song *et al*., 1995) and in recently formed natural polyploids like *Spartina anglica* (Salmon *et al*., 2005), *Tragopogon mirus* and *T. miscellus* (Soltis *et al*., 2004).

Technically, polyploidy by itself does not represent a change in genome size *per se* because it actually involves the addition of a second genome. That is, so long as the two genomes remain distinct (until rediploidization occurs), C-value will increase but genome size will not (Gregory, 2005). However, sequence elimination (which leads to diploidization) in the first generation of polyploid formation (Ozkan *et al*., 2001; Shaked *et al*., 2001) results in lower than expected C-value of polyploids i.e., the new polyploid C-value may be less than the expected sum of the parental genomes (Leitch and Bennett, 2004; Schneeweiss *et al*., 2006; Johnston *et al*., 2005; Ozkan *et al*., 2003).

When polyploids generally become functional diploids and stabilize with more DNA than before, polyploidy becomes a cause of increased C-value (Cavalier-Smith, 2005). As polyploidy is a major force of evolution and is common in plants (Grant, 1981), it is a major mechanism that results in C-value increase (Cavalier-Smith, 2005).

2.3.3.2 Aneuploidy

Aneuploidy is a difference in the number of individual chromosomes. The deviations from a standard chromosome number may be increasing or decreasing. The numerical deviations may involve a single chromosome, a single chromosome pair or more than one pair. The extra chromosomes or the missing chromosome could be homologs of members of the regular complement and the aneuploidy could occur at the diploid or polyploid level (Grant, 1981). Aneuploidy can be a cause of C-value variation (Greilhuber, 1998).

In some cases, the extra chromosomes are not a part of the regular complement called B-chromosomes (Grant, 1981). B-chromosomes are morphologically distinct and smaller than A-chromosomes and they fail to pair or recombine with any of the A-chromosomes at meiosis. They show numerical variation within and between individuals (Jones, 1995).

Intraspecific C-value variation can occur due to differences in the number of B-chromosomes such as maize (Rayburn *et al*., 1985) and *Cirsium acaule* (Bures *et al*., 2004).

2.3.3.3 Molecular factors as causes of DNA amount variation

In addition to chromosome numerical increase such as polyploidy, the wide variation in C-value observed among eukaryotic species is closely correlated with the amount of repetitive DNA. Major types of repetitive DNA include transposable elements, satellite DNAs and tandem repeats (Kidwell, 2002).

Transposable elements (TE) are DNA sequences that have the capacity to change genomic locations (Kidwell and Lisch, 2001). They comprise greater than 60% of plant genomes (Gregory, 2005). TE cause C-value increase. There is approximately linear relationship between C-value and the total amount of TE among eukaryotes whose genomes have been sequenced in detail (Kidwell, 2002). For example, genome of maize has tripled in size by transposable elements activity in its evolutionary history (SanMiguel and Bennetzen, 1998) and DNA content variation within natural populations of *Hordeum spontaneum* has been correlated with long terminal repeat retrotransposon (LTR) copy number variation within this species (Kalendar *et al*., 2000).

Plant chromosomes are rich in repetitive DNA, which comprises 70% or more of the total (Rees and Narayan, 1981). Some are arranged in tandem arrays at the sites of heterochromatin and others are dispersed at many locations in the chromosomes (Flavell, 1986). Different repetitive sequence families contribute significantly to variation in DNA content such as variation among *indica* and *japonica* varieties of rice (Ohmido *et al*.,

2000), variation among *Lathyrus* species (Rees and Narayan, 1981), sugar beet (Kubis *et al*., 1998) and *Secale* species (Flavell, 1986).

The factors mentioned above result in C-value increase. However, phylogeny has shown that C-value increases as well as decreases within a genus (Price *et al*., 2005; Johnston *et al*., 2005). Genome shrinkage counteracts the many mechanisms for genome growth (Knight *et al*., 2005). Deletion reverses the increase in C-value by loss of DNA (Bennetzen *et al*., 2005).

Although all of the mechanisms responsible for C-value variation are not known, a simple model has been formulated suggesting that C-value variation is generated by lineage-specific differences in the molecular mechanisms of DNA amplification and removal, creating major variations in DNA content that can serve as the substrate for fitness based selection (Bennetzen *et al*., 2005).

2.3.3.4 Other factors which cause DNA amount variation

In special cases, sex chromosomes cause intraspecific C-value variation. For example, the larger Y chromosome in *Silene latifolia* leads to larger C-value of the male plant (Meagher *et al*., 2005).

DNA amounts between polyploids of the same species and ploidy level may vary due to the difference of the DNA content of the constituent genomes. For example, in *Musa* the A and B genomes differ in their genome size. Thus, the triploids AAA, AAB, and ABB differ in C-value because they differ in the number of A and B genomes (Lysak *et al*., 1999).

2.3.4 Correlation of DNA amount with phenotype

DNA C-value is correlated with the nucleotype, which is in turn determined by the total quantity of DNA. Nucleotype is a term coined for the effects of DNA amount on the phenotype. It is correlated with nuclear volume, cell volume, mitotic cycle time and duration of meiosis (Bennett, 1972). Species with very large DNA content have very long duration of cell cycle and species with small genomes have less duration (Bennett, 1972; Price 1988).

According to the statistics obtained from data available in the plant functional database, there is a correlation between seed size and C-value. A larger range of C-value is found as seed mass increases, but all species with largest genomes always have largest seeds (Knight *et al*., 2005). There also exists negative correlation between C-value and body size. For example, species with low leaf area tend to have larger C-value (Knight *et al*., 2005). *Silene latifolia* shows negative correlation between C-value and flower size (Meagher *et al*, 2005).

C-value is also correlated to life form. Herbaceous perennials have largest C-values, annuals have low C-values and lowest C-values are found in ephemerals. Short life cycles are related to shorter mean cell cycle time and mean meiotic duration and lower DNA content than species with longer life cycles (Bennett, 1972). For example, biennial species of *Cirsium* possess larger nuclear DNA amounts than their perennial relatives (Bures *et al*., 2004).

2.4 Methods of genome quantification

The techniques used for genome quantification are flow cytometry and static cytometry. Flow cytometry analyses microscopic particles in suspension which are constrained to flow in a single file (Dolezel and Bartos, 2005) whereas static cytometry involves fixed materials (Vilhar *et al*., 2001).

2.4.1 Flow cytometry

Flow cytometry analyses stained microscopic particles in suspension, which are constrained to flow in a single file within a fluid stream through the focus of intense light. Pulses of scattered light and fluorescence are collected and converted to electric current pulses by optical sensors (Dolezel and Bartos, 2005). The flow cytometers have in-built computers where data can be stored and analyzed (Suda, 2004).

In a healthy tissue, a large majority of the cells will have 2N DNA amount (this phase is called G_1 in dividing cell and it is called G_0 if the cell is in the resting phase). A smaller proportion of healthy cells will be in the process of synthesizing DNA for cell division; hence these cells have between 2N and 4N DNA depending on how much of the DNA has been synthesized. This period of cell life is referred to as S-phase. Once a cell has twice its normal DNA content (4N, in the phase called G_2) it makes the final preparations for cell division and then divides into two cells (a phase called M for mitosis) each with 2N DNA (Eudey, 1996). (Fig 1)

Nuclei isolated from plant tissues and stained usually emit two distinct peaks of fluorescence, one corresponding to G_0 + G_1 phase cells (with 2C DNA amount) and the other G_2 + M phase cells (with a 4C DNA amount). The latter produce twice the

fluorescence, as they contain twice the DNA amount (Fig 2). Nuclei from some tissues display only one peak, e.g. non-dividing nuclei subject to a G_1 developmental hold form a single peak at the 2C DNA value. In some cases multiple peaks, corresponding to higher levels of ploidy, are seen. This has enabled flow cytometry to estimate ploidy levels in different tissues, cell cultures or plant populations (Bennett and Leitch, 1995).

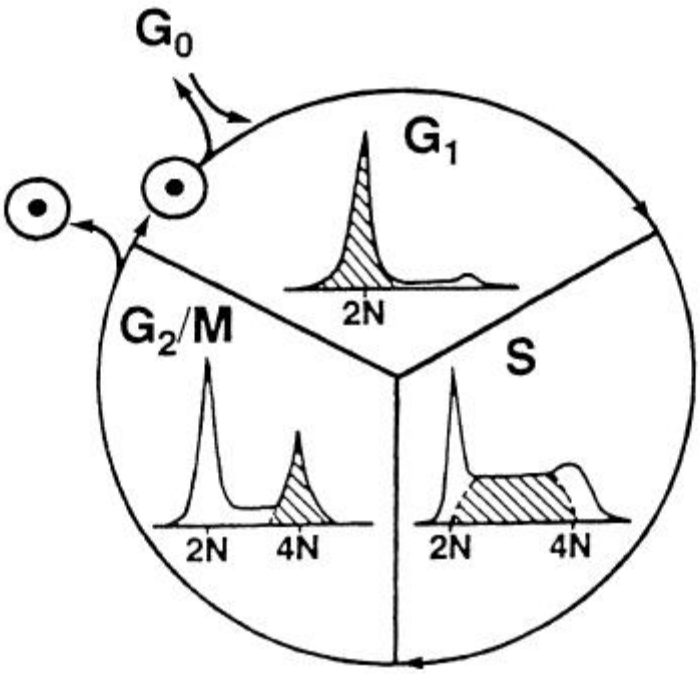

Figure 1. The cell cycle and the components of the DNA histogram: G_0 and G_1 have 2N amount of DNA; S-phase has between 2N and 4N component of DNA; and G_2/M has 4N amount of DNA. Source (Eudey, 1996)

The quality of the data set forming the DNA histogram is often assessed by the coefficient of variation (CV) for the G_0/G_1 peak and the CV of the G_2/M peak (Eudey, 1996). The fluorescent peaks should be distinct and have very low coefficients of variation, ideally not greater than 5% (Dolezel and Bartos, 2005). The CV of nuclei also

depends on fluorochrome used. DAPI (4', 6-diamidino-2-phenylindole) stain generally gives better histogram (Suda, 2004). Sharp peaks and low CVs indicate that the nuclei have been isolated cleanly and have not suffered significant damage or breakdown during staining and analysis (Bennett and Leitch, 1995).

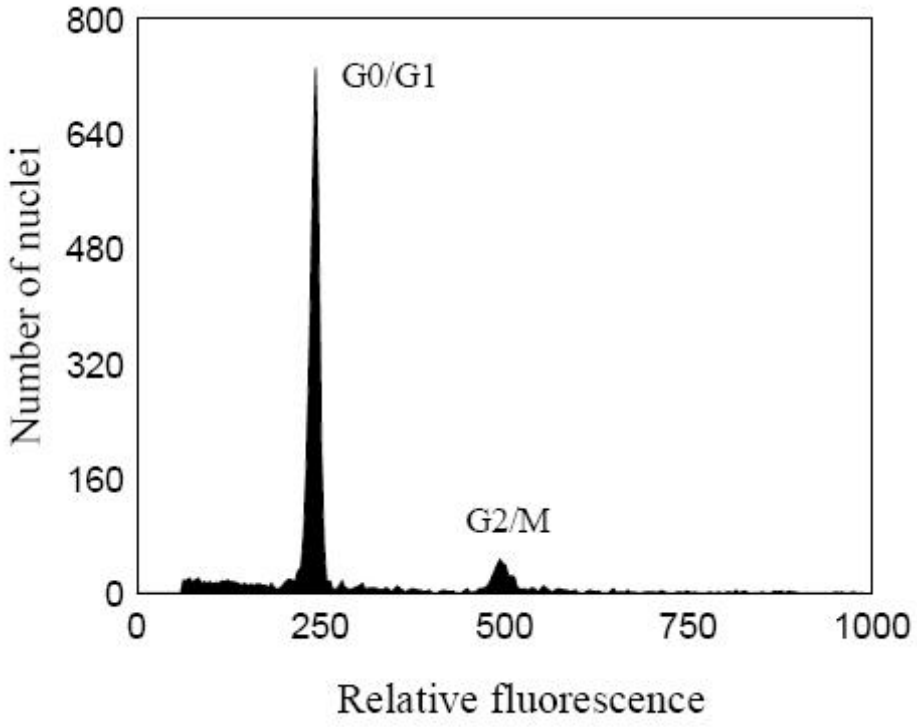

Figure 2. A histogram obtained from flow cytometric analysis of *Anthoxanthum alpinum* using DAPI. The prominent peak corresponds to the nuclei in G_0/G_1 phase (with 2C DNA content), the smaller peak corresponds to the nuclei in G_2/M phase (with 4C DNA content). Signals spanning the range correspond to the nuclei synthesizing DNA (S phase). Some background fluorescence (noise) appears on low channels (Source: Suda, 2004)

2.4.1.1 Methods of flow cytometry for plant DNA study

Nuclei are released by mechanically chopping small amount of fresh plant tissue in nuclei isolation buffer. Around 5000-10,000 nuclei are analyzed and this quantity is usually obtained from about 50 mg of tissue. The nuclei are stained and the sample is

passed down a flow cytometer. The relative fluorescence emitted from each nucleus, which is proportional to the DNA content, is measured and analyzed (Dolezel *et al*., 1998).

