

UNDERSTANDING THE WEEDY *CHENOPODIUM* COMPLEX IN THE NORTH  
CENTRAL STATES

BY

SUKHVINDER SINGH

DISSERTATION

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Doctoral Committee:

Professor Patrick J. Tranel, Chair  
Associate Professor Aaron G. Hager  
Associate Professor Geoffrey A. Levin  
Assistant Professor Matthew E. Hudson

## ABSTRACT

The genus *Chenopodium* consists of several important weed species, including *Chenopodium album*, *C. berlandieri*, *C. strictum*, and *C. ficifolium*. All of these species share similar vegetative morphology and high phenotypic plasticity, which makes it difficult to correctly identify these species. All of these weedy *Chenopodium* species have developed resistance to one or more classes of herbicides. An experiment was conducted to determine if there is variability in response of *Chenopodium* species present in the North Central states to glyphosate. Our results indicate variable responses within and among the *Chenopodium* species. Species such as *C. berlandieri* and *C. ficifolium* had higher levels of tolerance to glyphosate than did various accessions of *C. album*. In another experiment, 33 populations of *Chenopodium* sampled across six North Central states were screened with glyphosate. The results showed variable responses to glyphosate within and among the *Chenopodium* populations. In general, the *Chenopodium* populations from Iowa were more tolerant, but some biotypes from North Dakota, Indiana and Kansas also had significantly high tolerance to glyphosate.

Given there are species other than *C. album* that have high tolerance to glyphosate, and there are *Chenopodium* populations across the North Central states that showed tolerance to glyphosate, one intriguing question was to whether the *Chenopodium* populations were either biotypes of *C. album* were or are more closely related to other species such as *C. berlandieri*, *C. strictum* or *C. ficifolium*, which would indicate that there has been a species shift.

To investigate which species are prevalent in North Central states, 12 *Chenopodium* species were investigated using morphological DNA content values and sequence data. With the exception of morphological characters such as pericarp covering the seeds, calyx shape (keeled or not) and DNA content of the species, most of the morphological characters were not

phylogenetically informative. The morphological characters were useful in distinguishing some species from others, but with an increase in number of species under investigation, there was overlap of morphological characters within closely related species, and hence one cannot use morphological markers alone to differentiate the *Chenopodium* species.

Three DNA sequence based markers, ITS (nuclear), *matK* and *trnD-trnT* (chloroplast) were explored. The sequence data were subjected to maximum parsimony and Bayesian analyses. Irrespective of the method used for tree estimation, all three markers gave similar tree topologies, but ITS markers gave the greatest number of polymorphic sites. The ITS based phylogenetic tree was well resolved. Some closely related species such as *C. strictum*, *C. berlandieri* and *C. album* shared high sequence similarity among homologous genes, but these species were easily differentiated with the support of the genome size data. Samples of *C. strictum* had a 2C value of 2.0 pg, which was different than *C. album* (3.6 pg/2C). *Chenopodium* populations from North Central states were also tested with ITS markers, and DNA content values were also obtained from a few samples. Based on the sequence data, results indicated that all the field samples were closely related to *C. album*, and also the genome sizes of all the populations were close to the genome size values of different accessions of *C. album*. In conclusion, all the *Chenopodium* samples that were sampled for our experiment were closely related to *C. album*, and the increase in reports of common lambsquarters populations developing tolerance to glyphosate most likely is due to evolution within *C. album* and not because of a shift in weed species.

*I dedicate this dissertation to all the agricultural scientists and farmers for their efforts  
towards eradicating world hunger*

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# CHAPTER 1

## INTRODUCTION

Weeds pose a serious threat to agricultural and natural ecosystems, and managing them is one of the biggest challenges for farmers. Weeds represent the most important pest complex (Bridges, 1994) and are one of the main limiting factors in crop production (Avery, 1997). Today, due to weeds, the annual estimated loss in the US is around \$100 billion (Pimentel et al. 2005). There are several ways to manage weeds but one critical step for efficient weed management is prevention, which includes proper weed identification. Correct identification is important for weed control as different weed species respond differently to some weed management strategies. In fact, weeds that look similar can actually be unrelated and can exhibit different responses to the herbicides (Warwick and Black, 1981; Santelmann and Meade, 1961). By identifying weed species correctly, herbicide rates can be adjusted, thereby limiting potential adverse environmental impacts of herbicides. Another benefit of proper weed identification is that we can select for better herbicides to control a particular weed. Identification is also critical for determining which newly introduced weeds will pose possible threat to the crop (Anonymous, 2009). Several researchers systematically investigated common weed species in *Amaranthus* (Xu and Sun, 2001; Wetzal et al. 1999) *Setaria* (Dekker, 2003), and *Echinochloa* (Danquah et al. 2002) complexes, and this approach was successful in controlling weeds in economically important crops (Quakenbush and Anderson, 1985; Wang and Dekker, 1995).

The *Chenopodium* genus is not a well-understood complex and many species are highly polymorphic in habit, height, branching and leaf size (Basset and Crompton, 1982). Cole (1961) wrote that the confusion in *Chenopodium* identification mainly results from the abundance of morphologically similar species, existence of polymorphisms within the limits of many

individual species and of parallel variation between different species, occurrence of marked phenotypic plasticity complicating species identification, and presence of putative hybrids further complicating the variation pattern. Rahiminejad and Gornall (2004) also reported considerable genetic and morphological variation within the *Chenopodium* species thereby supporting the fact that this is a complex group. Thus, not only does this group lack good morphological characteristics to distinguish species, it also lacks informative sites that can be used to differentiate species at the biotype level, and the existence of different intra-specific chromosome numbers further complicates the classification (Maude, 1940).

In agricultural fields, most of the weedy *Chenopodium* species are referred to as common lambsquarters (*Chenopodium album* L.). Recently, it was reported that common lambsquarters, one of the worst weeds in the world (Holm et al. 1977), was difficult to control with the available herbicides in corn and soybean fields in the North Central states (Fischer et al. 2004; Conley et al. 2003). To more effectively manage the *Chenopodium* species in agronomic fields, it is important to understand the taxonomy of the complex and to develop markers that can help in proper species identification.

## **1.1 Introduction to the Genus *Chenopodium***

The genus *Chenopodium* consists of about 250 species (Giusti, 1970; Kadereit et al. 2005) and belongs to the Amaranthaceae, syn. Chenopodiaceae family (APG I 1998). With a few exceptions, the majority of them are annual weeds (Cole, 1961). The genus includes herbaceous and arborescent perennials, and is distributed throughout Asia, America and Europe (Ruas et al. 1999). ). The *Chenopodium album* classification has often functioned as a convenient taxonomic receptacle, loosely circumscribed to include material not readily assigned to other species of this

difficult genus (Wilson, 1980). The taxonomy of the genus *Chenopodium* has been a major point of controversy. Taxonomic problems mainly arise due to phenotypic plasticity (Kurashige and Agarwal, 2005; Zhou et al. 2005), parallel evolution (Duke and Crawford, 1979) and hybridization (Cole, 1961; Rahiminejad and Gornall, 2004). Bittrich (1993) included Chenopodiaceae as one of the six families that lack clear delimitation or valid synapomorphies. While studying the *Chenopodium* genus, Wahl (1954) wrote that “no group of comparable size and wide distribution known to the writer has suffered the lack of understanding of the taxa involved as has the genus *Chenopodium*, especially those members of its Section *Chenopodium* that are closely related to *C. album* and *C. berlandieri*.” In Illinois, there are about 20 species (USDA, NRCS. 2008) and some of them (*C. album*, *C. berlandieri*, *C. strictum*, *C. murale* and *C. hybridum*) are recognized as agricultural weeds. Some of these weeds are resistant to one or more classes of herbicides (Heap, 2010).