Table 1. Internal reference standards recommended for determination of absolute nuclear DNA amount. Source: Suda, 2004.

Recommended reference standard	2C-value (pg)
Raphanus sativus	1.11
Lycopersicum esculentum	1.96
Glycene max	2.50
Zea mays	5.43
Pisum sativum	9.09
Vicia faba	26.9

Flow cytometry can be used both for absolute DNA amount estimation and for differentiating between different ploidy levels based on the relative fluorescence of mixed ploidy samples.

In case of DNA amount estimation, the relative fluorescence is converted into absolute DNA amount by including an internal standard whose DNA amount is known (Bennet and Leitch, 1995) (Table 1). An ideal DNA reference standard should have a DNA amount close to the target species. Ratio between the 2C-values of an analyzed plant and the internal standard should be the smallest to minimize the potential linearity of flow cytometer measurements. However, at least 12% difference in 2C-values of the

internal standard and sample should be present to exclude bias due to very close or overlapping peaks (Suda *et al*., 2003).

Flow cytometry by itself does not provide the actual ploidy level of the samples; it only gives relative value to the samples co-analyzed. Thus, it is essential to count chromosome numbers in order to relate fluorescence value to the actual ploidy level of the samples (Bennett and Leitch, 2005a).

Plants exhibit large divergence in C-value even among closely related taxa with the same number of chromosomes and often have non-proportional changes of nuclear DNA amount with respect to ploidy level. To differentiate between the ploidy levels from flow cytometry i.e., relative DNA content estimation and the estimations from direct chromosome counting, the following terms have been proposed. To designate the ploidy levels estimated from flow cytometry, the term 'DNA ploidy' is used whereas 'Ploidy level' is used to designate the ploidy estimation from direct chromosome counting. Corresponding terms have been proposed regarding aneuploidy. To designate difference in number of individual chromosomes detected by flow cytometry, the term 'DNA-aneuploidy' is used, and the term 'aneuploidy' refers to difference in number of individual chromosomes detected by direct chromosome counting (Suda *et al*., 2006).

It has been found that DAPI is suitable dye for DNA-ploidy level estimation because of its high resolution with CV less than 3% (Suda, 2004). Thus, in plant studies involving flow cytometric DNA-ploidy level estimation, including aneuploidy detection, DAPI is used (Suda *et al*., 2004; Mandak *et al*., 2003). Arc-lamp is used as light source for DNA-ploidy level estimation with which DAPI fluorescence is particularly easy to excite and measure (Dolezel and Bartos, 2005).

Dolezel *et al*., (1998) have shown that DAPI is unreliable for estimation of plant C-value because it is base specific and that the DNA intercalating dye, propidium iodide (PI), can be used for reliable estimation of C-value in plants using flow cytometry (Dolezel *et al*., 1998). Argon ion laser at 488 nm is used as a light source for absolute C-value estimation (Suda *et al.*, 2003).

2.4.1.2 Applications of flow cytometry in plant studies

Flow cytometry has become a popular method for estimation of absolute DNA amount or C-value (Dolezel and Bartos, 2005). The ease of sample preparation and the ability to measure DNA quickly in a large number of cells make the technique ideal for studies that require large-scale measurement of C-value (Dolezel and Bartos, 2005).

C-value data is important. It has impacts on many areas of research and knowledge of it can be important when planning research (Bennett et al, 2000a). It is useful in systematics as comparing it with other characters may provide a way to elucidate evolutionary relationships (Ohri, 1998). It is useful when choosing organisms to be sequenced. Taxa initially chosen for sequencing projects all had relatively smaller genomes. *Arabidopsis* was chosen for sequencing because of its small C-value (Bennett and Leitch, 2005a). C-value is an indicator of the type of protocol to be used for AFLP studies. Species with very large and very small genomes have different protocols than the standard protocol (Fay *et al*., 2005).

Flow cytometry provides a fast and accurate way to look at changes in genome size during evolution and differentiation. The response to hybridization and polyploidy can be quantified on a genome-wide basis, using this method (Ozkan *et al*., 2003).

It is also used for differentiating between different genomes that constitute a polyploid. Lysak *et al*., (1999) found that genomes A and B of *Musa* differ in genome size and the triploids with AAA, AAB and ABB genomic constitution differ in their C-value and can be differentiated from one another by flow cytometry method (Lysak *et al*., 1999).

Flow cytometry could be useful to differentiate between cytotypes that are indistinguishable by morphological features but that differ in C-value. For example, two cytotypes of *Capsicum campilopodium* are indistinguishable by morpohological features. However, flow cytometric analysis revealed that they differ in DNA amount (Moscone *et al*., 2003).

Flow cytometry is used for sex detection if the plants have X and Y chromosome size difference, which will result in C-value difference of the two sexes (Meagher *et al*., 2005).

Even if C-value data is very important, there are many gaps in DNA C- value compilations regarding geographical and plant life form representation (Bennett *et al*., 2000a; Bennett and Leitch 2005b). Alpine environments are under represented. The samples whose C-value has been estimated are dominated by crops and their wild relatives, model species grown for experimental use and other species growing near laboratories in temperate regions (Bennett and Leitch, 2005b). The only C-value estimation in the database from Ethiopia is that of *Eragrostis tef* (Bennett *et al*., 2000a)

Flow cytometry is used for large scale screening of ploidy level in plants. This is useful to study population cytotype structure (Dolezel and Bartos, 2005). Cytotypes are any variety or race of a species whose chromosome complement differs in chromosome number from the standard complement of that species. Cytotype distribution over vast

geographical areas provides useful insight into population biology of multiploid taxa. Flow cytometry has made large-scale cytotype investigations possible. In addition it is used to detect aneuploidy (Suda, 2004).

Flow cytometry can be used to detect endopolyploidy in plants. For extensive use of cell culture techniques for plant breeding, increased knowledge of the degree of endopolyploidy in the explant tissue source is very valuable for the maintenance of the original ploidy level and the genotype of crop species (Kudo and Kimura, 2001).

It has also become a popular method for cell cycle analysis and determination of reproductive pathway (Dolezel and Bartos, 2005).

2.4.1.3 Artefactual data of flow cytometry

There are reports of intraspecific DNA content variation that cannot be explained by factors causing intraspecific variation. For example, there are numerous reports of these types of variation in *Helianthus annus*. Price and Johnston (1996) reported that the mean 2C DNA content of the first leaf of sunflower (grown under controlled conditions) is influenced by the quality and quantity of light. They concluded that the far-red light induced instability and reduction of nuclear DNA content, which may represent an adaptation for shade avoidance in competition with neighboring plants. Price *et al*., (1998) tested this in field conditions and presented the hypothesis: sunflower plant self regulates its DNA content in response to environmental stimuli. They also presented an alternative hypothesis that the variation in DNA content is due to differential accumulation in leaves of one or more secondary products which interfere with intercalation and/ or fluorescence of PI (Price *et al*., 1998). The latter hypothesis is soundable because in other studies reduced and distorted Feulgen staining was found to occur when tannins or similar

polyphenols are present in the cell and tissues to be analyzed (Greilhuber, 1998). Later, it was found that sunflower contains stain inhibitors (Price *et al*., 2000).

Noirot *et al*. (2000) studied many plant materials of coffee and showed how different concentrations of cytosolic components bring about dye accessibility differences to the sample nuclei. Any within species variation in phenol and caffeine contents leads to pseudo-intraspecific variations in C-value (Noirot *et al*., 2000). Caffeine and chlorogenic acid can also modify accessibility of the dye, propidium iodide, to the standard DNA. Since their contents vary between individuals and even on different dates in the same individual, they could wrongly implicate intraspecific C-value variation (Noirot *et al*., 2003).

Using different instruments and/or methods for the same plant species can also be a source of erroneous data. Dolezel *et al*. (1998) compared C-value results from four laboratories, which used different instruments and procedures. The C-value of the same species from the same cultivar showed up to 15.6% difference when estimated with different instruments and/or methods (Dolezel *et al*., 1998).

2.4.1.4 Best practice for avoiding artefactual data

Following the discovery that flow cytometry can give artefactual data, several solutions to the problem have been recommended, which are presented below.

It is recommended that simultaneous processing of nuclear samples from the target and standard (internal standardization) is necessary to minimize variation due to sample preparation. It also ensures similar dye accessibility to both the standard and sample (Price *et al*., 2000).

Simultaneous processing of individuals with highest and lowest values of intraspecific variation is another test, where the peaks of the two samples will be separated in case of true variation (Price *et al*., 2000). 2-mercaptoethanole is routinely added to staining buffers to avoid polyphenol oxidation (Suda, 2004).

Several extracts have to be measured per target and experiment should be repeated on different days (Noirot *et al*., 2000).

Determining the appropriate isolation and/ or staining buffer for the plant material is necessary (Dolezel and Bartos, 2005). Tris-$MgCl_2$ buffer proves to be successful in taxons with small genome while the two-step procedure and the alternatively simplified modification (see material and methods section) give optimal results in most plants (Suda, 2004).

To compare very small differences in C-value, the samples should be estimated using the same instrument, the same procedure (Dolezel et al, 1998) and even the same operator (Suda, 2004).

For some plant species, it may be difficult to analyze using leaf. So, other tissues like leaf petioles (*Viola*), young stems (*Lythrium*), flowering stems (*Ulticularia*), cotyledons (*Lavatera*), etc can be checked for acceptable histograms (Suda, 2004).

2.4.1.5 Correction on C-values using best practice

Erroneous reports on C-values have been identified in many literatures and these have been re-estimated using best practice outlined above (Bennett and Leitch, 2005a; Greilhuber, 2005).

For instance, the report of intraspecific variation reported for *Glycine max*, without using internal standard, was reinvestigated by using internal standard and no intraspecific

variation in C-value among cultivars was found. In case of *Dasypyrum villosum*, the large C-value difference reported was not found during reinvestigation (Greilhuber, 2005).

2.4.2 Static cytometry

In contrast to flow cytometry, which involves movement of stained nuclei in a flow chamber, there are static cytometric methods, which involve fixed materials.

One of these is densitometry, which comprises methods that measure DNA quantity in the nucleus from the optical density of the regions stained with Feulgen reaction procedure. The oldest method of densitometry measures optical density with instruments that combine a microscope with a photometer and have been variously referred to as microdensitometers, cytophotometers and microspectrophotometers (Vilhar *et al*., 2001).

An image analysis system has been integrated into microspectrophotometer and is called image cytometry. Image cytometry is a system which captures the microscope field by a microscope-mounted CCD (charge-coupled device) or digital camera connected to a computer and calculates optical density from the nucleus (Hardie *et al.*, 2002).

2.4.3 Advantages and disadvantages of flow cytometry and static cytometry

Microspectrophotometry is a well-established and accurate method for determining DNA content of plant nuclei. This method, however, is time consuming and the accumulation of a large data set can, therefore, be frustratingly slow. On the other hand, flow cytometry can potentially provide a method for rapidly determining DNA content of individual plants, plant organs, and plant tissues, based on the average of thousands of

nuclei (Michaelson *et al*., 1991). The ease of sample preparation has also made flow cytometry an attractive alternative to microspectrophotometry (Dolezel and Bartos, 2005). Microspectrophotometry is cost effective method for estimation of DNA C-values with acceptable accuracy for small number of samples (Bennett and Leitch, 1995). However, flow cytometry is a convenient method when a large number of samples have to be analyzed (Suda *et al*, 2003).

Many chromosomal changes and variations like aneuploidy, duplications and deletions, sex and supernumerary chromosomes can lead to detectable changes in DNA amount. If these are not identified before or after using flow cytometry, then interpretation of the results could be incorrect. Thus, although the speed of analysis of flow cytometer is appealing, cytological analysis, which can be time consuming, can be done simultaneously with genome analysis in case of microdensitometry (Bennett and Leitch, 1995).

Image cytometry also uses microscope slide preparations, allowing simultaneous cytological observations (Bennett and Leitch, 2005a). It gives accurate and reproducible results and can be used as an alternative to microdensitometry in plant nuclear DNA measurements (Vilhar *et al.*, 2001). Image cytometry had received less attention previously (Vilhar *et al.*, 2001). But now, it is becoming popular and is even replacing microdensitometry. Another advantage of an image cytometer is that it can use an existing microscope. Moreover, it costs less than a flow cytometer to set up and is also easier to service in countries that lack flow cytometer manufacturers support (Bennett and Leitch, 2005a).

2.5 Objectives of the study

General objective: Polyploidy and nuclear DNA amount determination in some selected afroalpine plants of Ethiopia.

Specific objectives:

1) Filling the gaps in chromosome number information of *R. stagnalis, R. oreophytus, D. caespitosa*, *C. cryptocephala* and *C. obliqua*

2) Provide nuclear DNA content data for *R. stagnalis, R. oreophytus, D. caespitosa and C. cryptocephala*

3. Materials and methods

3.1 Plant materials and sites of collection

This study was conducted on *Cotula cryptocephala* (Asteraceae)*, Cardamine obliqua* (Brassicaceae), *Deschampsia caespitosa* (Poaceae), *Ranunculus oreophytus* (Ranunculaceae) and *Ranunculus stagnalis* (Ranunculaceae) (Table 2 and Figure 3) that were collected from Afroalpine environment of Bale and Simen Mountains (Figure 4). Live plant materials were transplanted to the greenhouse at Addis Ababa University.

Initially, 30 accessions were collected but the study could be conducted on 15 accessions only since the rest of the plants died in the greenhouse due to the climate difference of the afroalpine environment and the greenhouse. *D. caespitosa* and *C. obliqua* were studied from Bale Mountains only and *R. stagnalis* was studied from Simen Mountains only whereas *R. oreophytus* and *C. cryptocephala* were studied from both mountains.

DNA amount estimation, DNA-ploidy estimation and chromosome counting were done for *R. stagnalis, R. oreophytus, D. caespitosa* and *C. cryptocephala* whereas only chromosome counting was done for *C. obliqua*.

Table 2. Plant materials studied with their accession numbers, locality, altitude and coordinate

				A		
				(		
1				4		
2				4		
3				3		
4	»	[illegible]	[illegible]	4110	13⁰15'19.5"N	38⁰13'0.7"E
5	»	RoSe6	Simen	3984	13⁰15'32.1"N	38⁰12'46.7"
6				37		
7				34		
8				39		
9				34		
10				34		
11				34		
12				35		
13				37		
14				38		
15				37		
16				37		

Figure 3. Four of the plant species used for the study A. *Cotula cryptocephala*, B *Ranunculus oreophytus*, C. *Ranunculus stagnalis*, D. *Deschampsia caespitosa*

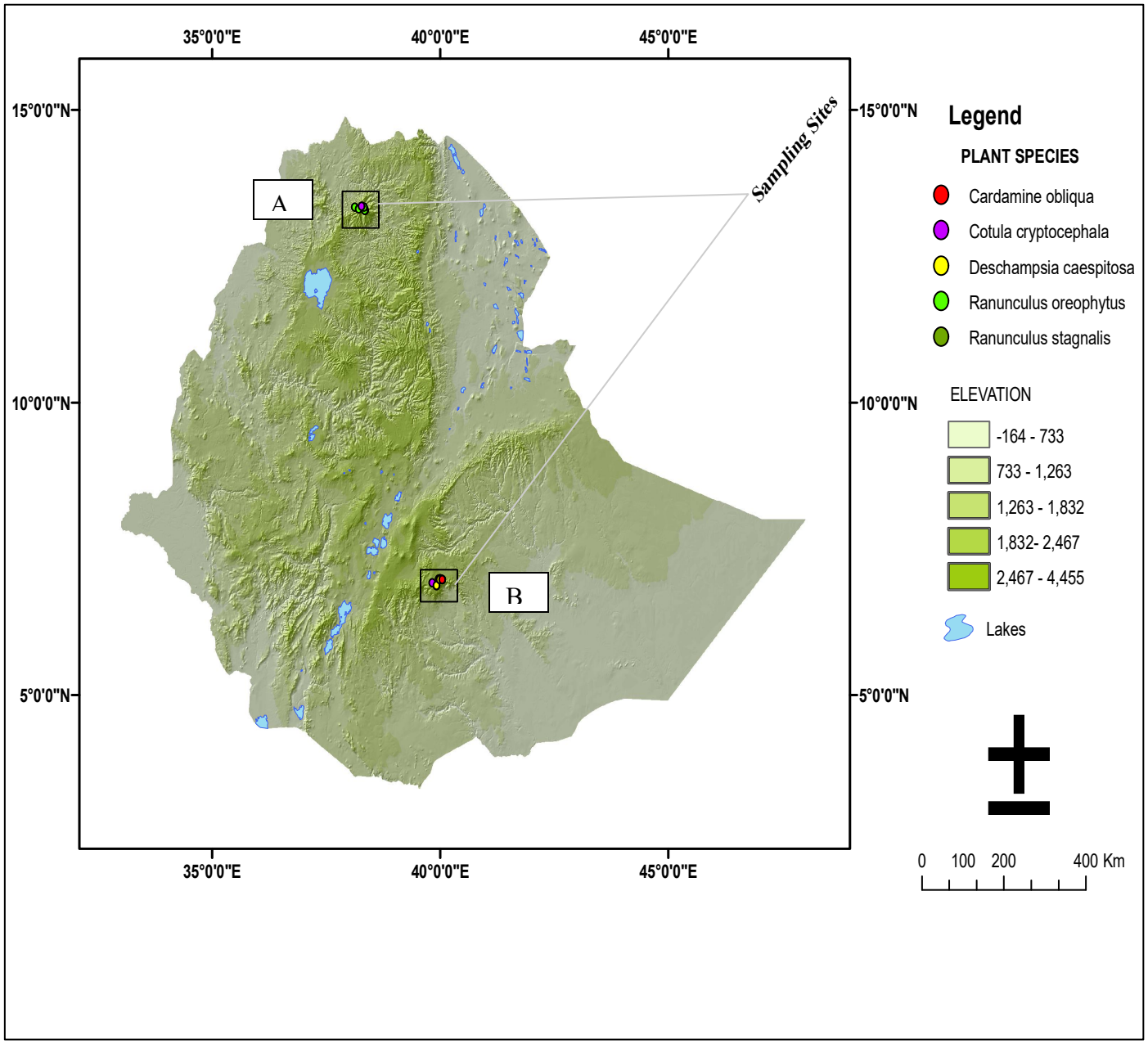

Figure 4. Map of Ethiopia showing sites from where the plant materials were collected. A. Simen Mountains and B. Bale Mountains

3.2 Methods

3.2.1 Chromosome counting

According to the procedure of somatic metaphase chromosome preparation of Dagne and Heneen (1992), actively growing roots were collected from potted plants and treated either with aqueous colchicine solution (0.1% w/v) or with 8- hydroxyquinline (0.002 M) for 3 to 4 hours. The roots were fixed in ethanol: acetic acid (3: 1) for 2 hours. Then they were rinsed in distilled water. Digestion with cellulase (3%) + pectinase (3%) solution was done for 1 hour and, in case of *D. caespitosa* for about 5 hour, in a water-bath at 37 ^{0}C. The enzyme solution was removed, roots rinsed in distilled water and the root tips were pipetted onto a glass slide. The water was blotted off with filter paper. The root tips were mashed with a forceps in a drop of fixative and spread by strongly blowing on the slide. The slides were allowed to air-dry at room temperature. Air-dried slides were stained in Giemsa stain, 6.8 PH for two hours and air-dried overnight before mounting in DPX mountant (Dagne and Heneen, 1992).

The chromosome observations were made under a light microscope and photographs were taken with 100x oil objective. At least three cells were counted per plant.

3.2.2 DNA-ploidy and C-value estimation

C-value estimation was done using CyFlow (Cytecs GmbH) and DNA-ploidy estimation was done using Ploidy Analyser PA-II (Partec GmbH).

Simplified two step procedure using Otto I buffer (0.1 M citric acid monohydrate, 0.5 % (v/v) Tween 20) and Otto II buffer (0.4M Na_2HPO_4 .$12H_2O$) (Otto, 1990) was used for

both the 2C-value and DNA-ploidy estimations, except that PI stain was used for the former and DAPI stain was used for the latter.

DNA-ploidy level was inferred based on the relative nuclei fluorescence of individuals within each species using DAPI stain. It was determined for all the 15 plant accessions listed in Table 2, except for *C. obliqua*. On the other hand, chromosome counts were made for only one representative accession from each species (Mandak *et al*., 2003). Absolute DNA amount was determined for all the 15 plant accessions listed in Table 2, except for *C. obliqua*.

Nuclei were mechanically isolated from the plant leaf tissue by chopping young, but not premature, intact leaf tissues (about 1 cm^2) of both the sample and standard together with a new razor blade in a Petridish containing 0.5 ml of ice-cold Otto I buffer (Otto, 1990). Then, the suspension was filtered through a 42-µm nylon mesh and incubated at room temperature for about 60 minutes. The isolated nuclei were stained with a fluorochrome by adding 1 ml of Otto II buffer supplemented with fluorochrome (DAPI, 4 µg/ml + RNase 50 µg/ml or Propidium iodide, 50 µg/ml + RNase, 50 µg/ml) and 2 µl/ml of ß-mercaptoethanol was added to avoid polyphenolics oxidation. The mixture was stored in the dark at room temperature for 5-15 minutes. The sample was passed down a flow cytometer and the relative fluorescence emitted from each nucleus, which is proportional to the DNA content, is measured and analyzed. Five thousand nuclei were analyzed (Suda *et al*., 2003).

By including an internal standard, the relative fluorescence is converted into absolute amounts.

Appropriate internal standards were selected for each species as follows. First, the 2C-value of the samples was estimated using *Pisum sativum* cv. Ctirad as a common standard for all the samples because it has the intermediate 2C-value among the recommended standards listed in Table 1. Then, internal standards were assigned to specific samples when the 2C-values of the sample and the standard are close but have some differences to avoid formation of very close or overlapping peaks of the sample and the standard (Suda *et al*., 2003).

Accordingly, *Pisum sativum* cv. Ctirad, 2C=9.09 pg was used as standard for *Cotula cryptocephala* and *Ranunculus oreophytus*; *Zea mays* cv. CE-777, 2C= 5.43 pg for *Deschampsia caespitosa* and *Vicia faba* cv. Inovec, 2C= 26.9 pg for *Ranunculus stagnalis*.

FloMax software (Quantum Analysis GmbH) was used to obtain the histograms of fluorescence, peak means and coefficients of variation (CV) (Figures 6-11).

The absolute DNA amount of a sample was calculated based on the values of the G_1 peak means as follows:

Sample 2C DNA content = [Sample G_1 peak mean / Standard G_1 peak mean] x Standard 2C DNA content (pg) (Dolezel and Bartos, 2005).

Standard deviation of the 2C DNA values of the samples was calculated as the square root of the sample variance: $SD = \sqrt{\partial^2}$

The variation within a species was calculated by dividing the largest 2C DNA value by the lowest 2C DNA value within that species.

4. Results

4.1 Chromosome count and Ploidy levels

The results of chromosome counts showed that *Cardamine obliqua* is an octaploid with 2n= 62, *Cotula cryptocephala* is an octaploid with 2n= 64, *Deschampsia caespitosa* is a diploid with 2n= 26, *Ranunculus oreophytus* has chromosome number of 2n= 32 and *Ranunculus stagnalis* 2n= 28 (Table 3 and Fig 5). The latter two species are both tetraploids.

Intraspecific DNA-ploidy level difference was not observed for any of the species *R. stagnalis, R. oreophytus, D. caespitosa*, and *C. cryptocephala*.

Table 3. Chromosome number, accession number and altitude from which chromosome numbers were counted

Accession no.	Species	Chr. no.	Altitude
CoBa9	*Cardamine obliqua*	62	3789
CcBa6a	*Cotula cryptocephala*	64	3476
RoBa8	*Ranunculus oreophytus*	32	3792
RsSe4	*Ranunculus stagnalis*	28	4094
DcBa4	*Deschampsia caespitosa*	26	3860