## **1.2 Biology and Ecology of *Chenopodium* Species**

According to the APG II system of 2003, the plants formerly treated as the family Chenopodiaceae are now categorized under the family Amaranthaceae, making Amaranthaceae a large family with 160 genera and 2,400 species. The Chenopodiaceae and Amaranthaceae families were two closely related but separate families in the order Caryophyllales until 1998 when, based on the similarities of the morphological and molecular data, APG I (1998) combined the two species. Traditionally, the two families were considered closely related based on the core floral formula consisting of 5 sepals, 0 petals, 5 stamens, and 2-3 carpels (Hershkovitz, 1989). Judd and Fergusen (1999) also gave similar facts with both families having apomorphic flowers with a single whorl of stamens present opposite to the tepals and

multiporate pollen grains. Rodman (1990) also supported the inclusion of two families together based on the autapomorphy of sieve-element plastids with peripheral ring-shaped bundles of proteins but lacking central position inclusion, unique to these two families. Other characteristics supporting the close relationship of the two families include anomalous secondary growth, presence of isoflavones (Sanderson et al. 1988) and pantoporate pollen (Herskovitz, 1989). So based on their similarities, these two families were always taxonomically close to each other in virtually all systems of classifications.

Molecular data, in addition to the existing morphological data, supported the combination of the two families into Amaranthaceae (APG II 2003). Molecular-level support has been provided by several researchers working on different gene regions. For instance, Rettig et al. (1992) reported the phylogenetic relationship between the two species, and based on the nucleotide subunit data of ribulose 1, 5-bisphosphate carboxylase /oxygenase (*rbcL*), supported the placement of Amaranthaceae and Chenopodiaceae as a monophyletic lineage. Similar results were also reported by Downie et al. (1997) by studying the phylogeny based on the sequence of partial chloroplast DNA ORF 2280 homolog. They concluded that phylogeny lacked the separation between two families.

Some of the most important genera included in Chenopodiaceae are *Chenopodium*, *Atriplex*, *Beta* and *Salsola*. A detailed description of the morphology of *Chenopodium* is given by Clemants and Mosyakin (2004). To provide a brief description, family members are mainly characterized as herbs, either annual or perennial, and farinaceous pubescent or glabrous. The stems are mostly striped with their orientation varying from erect to prostrate, and generally branched. The leaves of *Chenopodium* are alternate, petiolate or sessile, not fleshy; the blade can be linear, oblong, lanceolate, ovate, triangular, trullate, or rhombic, with the leaf base truncate,

cordate, hastate, or cuneate. The inflorescence is either spicate and terminal or axillary and glomerulate. The genus is generally characterized by small perfect or rarely unisexual flowers with the perianth five-parted (rarely three- or four-parted), and the segments rounded or keeled. The flowers have five or fewer stamens and the style usually has two (rarely five) stigmas (Standley, 1916; Clemants and Mosyakin, 2004; Judd and Fergusen, 1999). Fruits are utricles or achenes, often associated with persistent tepals; the pericarp appears smooth to papillate. Seeds of *Chenopodium* generally have flat margins but can also be rounded, flattened, or grooved with their surfaces appearing shiny to dull and smooth to alveolated (Bassett and Crompton, 1982)

### **1.3 Morphology-Based Studies in *Chenopodium* Species**

Species of *Chenopodium* are not easily defined because of lack of distinctive macroscopic morphological characters in the genus (Bassett and Crompton, 1982). Many species need more than vegetative material alone for identification. The first post-Linnean study on *Chenopodium* was done by Moquin-Tandon (1840) in his monograph of the family. Murr (1904-1927) contributed extensively to the *Chenopodium* complex. Brenan (1964) wrote about the taxonomy of *Chenopodium* for Flora Europaea. In the New World, the majority of the contribution to *Chenopodium* was made by Aellen (1929). Apart from Aellen, other researchers who contributed to *Chenopodium* studies include Watson (1874), Standley (1916), Aellen and Just (1943), and Wahl (1954), although they mainly contributed to revision of existing treatments of introduced and native American taxa (Bassett and Crompton, 1982).

The taxonomic classification of *Chenopodium* species has always proven to be very difficult and most of the researchers have used one or more morphological characteristics in combination to clear the confusion within the taxa. For instance, Aellen and Just (1943) and



Wahl (1954) made subsectional distinctions based on inflorescences and pericarps of the *Chenopodium* species. Similarly, Cole (1961) subdivided *Chenopodium* into 4 subsections based on seed coat markings. Iljin (1936) also differentiated *Chenopodium* taxa based on pericarp structures and keeling of the calyx lobes. Apart from seed coat and inflorescence, some researchers have also used leaf color as an additional characteristic (Moquin-Tandon, 1840).

Bassett and Crompton (1982) worked on clarification of taxonomic circumscriptions and the nomenclature and distribution of taxa in *Chenopodium* across Canada, mainly based on characteristics like seed testa and pericarp, along with the information related to the chromosome count in the species. Crawford and Reynolds (1974) took a different approach by using phenetic characters to understand the relationship among narrow-leaved *Chenopodium* species. They were successful in elucidating the relationship and reported certain affinities that were contrary to the literature present at that time. Such studies have also been successfully conducted in other plant groups also to gain insight into the variation pattern within the species (Crevello 1968; Gilmartin 1969; Ornduff and Crevello 1968; Heiser et al. 1965). Another parameter that has been studied to gain insight into the *Chenopodium* taxa is the pollen grain structure along with cytological and seed characteristics (Dvorak, 1983).

*Chenopodium album*, one of the worst weeds and widespread synanthropic plants, is also among the most polymorphic plant species (Clemants and Mosyakin, 2004). It is a loosely arranged aggregate with races still being insufficiently understood. Some authors have recognized numerous segregate intergrading species, while others have developed elaborate infraspecific hierarchies with numerous subspecies, varieties, forms, and even numerous subforms, or have combined both approaches. Neither approach has brought satisfactory and uncontroversial results (Clemants and Mosyakin, 2004). This lack of solid knowledge about the

*Chenopodium* species has led to the need for a set of molecular markers in order to gain additional information about the species.

#### **1.4 Molecular Markers for Phylogenetic Studies**

In the last three decades, molecular sequence data have revolutionized the field of systematics by providing additional approaches and giving more confidence to published morphology-based systematics or taxonomy. The potential of DNA data to reveal phylogenetic relationships was first discussed in 1965 by Zuckerkandl and Pauling. Earlier systematics studies focused on DNA-DNA hybridization (Bendich and Bolton, 1967). The potential of restriction fragment length polymorphisms (RFLP), as a marker was shown in the Compositae family (Jansen and Palmer, 1988). It was only after the advent of the PCR technique (Saiki et al. 1988) that DNA analysis came to be used very frequently.

Most molecular taxonomy data are generated by PCR amplification and sequencing of nuclear DNA markers (internal transcribed spacers, external transcribed spacer, transposable elements, simple sequence repeats, promoter sequences and single copy nuclear intron sequences), chloroplast DNA markers and mitochondrial regions. Soltis and Soltis (1998) have discussed the importance of different regions for studying angiosperms at different taxonomic levels.