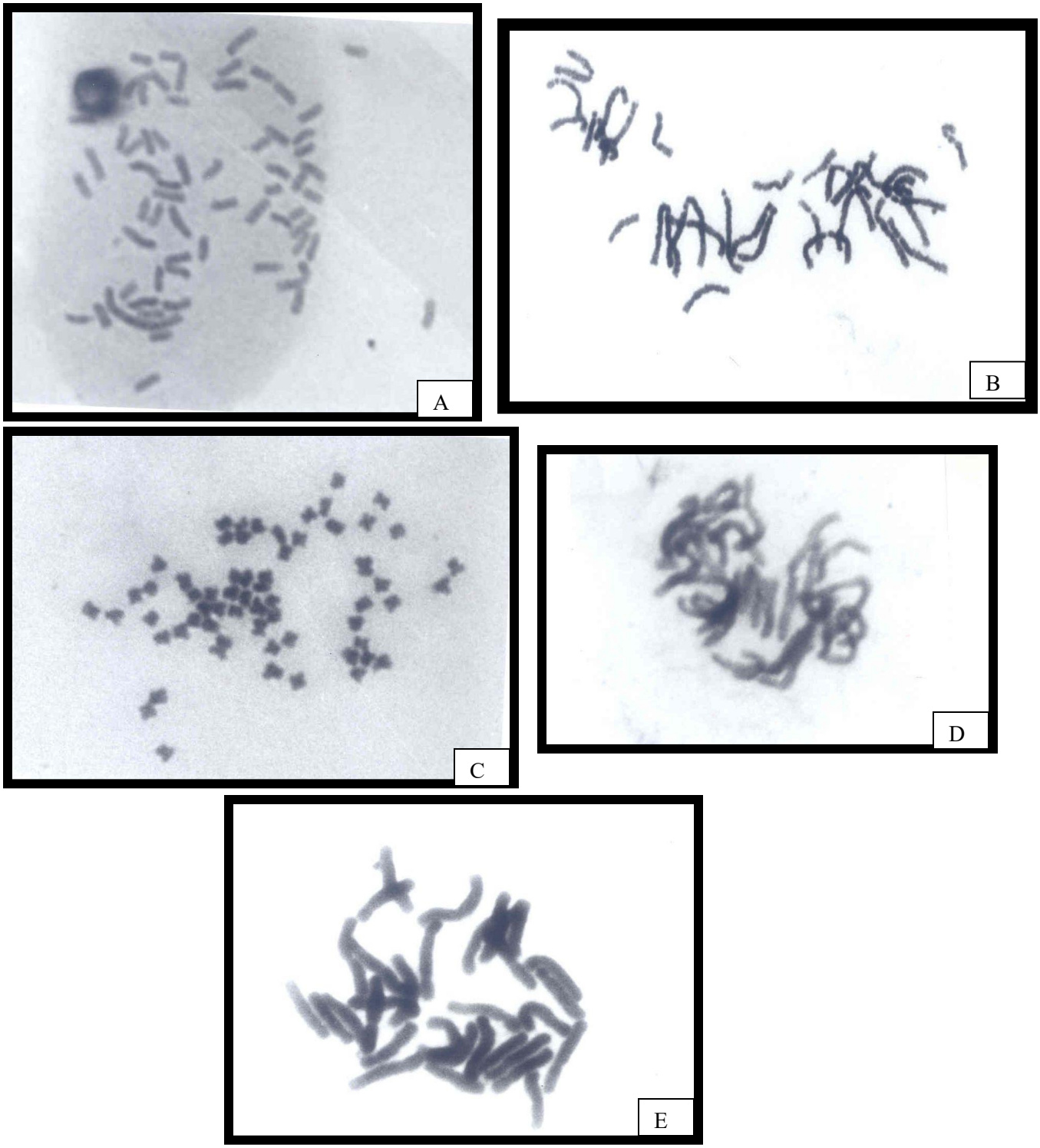

Figure 5. Chromosomes of the plant materials studied. A. *Cotula cryptocephala* (2n= 64), B. *Ranunculus stagnalis* (2n= 28), C. *Cardamine obliqua* (2n= 62), D. *Deschampsia caespitosa* (2n= 26) and E. *Ranunculus oreophytus* (2n= 32)

4.2 Intraspecific and interspecific 2C DNA value

Mean values of fluorescence for the samples and standards and coefficients of variation were obtained by the FloMax software (Quantum Analysis GmbH) (Figures 6-20).

The fluorescence data are presented in the form of histograms (Figures 6-20). In each figure, horizontal axis (fl1) shows the intensity of the fluorescence signal and the vertical axis (counts) shows the number of nuclei within the intensity range. In addition to the peaks of the sample and standard, figures 6 and 12 contain background fluorescence and figure 11 contains with background fluorescence and G_2 peak (4C DNA amount) of the standard. Figures 7, 8, 10 and 20 contain one G_2 peak each (4C DNA amount) of the standard in addition to the peaks of the sample and standard.

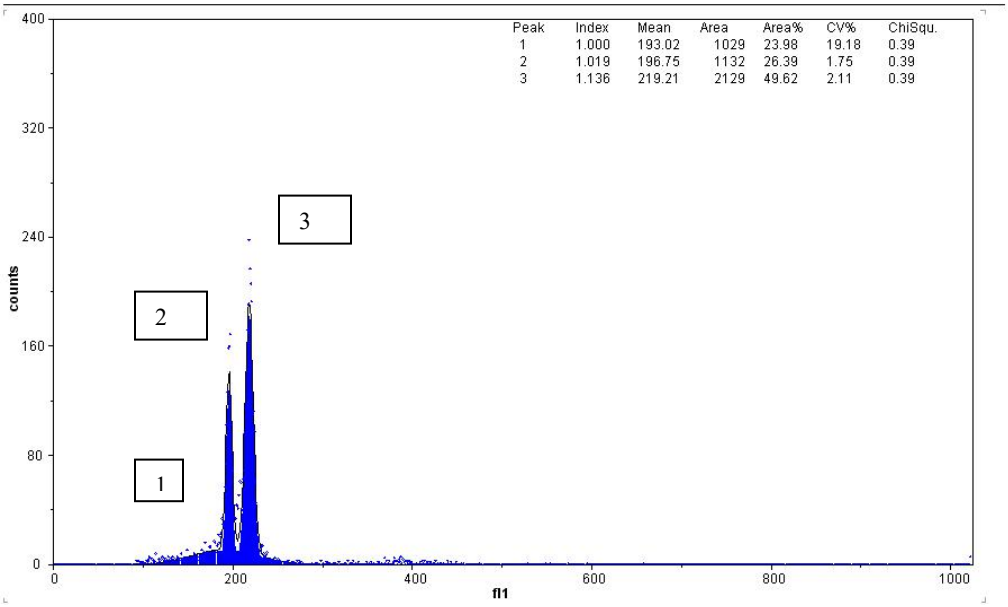

Figure 6. Histogram of *Cotula cryptocephala* accession CcBa6a obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to background fluorescence, peak 2 corresponds to the standard fluorescence and peak 3 corresponds to the sample fluorescence. The histograms are based on 5000 nuclei analyzed

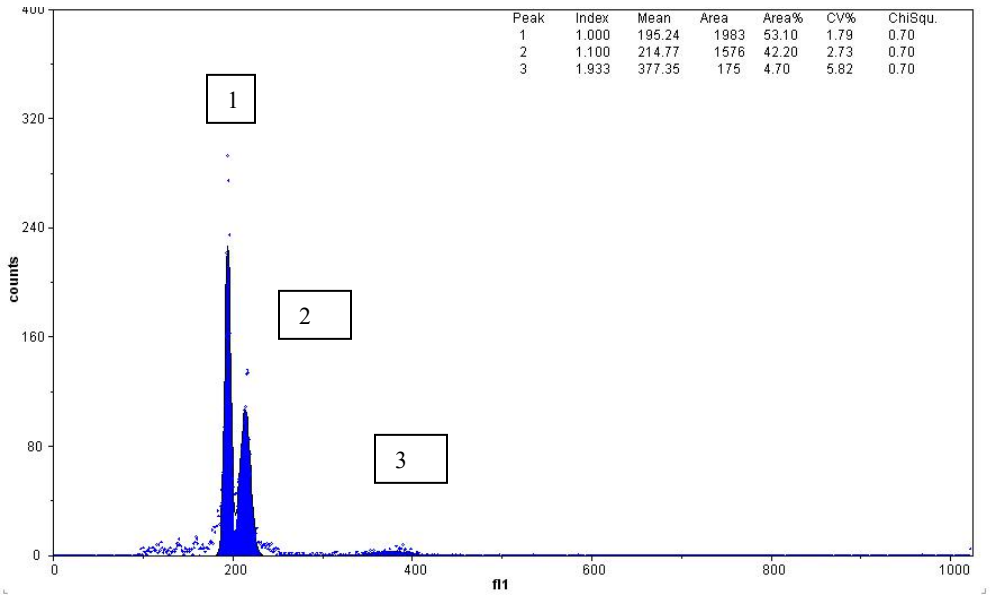

Figure 7. Histogram of *Cotula cryptocephala*, accession CcBa6b, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence, peak 2 corresponds to the sample fluorescence and peak 3 corresponds to fluorescence of G_2 nuclei of the standard (4C DNA amount). The histogram is based on 5000 nuclei analyzed

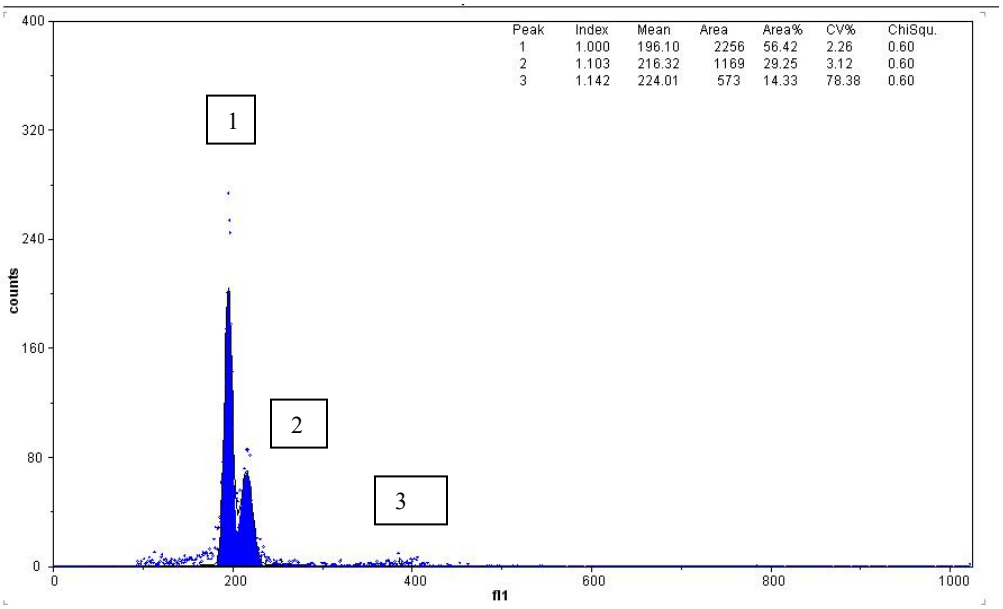

Figure 8. Histogram of *Cotula cryptocephala*, accession CcBa7, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence, peak 2 corresponds to the sample fluorescence and peak 3 corresponds to fluorescence of G_2 nuclei of the standard (4C DNA amount). The histogram is based on 5000 nuclei analyzed

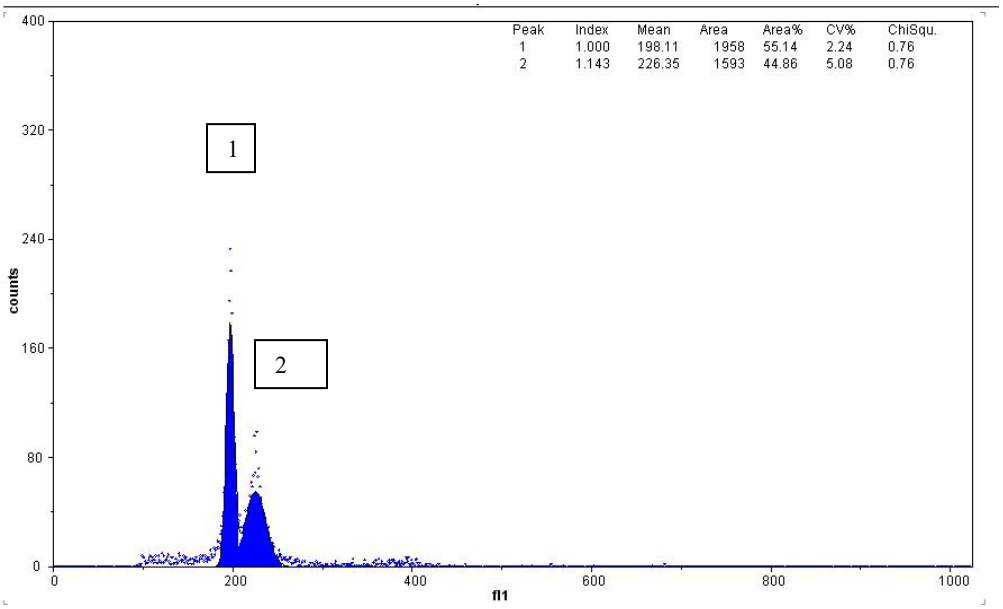

Figure 9. Histogram of *Cotula cryptocephala*, accession CcBa13, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence and peak 2 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

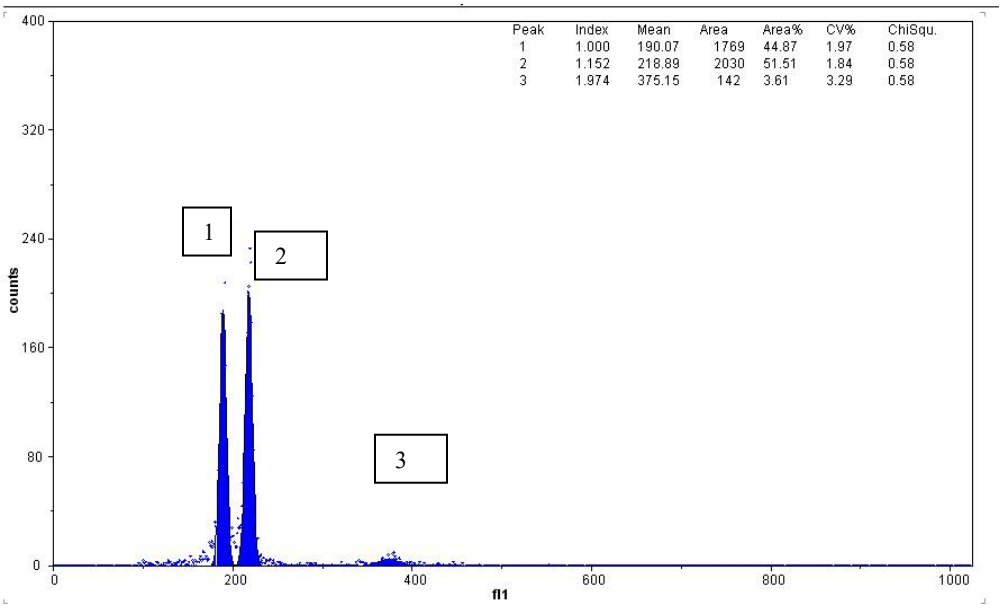

Figure 10. Histogram of *Cotula cryptocephala*, accession CcSe9, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence, peak 2 corresponds to the sample fluorescence and peak 3 corresponds to fluorescence of G_2 nuclei of the standard (4C DNA amount). The histogram is based on 5000 nuclei analyzed

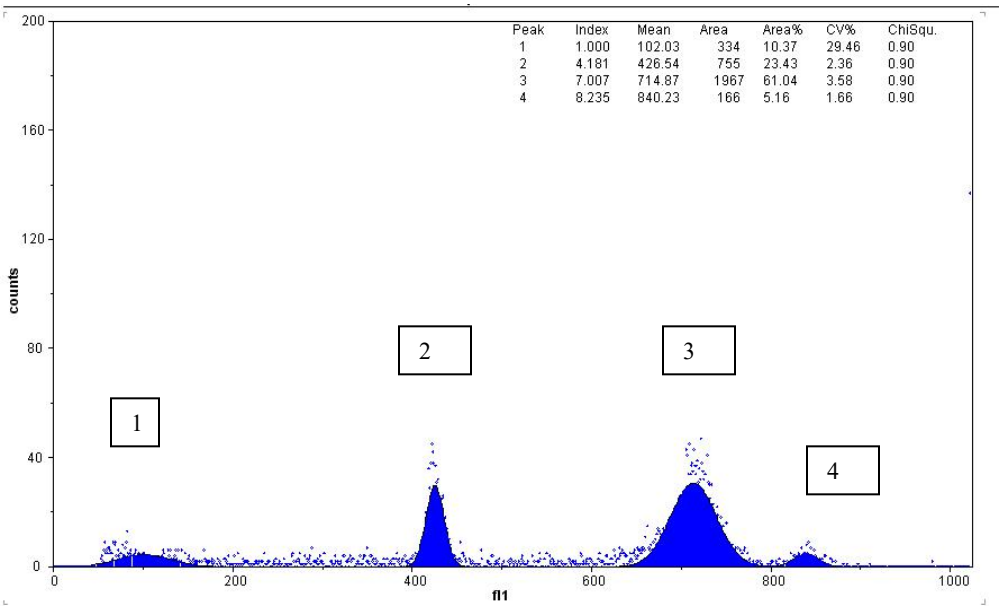

Figure 11. Histogram of *Deschampsia caespitosa*, accession DcBa4, obtained during DNA amount estimation using *Zea mays* (2c= 5.43 pg) as an internal standard. Peak 1 corresponds to background fluorescence, peak 2 corresponds to the standard fluorescence, peak 3 corresponds to the sample fluorescence and peak 4 corresponds to fluorescence of G_2 nuclei of the standard (4C DNA amount). The histogram is based on 5000 nuclei analyzed

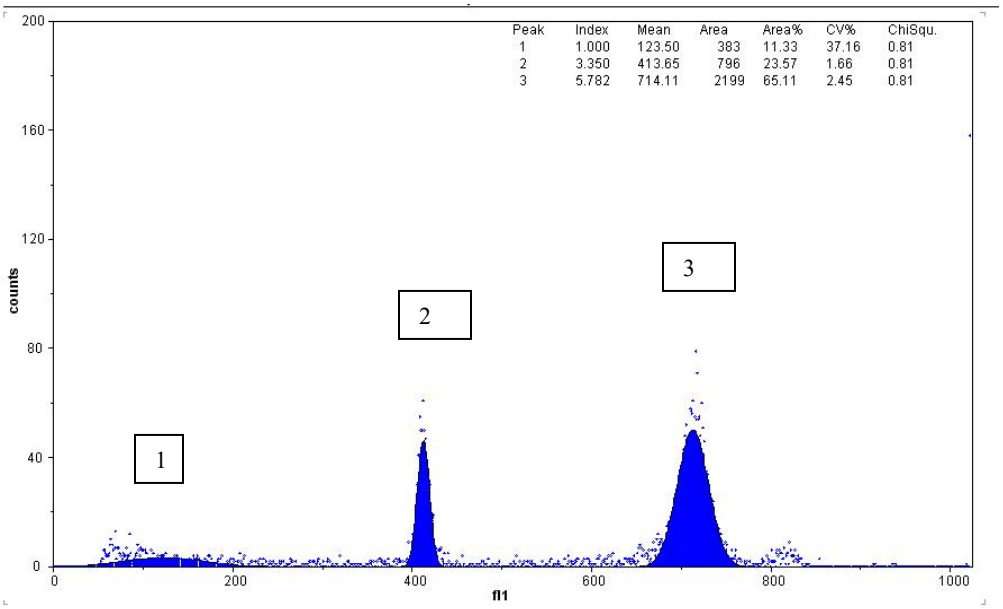

Figure 12. Histogram of *Deschampsia caespitosa*, accession DcBa8, obtained during DNA amount estimation using *Zea mays* (2c= 5.43 pg) as an internal standard. Peak 1 corresponds to background fluorescence, peak 2 corresponds to the standard fluorescence and peak 3 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

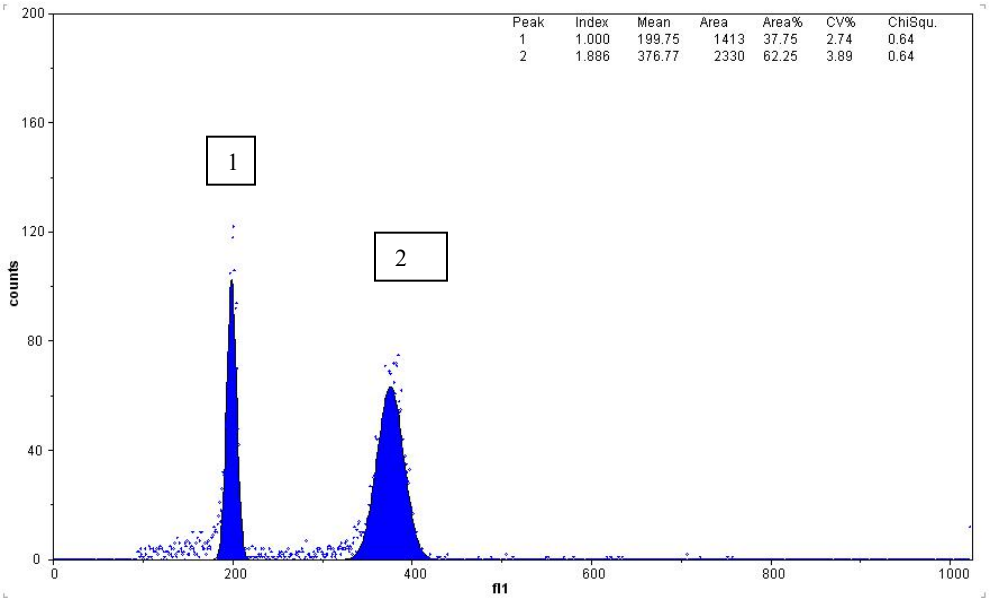

Figure 13. Histogram of *Ranunculus stagnalis*, accession RsSe3, obtained during DNA amount estimation using *Vicia faba* (2c= 26. 9 pg) as an internal standard. Peak 1 corresponds to the sample fluorescence and peak 2 corresponds to the standard fluorescence. The histogram is based on 5000 nuclei analyzed

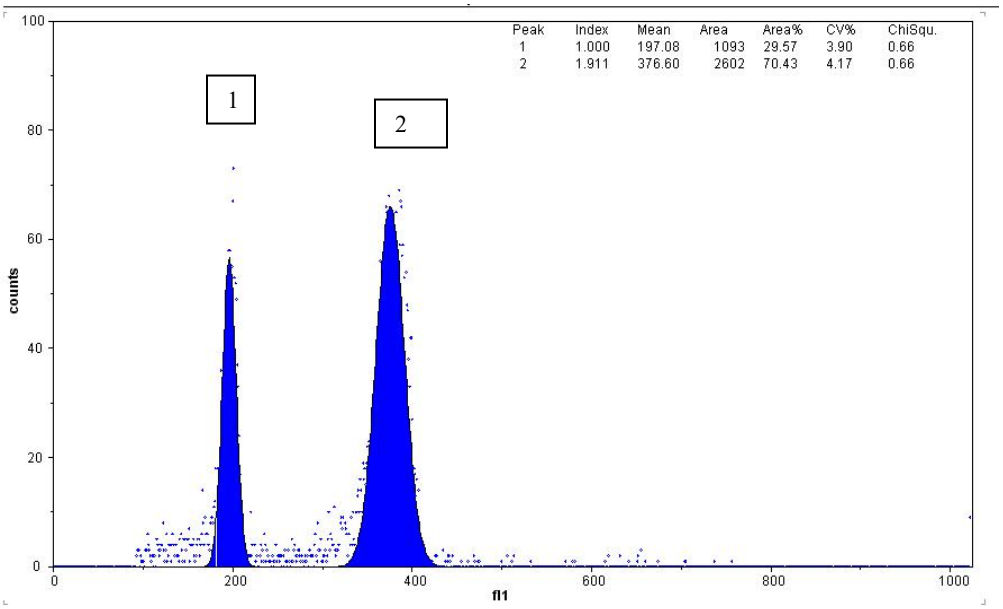

Figure 14. Histogram of *Ranunculus stagnalis*, accession RsSe4, obtained during DNA amount estimation using *Vicia faba* (2c= 26. 9 pg) as an internal standard. Peak 1 corresponds to the sample fluorescence and peak 2 corresponds to the standard fluorescence. The histogram is based on 5000 nuclei analyzed

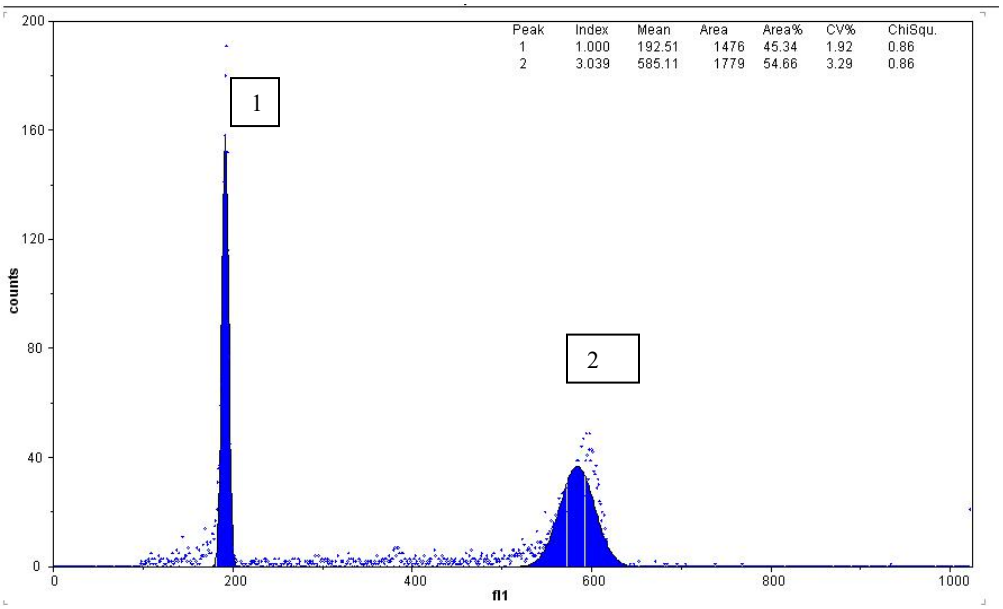

Figure 15. Histogram of *Ranunculus oreophytus*, accession RoBa2, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence and peak 2 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