Since the advent of the research done by Soltis and Soltis (1998), many new markers have been discovered, but the information provided still holds true for species and population level studies. The nuclear and chloroplast regions are more promising than mitochondrial region due to lack of mitochondrial markers that can be used at lower level taxonomy (Palmer, 1992).

The use of molecular data has proved to be a powerful approach for achieving well-delimited taxa in groups like the Ericaceae (Kron et al. 2002) and Asteraceae (Kim and Jansen, 1995). The independent information from DNA-based topologies can help in resolving relationships among morphologically intractable groups (Fior et al. 2006) and, along with morphological studies, can help in understanding the taxonomic complexities (Schönenberger and Conti, 2003). One main advantage of DNA markers is that, unlike morphological and biochemical markers, the DNA markers are practically unlimited in number and are not affected by plant developmental stage or growth environment (Winter and Kahl, 1995).

Molecular markers are developed from the organellar genomes (mitochondria, chloroplast) or regions of the nuclear genome and the utilities of these marker types is discussed in further detail below.

### **Mitochondrial DNA (mtDNA)**

Mitochondrial genomic regions are the less used organellar markers in phylogenetic analyses in plants. The reasons for their limited use are discussed in detail by Palmer (1992). Some of the limitations in using the mitochondrial DNA (mtDNA) are that it is very large and highly variable in size; sometimes chloroplast DNA sequences are present in mitochondrial genomes; large duplications are frequent; recombination can occur among repeats making the genome complex; and the nucleotide substitution rate is 3-4 times less than chloroplast DNA (cpDNA) and even less when compared to nuclear DNA (Wolfe et al. 1987, 1989; Palmer and Herbon, 1988; Laroche et al. 1995)

The utility of mtDNA is more in higher taxonomic studies but less in interspecies or low level taxonomic studies (Soltis and Soltis, 1998). An intron, *nad1*, which is present in NADH dehydrogenase has been used to understand the relationship of Polemoniaceae with other

families (Porter and Johnson, 1998). Although there are few studies at family and population levels (Davis et al. 1998; Luo and Boutry, 1995), mitochondrial regions from plants have not been investigated as the substitution rates are low. They have not been explored in detail as has been done in animals.

### **Chloroplast DNA (cpDNA)**

The chloroplast genome is smaller than the nuclear genome and is found in large numbers, making it easy to isolate. The chloroplast genome is divided into protein coding genes, introns and intergenic spacers. Most phylogenetic studies investigating plant evolution utilized chloroplast markers. The cpDNA is successful in such studies because it is structurally stable, non-recombinant, and highly conserved in genetic content among closely related species (Downie and Palmer, 1991). Chloroplast protein coding gene substitution rates are generally slower than those of the nuclear genome, making them good markers for high level taxonomic studies (Chase et al. 1993; Soltis and Soltis 1998), but noncoding introns and spacers are used frequently at low taxonomic levels, though sometime noncoding cpDNA also fails to provide significant phylogenetic information (Shaw et al. 2005, 2007; Small et al. 1998).

Among the coding genes, *rbcL* (Chase et al. 1993) was used in the earlier phylogenetic studies, but soon it was followed by other coding genes like *ndhF* (Olmstead and Palmer 1994; Clark et al. 1995; Kim and Jansen 1995), *atpB* (Hoot et al. 1995; Wolf 1997) and the more commonly used *matK* alone (Johnson and Soltis 1994; Shaw et al. 2005), or *matK* along with intron *trnK-matK-trnK* region (Johnson and Soltis 1994). Though the coding regions have been used extensively in studies at the family or higher taxonomic levels, the noncoding regions are used more at the lower taxonomic levels, making them more appropriate for species (Gielly and Taberlet, 1994).

A limitation of cpDNA is its slow rate of evolution (Wolfe et al. 1987), which hinders it in providing enough phylogenetic informative characters at lower taxonomic levels (Sang, 2002). Another problem is uniparental inheritance, which means it reveals information from only a single parent. So, if the samples consist of hybrids or polyploids, it will give false phylogeny since it cannot reveal hybrid history (Sang 2002; Small et al. 2004). The assumption of uniparental inheritance is also not without an exception. There are reports of biparental and paternal inheritance in angiosperms (Birky 1995; Corriveau and Coleman, 1988). Another assumption is that chloroplast genomes are non-recombinant; however, evidence from *Pinus contorta* suggests that recombination may occur (Marshall et al. 2001).

### **Nuclear Sequences**

Most of the studies using nuclear regions have relied on the nuclear ribosomal regions. At higher taxonomic levels, the slowly evolving rRNA genes are used (Soltis and Soltis, 1998; Kuzoff et al. 1998), while at lower taxonomic levels internal transcribed spacers (ITS) and external transcribed spacers (ETS) are more commonly used (Alvarez and Wendel 2003). In general, the eukaryotic ribosomal RNA genes are part of repeat units that are arranged in tandem and located at chromosomal sites known as nucleolar organizing regions. Each unit consists of a transcribed region having genes for 18S, 5.8S, 26S rRNA, internal transcribed spacer 1 and 2, external transcribed spacers (ETS1 and ETS2) and a non-transcribed spacer (NTS) region.

The ITS has been widely used mainly as it is biparentally inherited, can be amplified using universal primers, is present in thousands of rDNA repeats making it easy to amplify, has low genomic variability due to concerted evolution but high intra-genomic variability due to insertion and deletion mutations (indels) and it appears to largely evolve neutrally (Alvarez and Wendel, 2003). These qualities make ITS a popular region for phylogenetic studies, however

there are several concerns related to its use (for review Alvarez and Wendel, 2003; Baldwin et al. 1995; Dubcovsky and Dvorák, 1995; Feliner and Rossello, 2007). Briefly discussing the limitations, the first issue is with multiple rDNA repeats. The basic requirement for historical inference based on nucleic acid sequence is that the genes compared are orthologous and not paralogous. Orthologous sequences are preferred as their history reveals divergence events among species. However, if there is a gene duplication event, it leads to the formation of paralogous sequences between lineages. This paralogy in phylogenetic estimation will lead to incongruence in phylogeny (Alvarez and Wendel, 2003).

Another problem pertains to concerted evolution, a phenomenon that tends to homogenize the sequences in nrDNA arrays, however this process is not always completed. When there is a hybridization or introgression, the speed and direction of homogenization cannot be predicted. So analyzed at the species level, concerted evolution is probably responsible for complex patterns following the merging of ITS repeats due to hybridization (Feliner and Rosello, 2007)

Due to the concerted evolution, the duplicated ribosomal loci may degenerate into pseudogenes and this can lead to wrong inference. Moreover, if there are compensatory base mutations in positions located on stem structures, it violates the assumptions of neutrality and independence of characters (Liu and Schardl, 1994). Another common problem is with the alignment of the sequences. It has been observed that aligning ITS leads to hypothesizing indels. This can make the alignment of distantly species difficult and leads to incorrect homology assessment. If misalignment is not corrected, then along with compensatory base change, paralogy and lack of (complete) concerted evolution can increase homoplasy (Alvarez and

Wendel, 2003). To combat these limitations, single or low copy nuclear genes are often used in the place of the aforementioned markers (Sang 2002; Small et al. 2004).