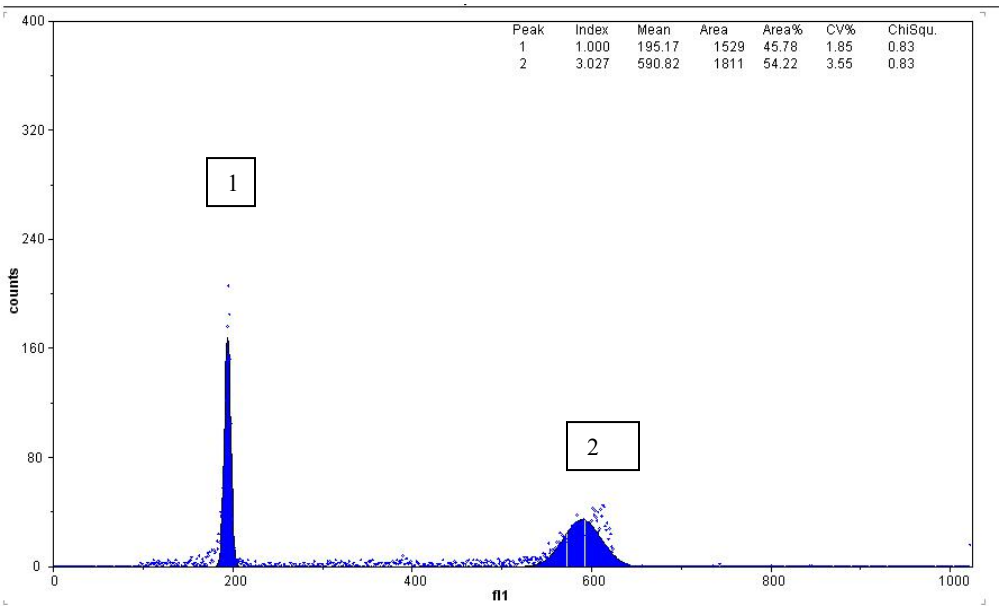

Figure 16. Histogram of *Ranunculus oreophytus*, accession RoBa4, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence and peak 2 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

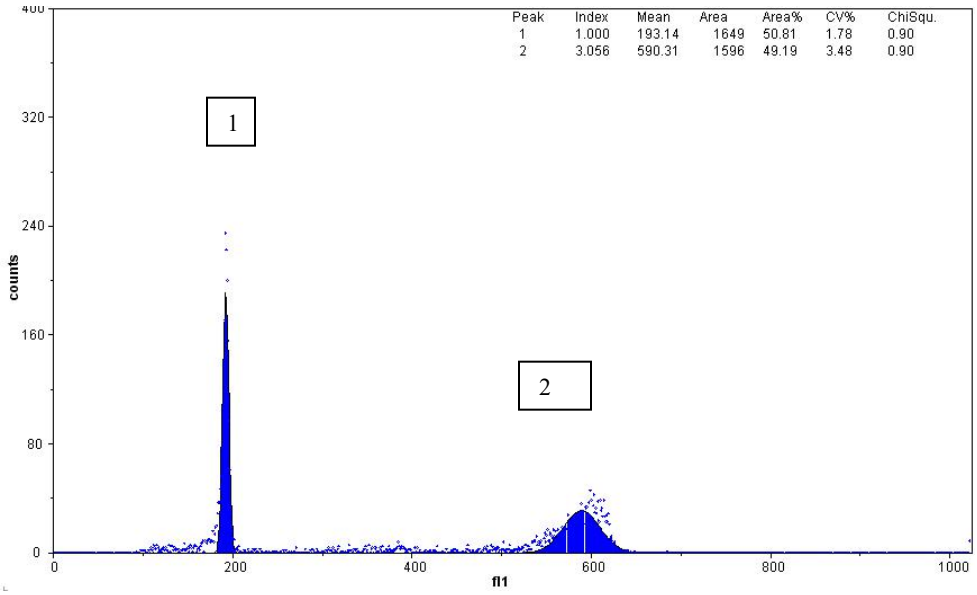

Figure 17. Histogram of *Ranunculus oreophytus*, accession RoBa6, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence and peak 2 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

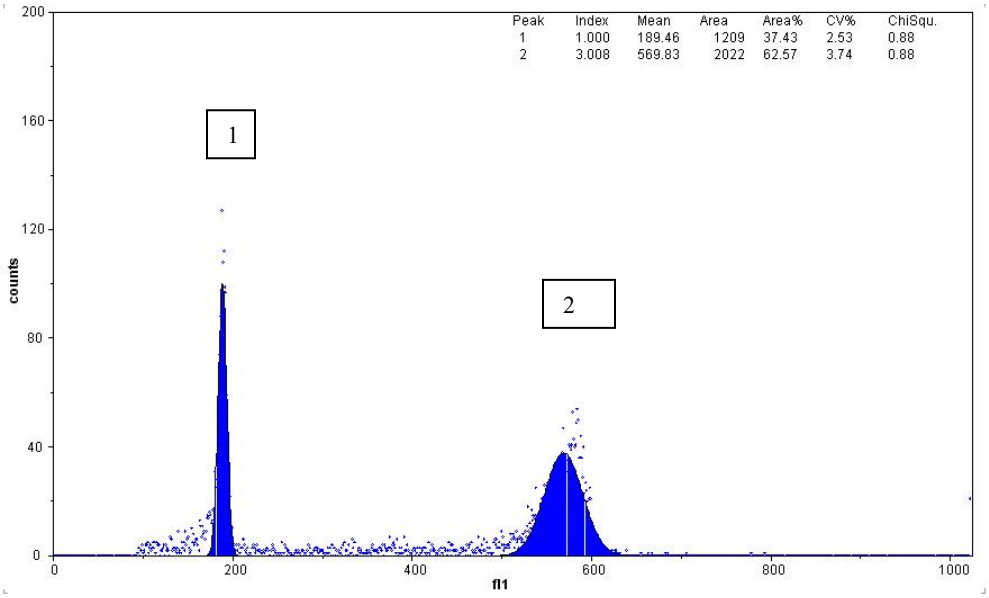

Figure 18. Histogram of *Ranunculus oreophytus*, accession RoSe1, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence and peak 2 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

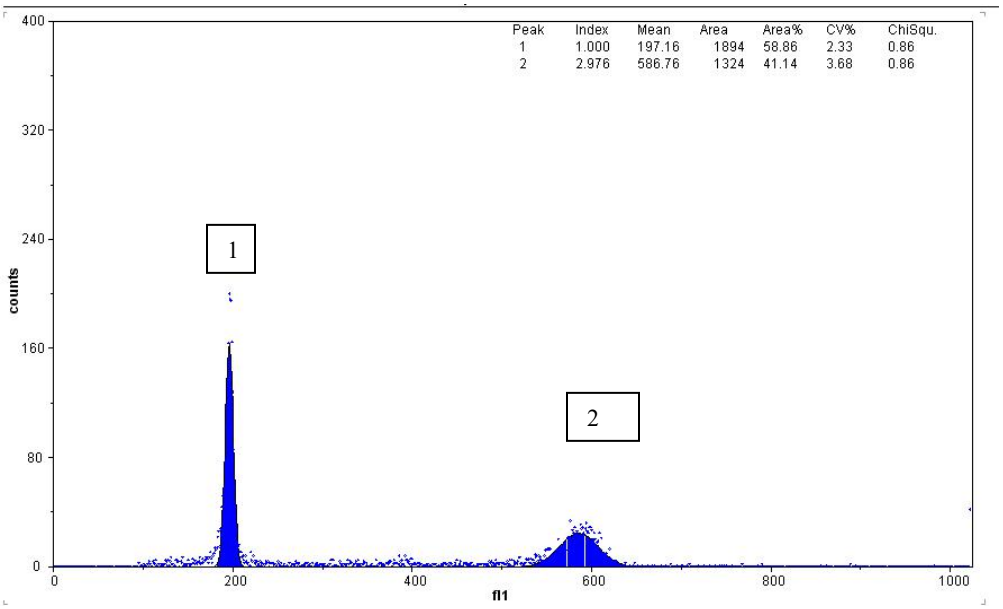

Figure 19. Histogram of *Ranunculus oreophytus*, accession RoSe6, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence and peak 2 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

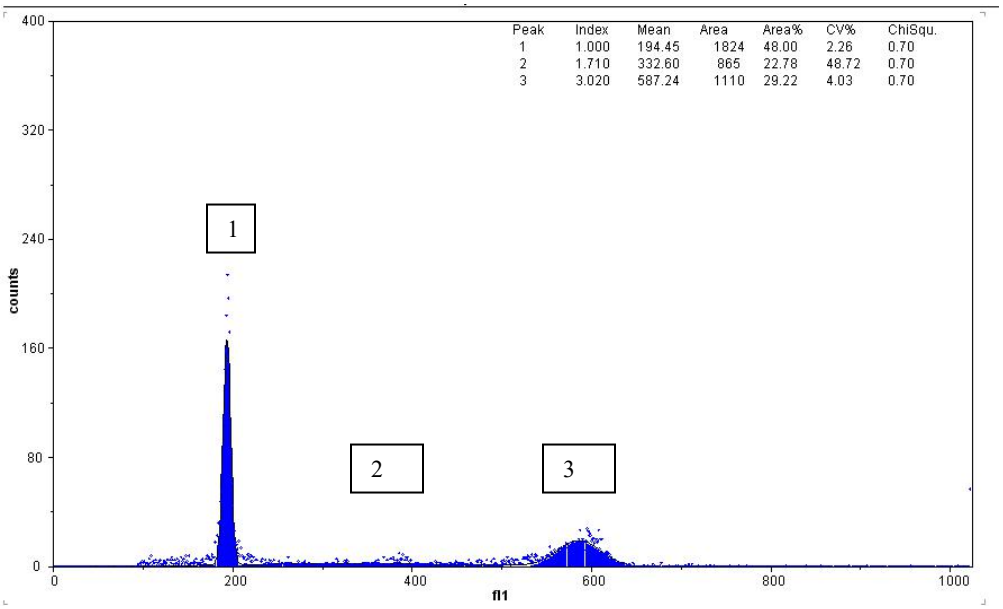

Figure 20. Histogram of *Ranunculus oreophytus*, accession RoBa8, obtained during DNA amount estimation using *Pisum sativum* (2c= 9.09 pg) as an internal standard. Peak 1 corresponds to the standard fluorescence, peak 2 corresponds to fluorescence of G_2 nuclei of the standard (4C DNA amount) and peak 3 corresponds to the sample fluorescence. The histogram is based on 5000 nuclei analyzed

Based on the fluorescence mean values obtained from the histograms (Figures 6-20), the absolute DNA amounts (2C-values) were obtained for the 15 accessions (Table 4). From these values, mean 2C-values, standard deviations and variations were obtained for each species (Table 5).

Little variation in intraspecific 2C-value were observed in *Cotula cryptocephala, Deschampsia caespitosa, Ranunculus stagnalis* and *Ranunculus oreophytus*, with standard deviation of 0.13 to 0.25 and only 1.013 to 1.047 fold variation (Table 5).

Table 4. 2C DNA values of accessions of different species including localities and altitudes of collection

Species	**Accession No.**	**2C DNA Value (pg)**	**Locality**	**Altitude (m.a.s.l)**
Cotula cryptocephala	CcBa6a	10.128	Bale	3480
	CcBa6b	9.999	Bale	3476
	CcBa7	10.027	Bale	3476
	CcBa13	10.386	Bale	3574
	CcSe9	10.468	Simen	3714
Deschampsia caespitosa	DcBa4	9.101	Bale	3860
	DcBa8	9.374	Bale	3792
Ranunculus stagnalis	RsSe3	14.261	Simen	4130
	RsSe4	14.077	Simen	4094
R. oreophytus	RoBa2	27.628	Bale	3860
	RoBa4	27.517	Simen	4110
	RoBa6	27.782	Simen	3984
	RoBa8	27.452	Bale	3792
	RoSe1	27.339	Bale	3476
	RoSe6	27.052	Bale	3963

Table 5. Mean 2C-value, standard deviation, variation and coefficient of variation

Species	Mean 2C-value (pg) ± standard deviation	Variation	Standard 2C-value	CV % sample	CV % standard
Cotula cryptocephala	10.2 ± 0.21	1.047	*Pisum sativum* 9.09 pg	1.84-3.12	1.79-2.46
Deschampsia caespitosa	9.2 ± 0.19	1.029	*Zea mays* 5.43 pg	2.45-3.58	1.65 -2.3
Ranunculus stagnalis	14.2 ± 0.13	1.013	*Vicia faba* 26.9 pg	2.74-3.9	3.89-4.06
Ranunculus oreophytus	27.5 ± 0.25	1.027	*Pisum sativum* 9.09 pg	2.45-4.03	1.78-2.52

5. Discussion

5.1 Chromosome count and Ploidy levels

One difficulty in polyploidy study is the problem of ascertaining basic chromosome numbers that is usually assumed to be half of the lowest somatic number but is adjusted as more numbers are discovered (Hedberg and Hedberg, 1977).

Hedberg and Hedberg, (1977) assigned basic chromosome number of 8 to the genus *Cardamine* and they have reported chromosome number of 62 for *Cardamine obliqua*, the same as reported here. Thus, this cytotype is an aneuploid with basic chromosome number of x=8.