### **Low Copy Nuclear Genes (LCNG)**

An alternate approach to ITS and cpDNA markers is to use single or low copy nuclear genes for phylogenetic analyses. Slow rates of sequence divergence in cpDNA or too much divergence in ITS create a problem for using these markers. The uniparental inheritance of cpDNA also cannot provide any information about hybrids, and in nrDNA the process of homogenization by concerted evolution creates a similar problem in assessing hybrids. Some of the main advantages of using single or low copy nuclear genes are biparental inheritance, and co-occurrence of introns and exons within the same gene, yielding characters that evolve at different rates thus can provide informative signals at different levels and relatively large number of markers makes them potentially good alternates to ITS and cpDNA markers (Alvarez et al. 2008).

At higher taxonomic levels LCNGs (such as phytochrome) have proven to be useful markers for understanding diversification in angiosperms (Matthews et al. 1995). There is a need to explore more LCNGs as they can potentially allow selection of genes with extremely conserved rates of evolution and consequently a robust reconstruction of deep branch relationships of plants (Sang, 2002). The studies at the intergeneric level also have shown promising results for LCNGs like the *Adhc* gene, which have proven to be better than cpDNA (*ndhF*) for constructing phylogeny. Similarly, the 4CL gene sequence is congruent with the chloroplast (*matK*) and mitochondrial (*nad5*) genes. Even at the interspecies level, *AdhC* proved to be more useful when studying the relationship of closely related tetraploid species as

compared to cpDNA which produced a weakly and poorly resolved phylogeny (Small et al. 1998).

However, there are some concerns too with using LCNG, including problems due to paralogy, lineage sorting, hybridization, requirement of large amount of high quality DNA, non universality of the primers and the need to clone to amplify all the copies of the alleles (Sang, 2002).

### **1.5 Pitfalls of Molecular Data**

The use of DNA as a taxonomic tool has been advocated by several researchers (Doyle, 1993; Hajibabaei et al. 2007; Tautz et al. 2003). However, there is a strong reaction against relying on DNA-based taxonomy (Miller et al. 1997). Some of the reasons for being vigilant when using DNA sequences as taxonomic tools are issues related to long branch attraction, pseudogenes, homology assessment, polyploidy and hybridization, alignment in sequences with indels, model selection to estimate nucleotide substitution rate and computation time for phylogenetic analysis. These are discussed separately.

#### **Long Branch Attraction (LBA)**

This is a phenomenon when rapidly evolving lineages, when included with slowly evolving lineages, are inferred to be closely related regardless of their actual evolutionary relationship or in other words due to false synapomorphies. The maximum parsimony (MP) method is more sensitive to LBA. When LBA is present, tree reconstruction methods are inconsistent, and they converge towards an incorrect solution as more data are considered. This artifact is serious as there were 112 hits on Web of Science that have discussed this problem. The LBA problem has been reviewed in detail by Bergsten (2005). He describes LBA as a phenomenon in which an amino acid in two species at a certain position looks identical but had



been independently acquired and will appear as synapomorphies. The simulation studies and Felsenstein four taxon case-study also showed that inference is difficult in the Felsenstein zone where the two long branched non sister taxa grouped together rather than with their true shorter branched sisters (Huelsenbeck, 1995).

## **Pseudogenes**

This is a problem when we are using nuclear genes or rDNA sequence data for phylogenetic analysis. The paralogues of ribosomal DNA can become pseudogenes when a single rDNA copy is dispersed to other genomic regions. The first step is to discriminate between paralogous rDNA, as paralogues may cause species to appear para- or polyphyletic if a gene tree is interpreted as a species tree (Buckler et al. 1997). Pseudogenes are more dependent on the molecular data used and less on analysis method. Good sequence alignment and rDNA secondary structure can help to identify pseudogenes in the given dataset.

## **Assessing Homology**

Hypothesis of homology is the basis of phylogenetic analysis (Phillips, 2006). The term homology was first introduced by Owen (1843) to express similarities in basic structure found between organs of animals that he considered to be more fundamentally similar than others. Homologies can be difficult to identify; this may be attributed in part to the existence of a finite number of character states and rates of change sufficient to yield independent expressions of the same state. We assign putative homology to molecular data using sequence alignments. The best alignment in context with phylogeny is the one that generates the most parsimonious tree when analyzed in conjunction with all relevant data. We should therefore be very careful when we are aligning the sequences and should check visually for shifts in sequences and manually edit them

if required. Another possibility is to give more weight to characters that change less frequently which can further help to reduce the effects of homoplastic similarity among the sequences.

### **Polyploidy and Hybridization**

One of the most significant findings of molecular data usage is that of polyploidy (genome duplication) and hybridization. Several studies have revealed that multiple rounds of polyploidy have occurred during angiosperm evolution. Polyploidy has long been recognized to be associated with novel morphologies and adaptations, but how genome duplication ultimately translates into novel evolutionary opportunity is not very clear. Interspecific hybridization followed by polyploidy can restore fertility to sterile hybrid lineages. There are several studies that indicate that there is doubling of chromosome numbers due to autopolyploidy or by allopolyploidy via a hybridization event. To uncover past hybridization or introgression events, comparison between phylogenies derived from nuclear and chloroplast genomes are important because discordance in relationships between the data sets may indicate that hybridization has occurred (Rieseberg, 1998; Doyle, 1992).

### **Multiple Sequence Alignment of Samples with Gaps**

When we do multiple sequence alignment of DNA sequences, it is very common to hypothesize gaps or indels in order to align orthologous sequences. If we use a program such as GapCoder, we can convert the gaps into binary characters and then easily analyze them using phylogenetic software like PAUP (Swafford, 1999). We should look at the sequences very carefully and edit the sequences in order to use gaps as phylogenetic informative characters.

## Model Selection

In the Maximum likelihood and in Bayesian analysis, model selection is really important. In molecular phylogenetics, models are used to estimate the evolutionary change at a given nucleotide site over the period. The primary aim of molecular phylogenetic inference is to approximate the progression of lineage divergences that produced a group of observed sequences. Most of our conclusions are based on phylogeny estimations, and without any prior knowledge of the actual evolutionary relationships between organisms, confidence in the performance of a model is necessarily a function of confidence in the suitability of the model and the data used (Kelchner and Thomas, 2007). Models are based on assumptions and some of them are: (i) mutations are independent and identically distributed; (ii) tree-like evolution: lineages arise in a divergent manner without reticulation; (iii) stationarity: mutational processes are consistent through time; (iv) reversibility: mutations can revert to a previous state; and (v) Markov process: mutation events are not influenced by a previous mutation at that site (Kelchner and Thomas, 2007). Such assumptions are often violated in reality; for example, prokaryote groups frequently share genes among lineages via lateral gene transfer and do not evolve in a tree-like fashion. Kelchner and Thomas (2007) have discussed key points that we should keep in mind when we select models for analysis. If the model is a poor approximation of reality due to absence of key parameters, the consequence can be systematic error that strongly influences the analysis, resulting in inaccurate but sometimes well-supported phylogeny estimation. Underfitting a model can lead to problems like long-branch attraction (discussed earlier) when sampling is inadequate and there is a faster substitution rate in one or more lineages. Model selection is important for phylogenetic analysis and there are tools that can help to select a better model (Sullivan and Joyce, 2005). In general, the model preferred is a parameter rich model. A

thorough review of importance and how to select a good model is also given by Sullivan and Joyce (2005).

### **Computation Time**

Another limitation in phylogenetic programs is of computation time. Distance based methods like UPGMA (Mitchner and Sokral, 1957) and minimum evolution (Kidd and Sgaramella, 1971) are fast but the information is lost in compressing sequences into distances. Maximum parsimony (Edwards and Cavali-Sforza, 1963) is fastest in character based tree but it can perform poorly depending upon the amount of homoplasy. Maximum likelihood (Edwards and Cavalli-Sforza, 1964; Felsenstein, 1981) is very good but on the other hand a very exhaustive method and can be only done with heuristic search if there are more samples. Bayesian analysis (Larget and Simon, 1999; Huelsenbeck and and Ronquist, 2001) can take days if the number of samples is more, so it comes to the point where we have to decide what kind of information we need from our data and accordingly we have to compromise with either time or accuracy.

Now, as we know that there are pros and cons of using DNA sequences in phylogenetic analysis, the best approach is to integrate morphological characteristics and molecular data. By incorporating unambiguous morphological characters and analyzing those along with molecular data, the bias of homoplasy within any individual data partition will decrease (Farris, 1983; Miller et al. 1997; Wahlberg et al. 2005). The utility of combined analysis has been well-defended by phylogenetic inferences from Doyle and Endress (2000) and Conard (2008). This type of combined analysis has proven to be helpful in distinguishing amongst the plant species.

## 1.6 Molecular Analysis in *Chenopodium*

Molecular studies have been done in *Chenopodium* using random amplified polymorphic DNA (RAPD) (Anderson 1999; Gangopadhyay et al. 2002), directed amplification of minisatellite DNA (DAMDA) (Rana et al. 2010), microsatellite markers (Mason et al. 2005), ribosomal DNA (Maughan et al. 2006) and plastid genes (Downie et al. 1997). Ruas et al. (1999) used RAPD markers to distinguish *C. album* from *C. berlandieri* and found 90 percent similarity between the two, which perhaps gives us a reason why these species are often misidentified. In Bolivia, Ruas et al. (1999) found that there was a 75 percent similarity between two accessions of *C. ambrosoides*, but these accessions could not be distinguished using morphological characters. In another study involving highland and lowland *C. quinoa*, Maughan et al. (2006) used the IGS region and revealed the presence of synapomorphic polymorphisms that separated the lowland from highland *C. quinoa*.

A majority of the research related to genetic diversity and phylogenetic studies in *Chenopodium* mainly emphasized domesticated species like *C. quinoa* and *C. berlandieri*. There are very few studies (Rana et al. 2010) that include the important weed species like *C. album*, *C. berlandieri*, *C. ficifolium*, *C. glaucum*, *C. murale* and *C. strictum*, and there is a clear lack of understanding of these species using both morphological and molecular data. Given the recent accounts of herbicide-tolerant common lambsquarters that have been reported, understanding this complex now is more important than ever. The following two sections will discuss herbicide tolerance in common lambsquarters.

## 1.7 Herbicide Resistance in Common Lambsquarters

Herbicide resistance in weed populations is an evolutionary process and refers to the evolution of a mechanism to withstand a normally effective herbicide dose as a result of selection pressure (Harper, 1956). In plants, resistance can occur naturally or can be induced by techniques such as genetic engineering or selection of variants produced by tissue culture or mutagenesis (Anonymous 1998). Resistance is different than tolerance in which the plants have the inherent ability to survive and reproduce after herbicide treatment and there is no role of selection or genetic manipulation of plants to be tolerant (Anonymous, 1998).

Resistance to herbicides is an ever-increasing problem and has become a threat to conventional agricultural practices in agriculture (Jasieniuk et al. 1996). Several factors like gene mutation, initial frequency of resistant alleles, inheritance, weed fitness in the presence and absence of herbicide, type of mating, and gene flow influence the evolution of herbicide resistance.

Since the observation in 1970 of triazine resistance in *Senecio vulgaris* L. (Ryan, 1970), herbicide resistance has increased dramatically. According to the latest report of International Survey of Herbicide Resistant Weeds, globally 195 species, of which 115 are dicots and 80 are monocots, have developed resistance to one or more classes of herbicides (Heap, 2010). In the US, there are 354 biotypes that are resistant to one or more classes of herbicides and Illinois ranks third with 18 resistant biotypes to photosystem system II (PSII), acetolactate synthase (ALS), protoporphyrinogen oxidase (PPO) and glycine herbicides. It is estimated that 4,062,200 acres of Illinois (Anonymous, 2010) land is affected with these resistant biotypes, with *Amaranthus tuberculatus* being the predominant species. This weed has evolved resistance to four classes of herbicides (Heap, 2010).

The first report of a weed resistant to atrazine and simazine appeared in 1970 when common groundsel (*Senecio vulgaris*) was reported resistant to simazine and atrazine (Ryan 1970). It was soon recognized that herbicide resistance in the weed species was passed down maternally and attributed to a change in the properties of photosystem II. However, the molecular basis of resistance, an amino acid change in the sequence of one of the reaction center proteins of photosystem II, was first reported in *Amaranthus hybridus* L. in which resistant plants had a serine to glycine substitution at position 264 on the D1 protein (Hirschberg and McIntosh, 1983). Another mutation at the amino acid position 219 has been identified which is also responsible for resistance to PS-II inhibitor (Dumont and Tardiff, 2002; Mengistu et al. 2000).

*Chenopodium* is on the list of top ten resistant weeds and is reflected by the fact that there are 42 resistant biotypes of *Chenopodium* in 17 countries (Heap, 2010). The majority of these biotypes are resistant to photosystem II inhibitors, followed by ALS inhibitors, synthetic auxins, ureas and amides. In the US, there are 21 biotypes that are resistant to photosystem II (19) and ALS inhibitors (2) and tolerant to glyphosate (Westhoven et al. 2008a). In 2009, within Illinois, there were 18 biotypes of resistant weeds and *Chenopodium album* was one of them.

The earliest case of herbicide resistance in the *Chenopodium* complex dates back to the 1970s in the Midwest when they evolved resistance to triazine herbicides (Bandeem and McLaren, 1976). In fact, triazine resistance in *Chenopodium* is quite notable and there are more than 19 biotypes of *Chenopodium* that are resistant to triazines (Bandeem and McLaren, 1976).

Herbicides that target photosystem II (triazine and phenylurea) inhibit plant growth by competing with the native plastoquinone, which is the binding site of the D1 protein in the photosystem II, thereby disrupting the linear electron transport from the electron donor,  $Q_A$ , to the mobile electron carrier,  $Q_B$  (Oettmeier, 1999). This results in a shortage of reduced  $NADP^+$

which is required for CO<sub>2</sub> fixation and the formation of free radicals which cause photooxidation of important molecules in the chloroplast, the latter being the major herbicidal action of these herbicides (Devine and Shukla, 2000).

A biotype of *Chenopodium album* in New Zealand has also developed resistance to synthetic auxins such as dicamba (James et al. 2005). A recent report from New Zealand confirmed that a biotype of *Chenopodium* was tolerant to 2400 g ha<sup>-1</sup> dicamba, which is equivalent to eight times the recommended field rate (Rahman et al. 2008). This is another major setback for weed control as dicamba mixed with other herbicides has proven to be very effective in controlling triazine resistant *Chenopodium album* (Ritter and Menbere, 2001). *Chenopodium album* has occasionally shown poor control with pre-emergence (PRE) herbicide treatments with dinitroaniline herbicides such as pendimethalin (Hagwood, 1989), but the results are not very consistent and the cases of tolerance to these herbicides are rare.

Within glyphosate-based cropping systems, weed scientists have confirmed that weed shifts have occurred, with common lambsquarters becoming more problematic (Culpepper, 2006). Control of common lambsquarters with glyphosate has been variable and there is an increase in the number of reports that show poor common lambsquarters control with glyphosate.