The only chromosome number reported earlier for *Cotula cryptocephala* is 2n= 80, with ploidy level 10x and basic chromosome number 8 (Hedberg and Hedberg, 1977). The chromosome number 2n= 64 and ploidy level 8x found in the present study is a new

report. This increases the number of afroalpine angiosperm species with intraspecific polyploidy i.e. species with two or more polyploid races, by this study.

The present chromosome number found for *Deschampsia caespitosa* (2n= 26) is in agreement with the earlier report. It is a diploid with x= 13 (Hedberg and Hedberg, 1977). This grass is probably a paleopolyploid (Hedberg and Hedberg, 1977).

The present chromosome number found for *Ranunculus oreophytus* and *R. stagnalis* are the same as the earlier report, (2n= 32, x= 8) and (2n= 28, x= 7), respectively (Hedberg and Hedberg, 1977). Both species are tetraploids but with different basic chromosome numbers. The occurrence of two or more basic chromosome numbers within a genus is not uncommon in the afroalpine flora (Hedberg and Hedberg, 1977). Differences in basic chromosome numbers within the same genus occur by aneuploid increase or decrease in the basic chromosome number during the evolutionary history of plants (Stebbins, 1971).

5.2 DNA-ploidy and ploidy levels

Since there is no observed difference in fluorescence intensity between different individuals of the same species for all the accessions of *C. cryptocephala, D. caespitosa, R. stagnalis,* and *R. oreophytus*, it can be interpreted as to that there is no intraspecific ploidy level difference, including aneuploidy difference, in these plants (Mandak *et al*., 2003).

For the study of other accessions of these species, the tedious work of chromosome counting will not be needed. Their chromosome ploidy levels can be inferred from their DNA ploidy only. Moreover, samples mixed with other ploidy levels can be easily identified using flow cytometry. Chromosome counts are needed only when a new DNA-

ploidy level is found thus reducing the number of accessions for which chromosome is counted (Suda, 2002). Based on the present DNA amount estimations, large-scale cytotype study of the species will also be possible, which would be impractical with chromosome counting only (Husband and Schemske, 1998; Suda *et al*., 2004).

5.3 2C DNA value

The quality of the dataset forming the DNA histogram is often assessed by the coefficient of variation of the peaks (Eudey, 1996). Broader peaks have relatively higher coefficients of variation (CV) than narrow peaks. The histograms (Figure 6-20) obtained in the present study are acceptable because the CV obtained for accessions of all the species analyzed are below 5%. CV values below 5% are acceptable in C-value estimation by using propidium iodide stain, which usually gives higher CV than estimation of ploidy with DAPI stain (Suda, 2004).

Although the ideal DNA flow histogram contains conspicuous peak corresponding to G_0/G_1 nuclei, smaller peak corresponding to G_2/M nuclei and S-phase nuclei in between, certain variation always exists. Additional fluorescence signals i.e. noise or background caused by damaged particles, non-specific fluorescence or clumped objects exist (Suda, 2004). In the present case, additional peaks were obtained on lower channels for figures 6, 11 and 12. These additional peaks are interpreted to correspond to background noise.

In figures 7, 8, 10 and 20, peaks corresponding to the G_2 nuclei of the standard were obtained. These peaks have double the fluorescence of the standard G_1 peak representing G_2 nuclei with 4C DNA amount (Suda, 2004). In figure 11, two additional peaks, the one on lower channel corresponding to background noise and the other corresponding to G_2 nuclei have been obtained.

Among angiosperms, 2C-value ranges from 0.32 pg in *Arabidopsis thaliana* (Brassicaceae) to 254.8 pg in *Fritillaria assyriaca* (Liliaceae) and the mean is 13.05 pg (Schneeweiss *et al*., 2006). 2C-values of ≤ 7 pg are defined as small genomes, between 7 pg and 28 pg as intermediate genomes and ≥ 28 pg as large genomes (Leitch *et al*., 2005). According to the above-indicated ranges, the mean 2C-values of *C. cryptocephala, D. caespitosa*, *R. stagnalis* and *R. oreophytus* are intermediate in size (Table 5).

The 2C DNA values obtained in the present study show slight variation within species; with only 1.013 to 1.047 fold variation and standard deviation ranging from 0.13 to 0.25 (Table 5). In one study, the 2-3 % variation in *Allium cepa* cultivars was not considered as intraspecific variation (Bennett *et al.*, 2000b). On the other hand, in another study, the very small intraspecific C-value variation with standard error of 0.027 within natural populations of *Hordeum spontaneum* has been found to be real intraspecific variation and was correlated with long terminal repeat (LTR) retrotransposon copy number variation within this species (Kalendar *et al*., 2000). In still another study, DNA content estimated in 12 lines of soybean using best practice exhibited variation up to 1.046 fold that was considered as true intraspecific variation and was strongly correlated with seed weight and leaf size (Greilhuber, 2005).

The small variations in 2C DNA amount observed in the present study could be due to experimental errors. An alternative possibility is that this variation represents genuine intraspecific variation. However, in order to determine which factor is responsible, data should be collected from more individuals, and simultaneous processing of the individuals from the same species with highest and lowest values should be done. This would minimize experimental error due to differences in nuclei isolation and staining and

reveal whether the variations are due to experimental errors or whether it is real intraspecific variation (Price *et al*., 2000). If the variation of the plants is proved to be real intraspecific variation, molecular investigations can reveal the reason behind this variation.

A 10.43 pg 2C-value estimate for the diploid (2n= 26) *D. caespitosa* from New Zealand has been reported (Murray *et al*., 2005), using a different procedure and flow cytometry instrument than used in the present study. There exists a difference of about 1 pg of DNA amount between this report and the present estimation (9.2 pg). This difference could be due to differences in procedures and instrument used by the two studies or it could be real intraspecific variation. To make comparisons involving small differences in C-value, the estimations have to be performed in the same laboratory using the same method and instrument to avoid any potential variations that could arise due to differences in the instrument and methods used. Up to 15.6% differences have been found in the analysis done with different procedures and/instrument on the same cultivar (Dolezel *et al*., 1998). Thus, unless both materials are analyzed in the same lab using the same procedure and instrument, it is not possible to make a reliable inference whether the differences observed are genuine intraspecific variation or simply due to experimental error.

The 2C DNA amount of the tetraploid *D. caespitosa*, 2n= 52 which was collected from Antarctic and Atlantic area was reported to be 18 pg. This was estimated by Feulgen microdensitometry (Bennett and Leitch, 2005b). This value is about double that of the diploid *D. caespitosa* (2n= 26) of the afroalpine environment, which according to the estimation made in this study using flow cytometry technique has a mean 2C-value of 9.2

pg (Table 5). The DNA amount of the tetraploid species is about double that of the diploid species, which presents an interesting contrast to significant genome downsizing in polyploids. A number of studies have shown that in the process of diploidization, polyploids reduce their C-value and as a result the DNA amount of the diploid is significantly lower than twice the DNA amount of their diploid progenitors (Leitch and Bennett, 2004; Schneeweiss *et al*., 2006; Johnston *et al*., 2005; Ozkan *et al*., 2003). It could either be a recently formed polyploid (neopolyploid) such as *Spartina anglica*, *Tragopogon mirus* and *T. miscellus* (Soltis *et al*., 2003) or the mechanisms of genome downsizing are absent as in the case of cotton (Liu *et al*., 2001). However, both the diploid and tetraploid cytotypes should be analyzed for DNA amount using the same method and standard in order to get a better picture about the magnitude of differences in their C-values.

6. Summary and Recommendations

In the present study, flow cytometric technique was employed to estimate DNA amounts of ecotypes of the afroalpine species *Cotula cryptocephala, Deschampsia caespitosa*, *Ranunculus oreophytus* and *Ranunculus stagnalis*. The result showed no significant intraspecific variation for any of the species investigated. However, it is not possible to establish whether the small intraspecific variations observed are genuine or simply artifactual due to experimental error.

The chromosome number found for *C. cryptocephala* is a new report whereas chromosome numbers for the rest of the species investigated are confirmations of earlier reports.

When relating the DNA amounts to chromosome counts, the present study has produced estimates of DNA amounts for diploid form of *D. caespitosa*, tetraploid forms of *R. oreophytus* and *R. stagnalis* and octaploid form of *C. cryptocephala*. This information is very useful for future large-scale screening of possible intraspecific ploidy level variation for the above species without reverting to chromosome counting.

Based on the findings of the present study, the following can be recommended. In order to determine whether the observed intraspecific variations in DNA amount, though small, are genuine or artifactual, more individuals will have to be studied and the simultaneous processing of the individuals from the same species with highest and lowest values should be carried out.

In case the above-recommended study confirms that these variations are genuine, further molecular investigation is recommended to determine the actual molecular factor(s) responsible. It can further be investigated if there are any correlations between DNA amount differences and morphological or environmental differences. For example, C-value estimations and morphometry study done for caulescent and acaulescent specimens of *R. oreophytus* could solve the debate whether caulescent morphology of *R. oreophytus* is an environmental effect or the caulescent plant is a different variety which arose by hybridization with *R. multifidus*.

In order to get better understanding about C-value evolution in the genera studied in the present case, for each genus, those species not included in the present study should be investigated in the future with regard to C-value estimation, ploidy level and chromosome counts.

At present, data on the C-value of afroalpine flora in general are very scarce. Thus, further morphological, cytological, flow cytometric and molecular investigations of the afroalpine flora will provide useful data that would answer many important biological and evolutionary questions like genome evolution in harsh environment such as the afroalpine.

7. References

Bennett, M.D and Leitch, I.J (1995). Nuclear DNA amounts in Angiosperms. *Annals of Botany.* **76**:113-176.

Bennett, M.D and Leitch, I.J. (2005b). Angiosperm DNA C-values database (release 6.0). http://www.rbgkew.org.uk/cval/homepage.html

Bennett, M.D. (1972). Nuclear DNA content and minimum generation time in herbaceous plants. *Pro. R. Soc .Lond. B.* **181**:109-135.

Bennett, M.D. and Leitch, I.J. (2005a). Nuclear DNA amounts in angiosperms-progress, problems and prospects. *Annals of Botany.* **95**: 45–90.

Bennett, M.D., Johnston, S., Hodnett, G.L, and Price, H.J. (2000b) *Allium cepa* L. cultivars from four continents show nuclear DNA constancy. *Annals of Botany.* **85**: 351-357.

Bennett, M.D., Bhandol, P. and Leitch, I.J. (2000a). Nuclear DNA amounts in angiosperms and their modern uses—807 new estimates. *Annals of Botany.* **86**: 859–909.

Bennetzen, J. and Kellogg, E.A (1997). Do plants have a one-way ticket to genomic obesity? *Plant Cell* **9**: 1509-1514.

Bennetzen, J., Ma, J. and Devos, K.M. (2005). Mechanisms of recent genome size variation in flowering plants. *Annals of Botany.* **95**: 127–132.

Bretagnolle, F. and Thompson, J.D. (1995). Gametes with the somatic chromosome number: Mechanisms of their formation and role in the evolution of autopolyploid plants. *New Phytol.* **129**:1-22.

Bures, P., Wang, Y., Horova, L. and Suda, J. (2004). Genome size variation in central European species of *Cirsium* (Compositae) and their natural hybrids. *Annals of Botany.* **94**: 353–363.

Cavalier-Smith T. (2005). Economy, speed and size matter: evolutionary forces driving nuclear genome miniaturization and expansion. *Annals of Botany.* **95**: 147–175.

Comai, L., Tyagi, A.P., Winter, K., Holmes D.R., Reynolds, S.H., Stevens, Y., and Byers, B. (2000). Phenotypic instability and rapid gene silencing in newly formed *Arabidopsis* allotetraploids. *Plant Cell* **12:** 1551–1567.

Cox, A.V., Abdelnour, G.J., Bennett, M.D. and Leitch, I.J. (1998). Genome size and karyotype evolution in the slipper orchids (Cypripedioideae: Orchidaceae). *American Journal of Botany.* **85**: 681-687.

Dagne, K. and Heneen, W.K (1992). The karyotype and nucleoli of *Guizotia abyssinica* (Compositae). *Hereditas.* **117**: 73-83.

Dolezel, J. and Bartos, J. (2005). Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany.* **95**: 99-110.

Dolezel, J., Greilhuber, J., Lucretti, S., Meister, A., Lysak, M.A., Nardi, L., and Obermayer, R. (1998). Plant genome size estimation by flow cytometry: Inter-laboratory comparison. *Annals of Botany.* **82** (Supplement A): 17-26.

Edwards, S., Tadesse, M., Demissew, S. and Hedberg, I (2000). *Flora of Ethiopia and Eriteria*. Volume 2, part 1, Addis Ababa University.

Eudey, T.L. (1996). Statistical considerations in DNA flow cytometry. *Statistical Science*. **11**: 320-334.

Fay, M.F., Cowan, R.S. and Leitch, I.J. (2005). The effects of DNA amount on the quality and utility of AFLP fingerprints. *Annals of Botany.* **95**: 237–246

Finnegan, E.J. (2001). Epialleles-a source of random variation in times of stress. *Current Opinion in Plant Biology*. **5**: 101-106.