### **1.8 Glyphosate Tolerance in Common Lambsquarters**

Glyphosate is the most commonly used broad-spectrum herbicide and is effective in controlling many weeds. It acts by blocking the shikimate pathway through inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs). Inhibition of EPSPS results in reduced biosynthesis of aromatic amino acids tyrosine, phenylalanine, and tryptophan in sensitive plant species (Amrhein et al. 1980; Kishore and Shah, 1988). Glyphosate competes with



phosphoenolpyruvate (PEP) for the binding site on the EPSPs enzyme (Sikorski and Gruys, 1997). Upon inhibition of EPSPS, shikimic acid and shikimate 3-phosphate levels increase in plants and this increase is linked to a decline in carbon fixation intermediates and a reduction of photosynthesis (Duke et al. 2003).

In the last decade, glyphosate resistant (GR) and tolerant populations have increased (Powles, 2008). Currently, 16 weed species have evolved resistance to glyphosate and some of the common weeds species are *Ambrosia*, *Amaranthus*, *Conyza*, *Lolium*, *Conyza* and *Sorghum*. Apart from resistant weeds, farmers have reported tolerance to glyphosate in different populations of common lambsquarters. Similar reports have been given by researchers across Midwestern states confirming that common lambsquarters has some level of tolerance to glyphosate (Westhoven 2008a). With over-reliance on glyphosate in GR cropping systems, there are chances of these tolerant weeds developing resistance due to high selection pressure. Several factors, such as rapid growth of common lambsquarters plants to maturity, ability to germinate under different environmental conditions (Chu et al. 1978; Cummings 1963; Hilgenfield et al. 2004 ), indeterminate growth, environmental plasticity under field conditions (Kurashige and Agarwal, 2005), high seed production (Conn and Deck, 1995; Holm et al. 1977), long seed viability (Conn and Deck, 1995; Madsen, 1962), delayed germination, discontinuous dormancy and germination under low temperature conditions ahead of the crop make it a problematic weed (Chu et al. 1978; Wiese and Binning, 1987). Although resistance in agronomic fields is not yet a problem, the threat is always there; so even if a single plant survives, its progeny can establish as a resistant weed.

Within GR based cropping systems, weed scientists have confirmed that weed shifts have occurred, with common lambsquarters becoming more problematic (Culpepper, 2006). The

incidence of lambsquarters has increased under no-tillage systems (Sosnoskie et al. in 2006). Similar results were also reported in seed bank studies by Cardina et al. (2002). Field surveys conducted in Indiana from 2003 to 2005 reported that common lambsquarters plants were present in 11 percent of randomly sampled soybean fields (Westhoven et al. 2008b). Similar results have also been reported by researchers across other Midwestern states (Curran, 2005; Harder et al. 2007; Loux and Stachler, 2003; Kniss et al. 2006; Schuster et al. 2007). Season long interference by common lambsquarters in soybean fields has decreased soybean yield by 20 to 61 percent (Crook and Renner, 1990; Conley et al. 2003). Common lambsquarters became one of the dominant weed species in long-term experiments evaluating weed species shifts in glyphosate-resistant cropping systems. Results from these experiments indicate the dominance of common lambsquarters in soybean-corn rotation in the fields (Jeschke and Stoltenberg, 2006).

## **1.9 Research Objectives**

The weedy *Chenopodium* complex is not well understood within the North Central states and there are ongoing reports that the complex is becoming more difficult to control with glyphosate. We conducted greenhouse and laboratory studies on this weedy *Chenopodium* complex to determine the species present in the Midwest and the response of these populations to glyphosate. We test the hypothesis that there is no difference in the response to glyphosate of different *Chenopodium* spp. across the Midwest. Based on our testing hypothesis, we had three main objectives:

1. Determine the responses to glyphosate of different *Chenopodium* species and populations present in the North Central states.
2. Develop molecular markers to identify different species of *Chenopodium* spp.

3. Determine which species are present in North Central states using molecular markers.

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**CHAPTER 2**

**RESPONSES OF *CHENOPODIUM* SPECIES AND COMMON LAMBSQUARTERS  
POPULATIONS FROM NORTH CENTRAL STATES TO GLYPHOSATE**

**2.1 Abstract**

Experiments were conducted to determine the responses to glyphosate of 12 accessions representing 7 *Chenopodium* species, and 33 populations of *Chenopodium* from agricultural fields across the North Central states. Glyphosate responses of *Chenopodium* species were variable within and among the different species. One interesting finding was that *C. ficifolium*, *C. berlandieri* 2 and *C. simplex* were more tolerant to glyphosate than the four accessions of *C. album*, which is presumed to be the most common weed species in this genus. Variability to glyphosate was observed among the accessions of *C. album* and *C. berlandieri*. *C. album* 4 had more tolerance than other *C. album* accessions, and *C. berlandieri* 2 had significantly higher tolerance to glyphosate than *C. berlandieri* 3 and *C. bushianum*. When the 33 populations were evaluated for response to glyphosate, we observed variability within and among the populations from different North Central states. The populations from Iowa were more tolerant to glyphosate, but high tolerance was also observed in a few populations (IN6, IL3, IL7, IL8 KS21 and ND7) from Indiana, Illinois, Kansas and North Dakota. Our results indicate that if there are mixed populations of different *Chenopodium* species or biotypes in a field, the control of these weeds will be difficult due to the differential tolerance of the plants to glyphosate.

## 2.2 Introduction

The genus *Chenopodium* includes about 150 plant species and most of them are annual, herbaceous flowering plants, commonly known as goosefoot. The genus is distributed throughout Asia, America and Europe (Ruas et al. 1999). Although some species of the genus have been domesticated and used for their seeds (*C. quinoa*, *C. berlandieri* subsp. *nuttalliae*, *chia* and *huautzontle*) and vegetative parts (*C. album* and *C. giganteum*) in Asia and Europe (Pratap et al. 1998), the genus is more commonly known for having some of the most troublesome weed species (Cole, 1961).

*Chenopodium album* is among the most important weed species in this genus, and is considered among the ten most important weeds in the world (Holm et al. 1977). Characters that make *C. album* a successful weed include its ability to germinate under different environmental conditions (Chu et al. 1978; Cummings 1963; Hilgenfield et al. 2004 and Schuster et al. 2007), phenotypic plasticity under different environments, and prolific seed production (Holm et al. 1977). *Chenopodium* seeds can also remain dormant and viable in the soil for many years (Conn and Deck 1995; Madsen 1962), which maximizes their fitness while reducing their losses due to sib competition in the fields (Hyatt and Evans 1998).

There are about 20 species of *Chenopodium* that are present in Illinois (USDA, NRCS 2008), and some of the species (*C. album*, *C. berlandieri*, *C. bushianum*, *C. strictum*, *C. murale*, *C. ficifolium* and *C. hybridum*) are recognized as agricultural weeds. Several variants of these species also have been reported by Aellen (1929a, 1929b), who recognized 34 sub-species, varieties and forms of common lambsquarters in North America.

Due to taxonomic difficulties in identifying *Chenopodium* species, most of the weedy *Chenopodium* species in agricultural fields are generally referred to as common lambsquarters

(*Chenopodium album* L.). In the last decade, there have been increased reports of common lambsquarters being a problem in corn and soybean fields across North Central states (Conley et al. 2003; Fischer et al. 2004). The incidence of lambsquarters has increased under no-tillage systems (Cardina et al. 2002; Sosnoskie et al. 2006). Common lambsquarters became one of the dominant weed species in long-term experiments evaluating weed species shifts in glyphosate-resistant cropping systems. Results from experiments with soybean-corn rotation indicate the dominance of common lambsquarters in these fields (Jeschke and Stoltenberg, 2006). Field surveys conducted in Indiana from 2003 to 2005 reported that common lambsquarters plants were present in 11 percent of randomly sampled soybean fields (Westhoven et al. 2008a). Season-long interference by common lambsquarters in soybean fields has decreased soybean yield by 20 to 61 percent (Conley et al. 2003; Crook and Renner, 1990).

Within *Chenopodium*, there are 42 resistant biotypes in 17 countries (Heap, 2010) and the majority of these biotypes are resistant to photosystem II inhibitors, followed by acetolactate synthase (ALS) inhibitors, synthetic auxins, ureas and amides (Heap, 2010). In the US, there are 21 biotypes that are resistant to either Photosystem II (19) or ALS inhibitors (2) and there are also reports of tolerance in some biotypes to glyphosate (Westhoven 2008a, b). A recent survey conducted by weed extension specialists in 2007 reported that common lambsquarters is one of the seven genera or species that represents 80 percent of the herbicide resistant biotypes (Scott et al. 2009).

Inter- and intra-specific differences in response to herbicides are common in weeds as well as in crops (Hayes, 1959; Smith and Illnicki, 1973; Wax et al. 1974). Within weed species, there are several studies that have shown differential responses of weeds to herbicides. Some of the commonly studied genera include *Amaranthus* (Coetzer et al. 2002; Xu and Sun, 2001),

*Chenopodium* (Warwick and Black, 1981), *Echinochloa* (Damalas et al. 2008), *Setaria* (Wang and Dekker, 1995) and *Trifolium* (Smith, 1985). This difference in response to herbicides can be attributed to differences in the physiology of weeds affecting herbicide absorption or translocation (Baylis, 2000; Klingman, 1961), differences in plant morphology (Damalas et al. 2008), and the variability in the species to metabolize the herbicides to non-toxic products (Smith, 1985).

The incidence of common lambsquarters resistant to triazine herbicides and ALS inhibitors is reported to be more common than to glyphosate, but in recent years, there have been several reports demonstrating less control of some *Chenopodium* populations by glyphosate (Westhoven et al. 2008a). Similar reports of reduced sensitivity to common lambsquarters have also been documented by weed scientists across the Midwestern states (Curran, 2005; Harder et al. 2007; Kniss et al. 2006; Loux et al. 2004; Schuster et al. 2007).

Although there are several reports of herbicide tolerance in the *Chenopodium* genus, very few studies have been conducted that look at the responses of different weedy *Chenopodium* species and common lambsquarters populations to glyphosate under controlled environmental conditions. Such a study would be important to test the hypothesis that there will be species shifts in fields having species with different herbicide tolerances. Thus, the aim of the study was to determine the responses to glyphosate of different *Chenopodium* spp. and populations present in the North Central states.

## 2.3 Materials and Methods

### Plant Materials

The plant materials selected for this study were obtained from the North Central Regional Plant Introduction Station (NCRPIS), Ames, Iowa. *Chenopodium* species prevalent in North Central states were selected based on the information provided in the Flora of North America (Clemants and Mosyakin, 2004). In the present study, seeds were obtained for 12 accessions representing eight *Chenopodium* species, and the information about the species and their accession numbers is provided in Table 2.1.

For the study including common lambsquarters populations, seeds were provided by colleagues from six North Central states (Illinois, Indiana, Iowa, Kansas, Missouri and North Dakota). In a preliminary study, randomly pooled seeds from plants within a single field showed very limited variability in their response to glyphosate; therefore, the seeds from 2 to 5 plants from a single field were pooled and used for herbicide screening. This method was useful in evaluating a large number of seed lots under limited space and resources. The information regarding the common lambsquarters populations from fields across the North Central states is presented in Table 2.2.



**Table 2.1.** List of the *Chenopodium* species used in glyphosate screening. The seeds were obtained from North Central Regional Plant Introduction Station, Ames, Iowa.

| S.No | Genus                   | Accession No. |             |                |
|------|-------------------------|---------------|-------------|----------------|
|      |                         | (NCRPIS)      | Origin      | Year collected |
| 1    | <i>C. album 1</i>       | Ames 23855    | Poland      | 1997           |
| 2    | <i>C. album 2</i>       | PI 605700     | MI, USA     | 1994           |
| 3    | <i>C. album 3</i>       | PI 262168     | France      | 1959           |
| 4    | <i>C. album 4</i>       | PI 605701     | CO, USA     | 1983           |
| 5    | <i>C. ficifolium</i>    | Ames 25246    | Switzerland | 1999           |
| 6    | <i>C. murale</i>        | PI 614895     | Portugal    | 1998           |
| 7    | <i>C. bushianum</i>     | PI 608030     | IL, USA     | 1995           |
| 8    | <i>C. berlandieri 2</i> | PI 595316     | IA, USA     | 1995           |
| 9    | <i>C. berlandieri 3</i> | PI 612858     | UT, USA     | 2000           |
| 10   | <i>C. simplex</i>       | Ames 21981    | MI, USA     | 1994           |
| 11   | <i>C. strictum1</i>     | Ames 23893    | Germany     | 1997           |
| 12   | <i>C. rubrum1</i>       | Ames 23860    | Poland      | 1997           |

**Table 2.2.** List of the *Chenopodium* populations from North Central states.

| <b>Population</b> | <b>Location</b>     | <b>Crop</b>   |
|-------------------|---------------------|---------------|
| IA20              | N. Harding Co., IA  | Soybean       |
| IA21              | S. Harding Co., IA  | Soybean       |
| IA22              | W. Story Co., IA    | Soybean       |
| IA23              | S. Story Co., IA    | Soybean       |
| IA24              | Adair Co., IA       | Soybean       |
| KS20              | S. Riley Co., KS    | Soybean       |
| KS21              | E. Doniphan Co., KS | Soybean       |
| KS22              | C. Doniphan Co., KS | Soybean       |
| MO10              | Platte Co., MO      | Corn          |
| MO6               | Boone Co., MO       | Pumpkin       |
| ND14              | Ransom Co., ND      | -             |
| ND2               | Steele Co., ND      | -             |
| ND3               | Trail Co., ND       | -             |
| ND5               | Grand Forks Co., ND | -             |
| ND 7              | Griggs Co., ND      | -             |
| ND9               | Barnes Co., ND      | -             |
| IL1               | Champaign Co., IL   | Soybean       |
| IL2               | Clark Co., IL       | Soybean       |
| IL3               | Kankakee Co., IL    | Soybean       |
| IL4               | Piatt Co., IL       | Soybean       |
| IL5               | Putnam Co., IL      | Between field |
| IL6               | Ogle Co., IL        | Corn          |
| IL7               | Iroquois Co., IL    | Soybean       |
| IL8               | LaSalle Co., IL     | Soybean       |
| IL9               | Lee Co., IL         | Corn          |
| IL10              | Livingston Co., IL  | Soybean       |
| IN2               | Benton Co., IN      | Soybean       |
| IN3               | Fountain Co., IN    | Soybean       |
| IN4               | Fountain Co., IN    | Soybean       |
| IN5               | Huntington Co., IN  | Soybean       |
| IN6               | Jackson Co., IN     | Soybean       |
| IN9               | Montgomery Co., IN  | Soybean       |
| IN11              | Ripley Co., IN      | Soybean       |

**- Data not available for the crops**

Common lambsquarters seeds have high dormancy (Williams 1962), so before using the seeds for herbicide evaluation, the seeds were treated to break the dormancy. This procedure involved treating the seeds with 1:1 bleach: distilled water and intermittently vortexing for 10 min. Then the seeds were rinsed twice with distilled water before suspending the seeds in autoclaved 0.2 percent agar. The treated seeds were then stored at 4°C for at least a month before sowing in the greenhouse.