Flavell, R.B. (1986). Repetitive DNA and chromosome evolution in plants. *Phil. Trans. R. Soc. Lond. B.* **312**:227-242.

Grant, V. (1981). *Plant Speciation*. (2nd ed.). Columbia University Press, New York.

Gregory, T.R. (2005*)***.** The C-value enigma in plants and animals: a review of parallels and an appeal for partnership. *Annals of Botany.* **95**: 133–146.

Greilhuber, J. (1998). Intraspecific variation in genome size: a critical reassessment. *Annals of Botany.* **82** (Supplement A): 27–35.

Greilhuber, J. (2005). Intraspecific variation in genome size in angiosperms: identifying its existence. *Annals of Botany.* **95**: 91-98

Greilhuber, J., Dolezel J., Lysak M.A. and Bennett, M.D. (2005). The origin, evolution and proposed stabilization of the terms 'Genome Size' and 'C-Value' to describe nuclear DNA contents. *Annals of Botany.* **95**: 255–260.

Hardie, D., Gregory, T., and Hebert, P. (2002). From Pixels to Picograms: A Beginners' Guide to Genome Quantification by Feulgen Image Analysis Densitometry. *J. Histochem. Cytochem.* **50**: 735–749

Hedberg, O. (1957). Afroalpine Vascular Plants- a Taxonomic Revision. *Symb.Bot.Ups*. **15**

Hedberg, O. (1970). Evolution of the afroalpine flora. *Biotropica* **2**: 16-23

Hedberg, O. (1995). Features of Afroalpine Plant Ecology. *Acta Phytogeogr. Suecica* **49**

Hedberg, O. and Hedberg, I. (1977). Chromosome number of the afroalpine and afromontane angiosperms *Bot. Notiser.* **130**: 1-24

Husband, B.C. and Schemske, D.W. (1998). Cytotype distribution at diploid-tetraploid contact zone in *Chamerion* (*Epilobium*) *Angustifolium* (Onargaccae). *Annals of Botany.* **85**: 1688-1694.

Johnston, J.S., Pepper, A.E., Hall, A.E., Chen, Z.J., Hodnett, G., Drabek, J., Lopez, R. and Price, H.J. (2005). Evolution of genome size in *Brassicaceae*. *Annals of Botany.* **95**: 229–235.

Jones, R.N. (1995). B-chromosomes in plants. *New phytol*. **131**: 411-434

Kalendar, R., Tanskanen, J., Immonen, S., Nevo, E., and Schulman, A.H (2000). Genome evolution of wild barley (*Hordeum spontaneum*) by BARE-1 retrotransposon dynamics in response to sharp microclimate divergence. *Proc. Natl. Acad. Sci.* **97**: 6603-6607.

Kidwell, M.G. (2002). Transposable elements and the evolution of genome size in eukaryotes. *Genetica.* **115**: 49-63

Kidwell, M.G. and Lisch, D.R. (2001). Perspective: Transposable element, parasitic DNA and genome evolution. *Evolution* **55**: 1-24.

Knight, C.A., Molinari, N. and Petrov, D.A. (2005). The large genome constraint hypothesis: evolution, ecology and phenotype. *Annals of Botany.* **95**: 177–190.

Kubis S., Schmidt, T., Heslop-Harrison, J.S. (1998). Repetitive DNA elements as a major component of plant genomes. *Annals of Botany.* **82** (Supplement A): 45-55.

Kudo, K. and Kimura, Y. (2001). Patterns of endopolyploidy during seedling development in cabbage (*Brassica oleracea* L.). *Annals of Botany.* **87**: 275-281.

Leitch, I.J, Soltis, D.E, Soltis, P.S and Bennett, M.D. (2005). Evolution of DNA amounts across land plants (*Embryophyta*). *Annals of Botany.* **95**: 207–21.

Leitch, I.J. and Bennett, M.D. (2004). Genome downsizing in polyploid plants. *Biol. J. Linn. Soc.* **82**: 651-663.

Leitch, I.J., Chase, M.W. and Bennett, M.D. (1998). Phylogenetic analysis of DNA C-values provides evidence for a small ancestral genome size in flowering plants. *Annals of Botany.* **82**: 85-94.

Liu, B., and Wendel, J.F. (2002). Non-Mendelian phenomena in allopolyploid genome evolution. *Current Genomics*. **3**: 1-17.

Liu, B., Brubaker, C.L., Mergeai, G., Cronn, R.C., and Wendel, J.F. (2001). Polyploid formation in cotton is not accompanied by rapid genomic changes. *Genome.* **44:** 321–330.

Lysak, M.A., Dolzelova, M., Horry, J.P., Swennen, R. and Dolezel, J. (1999). Flow cytometric analysis of nuclear DNA content in *Musa*. *Theor. Appl. Genet.* **98**: 1344-1350

Mandak, B., Pysek, P., Lysak, M. A., Suda, J**.**, Krahulcova, A. and Bímova K. (2003). Variation in DNA-ploidy levels of *Reynoutria* taxa in the Czech Republic. *Annals of Botany.* **92**: 265-272.

Meagher, T.R., Gillies, A.C.M. and Costich, D.E. (2005). Genome size, quantitative genetics, and the genomic basis for flower size evolution in *Silene latifolia*. *Annals of Botany.* **95**: 247–254.

Mesfin, T. (2004). *Flora of Ethiopia and Eriteria*. Hederg, I., Friss, I., and Edwards, S. (eds.). Volume 4, part 2, Addis Ababa University.

Michaelson, M.J., Price, H. J., Ellison, R. and Johnston, J.S. (1991). Comparison of plant DNA contents determined by Feulgen Microspectrophotometry and laser flow cytometry. *American Journal of Botany.* **78**: 183-188.

Moscone, E.A., Baryani, M., Ebert, I., Greilhuber, J., Ehrendorfer, F. and Hunziker, A.T. (2003). Analysis of nuclear DNA content in *Capsicum* (Solanaceae) by flow cytometry and Feulgen densitometry. *Annals of Botany.* **92**: 21–29.

Murray, B.G., De Lange, P.D. and Ferguson, A.R. (2005). Nuclear DNA variation, chromosome numbers and polyploidy in the endemic and indigenous grass flora of New Zealand. *Annals of Botany.* **96**: 1293-1305.

Noirot, M., Barre, P., Duperray, C., Hamon, S. and De Kochko, A. (2005). Investigation on the causes of stoichiometric error in genome size estimation using heat experiments: Consequences on data interpretation. *Annals of Botany.* **95**: 111–118.

Noirot, M., Barre, P., Duperray, C., Louarn, J. and Hamon, S. (2003). Effects of caffeine and chlorogenic acid on propidium iodide accessibility to DNA: consequences on genome size evaluation in coffee tree. *Annals of Botany.* **92**: 259–264.

Noirot, M., Barre, P., Louarn, J., Duperray, C. and Hamon, S. (2000). Nucleus– cytosol interactions: a source of stoichiometric error in flow cytometric estimation of nuclear DNA content in plants. *Annals of Botany.* **86**: 309–316.

Ohmido, N., Kijima, K., Akiyama, Y., Jong, J.H. and Fukui, K. (2000). Quantification of total genomic DNA and selected repetitive sequences reveals concurrent changes in different DNA families in *indica* and *japonica* rice. *Mol. Gen. Genet.* **263**: 388-394.

Ohri, D. (1998). Genome size variation and plant systematics. *Annals of Botany.* **82** (Supplement A): 75-83.

Otto, F. (1990). DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. **In**: Crissman H.A. & Darzynkiewicz Z. (eds.). *Methods in Cell Biology. Vol. 33.* Academic Press, New York.

Ozkan, H., Levy, A., and Feldman, M. (2001). Allopolyploidy-Induced Rapid Genome Evolution in the Wheat (*Aegilops–Triticum*) Group. *Plant Cell* **13:** 1735-1747.

Ozkan, H., Tuna, M., and Arumuganathan, K. (2003). Nonadditive changes in genome size. *Journal of Heredity* **94**: 260–264

Phillips, S. (1995). *Flora of Ethiopia and Eriteria*. Hederg, I., Friss, I., and Edwards, S. (eds.). Volume 7, Addis Ababa University.

Price, H.J (1988). DNA content variation among higher plants. *Ann. MO Bot. Gard*. **75**: 1248-1257

Price, H.J and Johnston, J.S. (1996). Influence of light on DNA content of *Helinathus annuus* L. *Proc. Natl. Acad. Sci.* **93**: 11264–11267.

Price, H.J, Hodnet G. and Johnston, J.S. (2000). Sunflower (*Helianthus annuus*) leaves contain compounds that reduce nuclear propidium iodide fluorescence. *Annals of Botany.* **86**: 929–934.

Price, H.J., Dillon, S.L., Hodnett, G., Rooney, W.L., Ross, L. and Johnston, J.S. (2005). Genome evolution in the genus *Sorghum* (Poaceae). *Annals of Botany.* **95**: 219–227.

Price, H.J., Morgan, P.W. and Johnston, J.S. (1998). Environmentally correlated variation in 2C nuclear DNA content measurements in *Helianthus annuus* L. *Annals of Botany.* **82** (Supplement A): 95–98.

Ramsey, J. and Schemske, D.W. (2002). Neopolyploidy in flowering plants. *Annu. Rev. Ecol. Syst*. **33**: 589-639.

Rayburn, A.L., Price, H.J., Smith, J.D., and Gold, J.R. (1985). C-band hetechromatin and DNA content in maize (*Zea mays*). *Am. J. Bot.* **72**: 1610-1617

Rees, H. and Narayan, R. (1981). Chromosomal DNA in higher plants. *Phil. Trans. R. Soc. Lond.* B. **292**: 569-578.

Salmon, A., Ainouche, M.L. and Wendel, J.F. (2005). Genetic and epigenetic consequences of recent hybridization and polyploidy in *Spartina* (*Poaceae*). *Mol. Ecol.* **14**: 1163- 1175.

SanMiguel, P. and Bennetzen, JL. (1998). Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. *Annals of Botany.* **82** (Supplememt A): 37–44

Schneeweiss, H. W., Greilhuber, J. and Schneeweiss, G. M. (2006). Genome size evolution in holoparasitic *Orobanche* (Orobanchaceae) and related genera. *American Journal of Botany.* **93**: 148–156.

Shaked, H., Kashkush, K., Ozkan, H., Feldman, M., and Levy, A.A. (2001). Sequence elimination and cytosine methylation are rapid and reproducible responses of the genome to wide hybridization and allopolyploidy in wheat. *Plant Cell.* **13**: 1749–1759.

Singh, B.D. (1993). *Plant Breeding: Principles and Methods*. Kalyani Publishers, New Delhi.

Soltis, D. E., Soltis, P.S. and Tate, J.A. (2003). Advances in the study of polyploidy since plant speciation. *New Phytol.* **161**: 173-191.

Soltis, D.E. and Rieseberg, L.H. (1986) Autopolyploidy in *Tolmiea menziesii* (Saxifragaceae): Genetic insights from enzyme electrophoresis. *American Journal of Botany.* **73**: 310-318.

Soltis, D.E., Soltis, P.S., Pires, J. C., Kovarik, A., Tate, J. A., and Mavrodiev, E. (2004). Recent and recurrent polyploidy in *Tragopogon* (*Asteraceae*): cytogenetic, genomic and genetic comparisons. *Biol. J. Linn. Soc.* **82:** 467-474.

Soltis, P.S., and Soltis, D. E. (2000). The role of genetic and genomic attributes in the success of polyploids. *Proc. Natl. Acad. Sci.* **97:** 7051–7057.

Song, K., Lu, P., Tang, K., and Osborn, T.C. (1995). Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. *Proc. Natl. Acad. Sci.* **92:** 7719–7723.

Stebbins, G.L. (1971). *Chromosomal Evolution in Higher Plants*. Addison-Wesley, New York.

Suda, J. (2002). New DNA-ploidy level in *Empetrum* (Empetraceae) revealed by flow cytometry. *Annales Botanici Fennici.* **39**: 133-141.

Suda, J. (2004): An employment of flow cytometry into plant biosystematics. Ph.D. Thesis, Charles University in Prague, Faculty of Science, Department of Botany.

Suda, J., Krahulcova, A., Travnicek, P., and Krahulec, F. (2006). Ploidy level versus DNA-ploidy level: an appeal for consistent terminology. *Taxon* **55**: 447-450.

Suda, J., Kyncl, T., and Freiova, R. (2003). Nuclear DNA amounts in Macronesian angiosperms. *Annals of Botany.* **92:** 153-164.

Suda, J., Malcova, R., Abazid, D., Banas, M., Prochazka F., Sida O. and Stech, M. (2004). Cytotype distribution in *Empetrum* (*Ericaceae*) at various spatial scales in the Czech Republic. *Folia Geobotanica.* **39**: 161-171.

Vilhar, B., Greilhuber, J., Koce, J.D, Temsch, E.M and Dermastia, M. (2001). Plant genome size measurement with DNA image cytometry. *Annals of Botany.* **87**: 719-728.