### **Growth Conditions**

*Chenopodium* seeds were first grown in flats using a medium that was a non sterilized mixture of LC1<sup>1</sup> and soil:peat:sand in 1:1 ratio with osmocote, a 14:14:14 slow release fertilizer, added to the soil. At the cotyledon stage, the plants were transferred into sectioned flats. The seedlings were maintained in sectioned flats, one seedling in each section having approximately 10 g of the above-mentioned medium, until they were 4-5 cm tall. Plants were again transferred into 20 cm round plastic pots containing the same non-sterile medium. It has been reported that sensitivity of *Chenopodium* seedlings to glyphosate is greatly reduced when the plants were grown in steamed soil (Schafer et al. 2009).

The greenhouse room was maintained at 28/22°C day/night, with supplemental light (minimum of 800  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  photon flux at the plant canopy) provided by mercury halide and sodium vapor lamps programmed for a 16-hr photoperiod. Plants were watered daily.

### **Glyphosate Applications**

To study the responses of different *Chenopodium* species and common lambsquarters populations, 5 seedlings from each species or biotype from North Central states were treated at

10 to 14 cm height with the potassium salt of glyphosate (Roundup WEATHERMAX<sup>2</sup>) at 0, 800, 1600 or 3200 g a.e. ha<sup>-1</sup> along with 2.5% ammonium sulfate (AMS). These rates correspond to roughly 0X, 1X, 2X and 4X the recommended rate used in fields to control weeds. In a preliminary study (data not shown), glyphosate at 800 g ha<sup>-1</sup> controlled 95 percent of our sensitive biotype (a population from North Dakota referred as ND5), which was also used as sensitive control in the greenhouse experiments.

Herbicide treatments were applied with a compressed-air, moving-nozzle laboratory sprayer equipped with an 80° flat fan TeeJet<sup>3</sup> nozzle delivering 187 liters·ha<sup>-1</sup> of water at 207 kPa. The nozzle was maintained at 40-45 cm above the plant canopy to ensure proper coverage with herbicide.

Two weeks after glyphosate applications, the treated plants were evaluated for herbicide injury. The aboveground biomass of each common lambsquarters seedling was harvested to determine the decrease in dry weight due to the herbicide injury. The harvested seedlings were oven dried at 68°C for 72 hrs and weighed.

### **Statistical Analysis**

The experiment was conducted as a completely randomized design. To study the responses to glyphosate of different *Chenopodium* species, the experiment was replicated twice. Based on the homogeneity of variance test (Levene's test), which determines the interaction between two replications, no significant effect of replications ( $p=0.05$ ) was found, and the data from the two replications were pooled. Using the Proc GLM procedure in SAS<sup>4</sup>, the mean differences between the *Chenopodium* species and populations were calculated based on the LSD values ( $\alpha=0.05$ ).

A separate study was conducted to determine if there is variability in *Chenopodium* populations from the North Central states. For this study, 33 populations of *Chenopodium* were selected from six states (Illinois, Indiana, Iowa, Kansas, Missouri and North Dakota) and they were compared to a glyphosate susceptible population (ND5). The experiment was replicated twice, however for the second run, due to the limitation of conducting an experiment involving a large number of populations, we reduced the number of populations by selecting 19 populations from the original 33 that spanned the range of responses observed in the first run. The data from two replicates for populations across North Central states were pooled as the homogeneity of variance test ( $p=0.05$ ) was not significant for the two runs. The Spearman rank correlation between the two replications was 0.84 which was significant at  $p<0.001$ , indicating similar response of *Chenopodium* populations to glyphosate in both the replications.

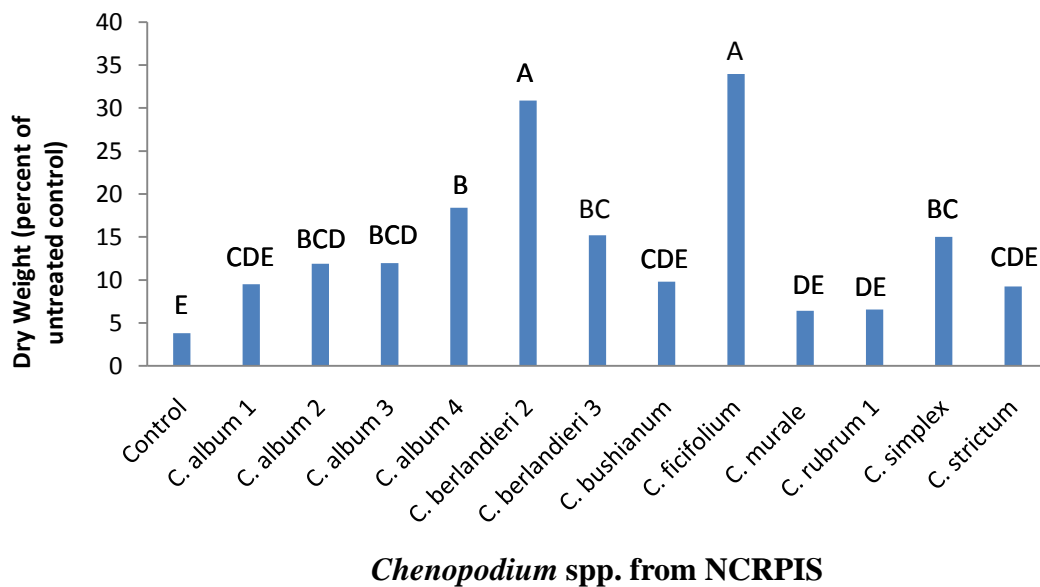
To compare the responses of different plants to glyphosate, the more common procedure is to analyze data using nonlinear logistic model. However, in this experiment we cannot use data to fit a logistic model as there were too few low doses, which resulted in failure of the model to converge. Low doses (between 0 and 800 gm a.e ha<sup>-1</sup>) were not used as our objective was to determine variable response in species and not to quantify levels of resistance by calculating the ED<sub>50</sub> values.

Results are presented as dry weight (percent of untreated control) for comparing the effect of various doses of glyphosate (0, 800, 1600 and 3200 g a.e ha<sup>-1</sup>) on different *Chenopodium* species and populations across the North Central states.

## 2.4 Results and Discussions

### Interspecific Variability Within *Chenopodium* Species to Glyphosate

Glyphosate injury to *Chenopodium* species increased with increasing glyphosate dose. Injury symptoms included stunting and chlorosis of treated plants within 10 days after treatment (DAT) and eventual plant death within 14-16 DAT in the susceptible plants; however the tolerant plants slowly recovered from injury. These findings were similar to other studies on common lambsquarters (Schuster et al. 2007). Figure 2.1 and Table 2.3 present the mean values of dry weight, expressed as percent of untreated control averaged across 800, 1600 and 3200 g a.e ha<sup>-1</sup> of glyphosate.

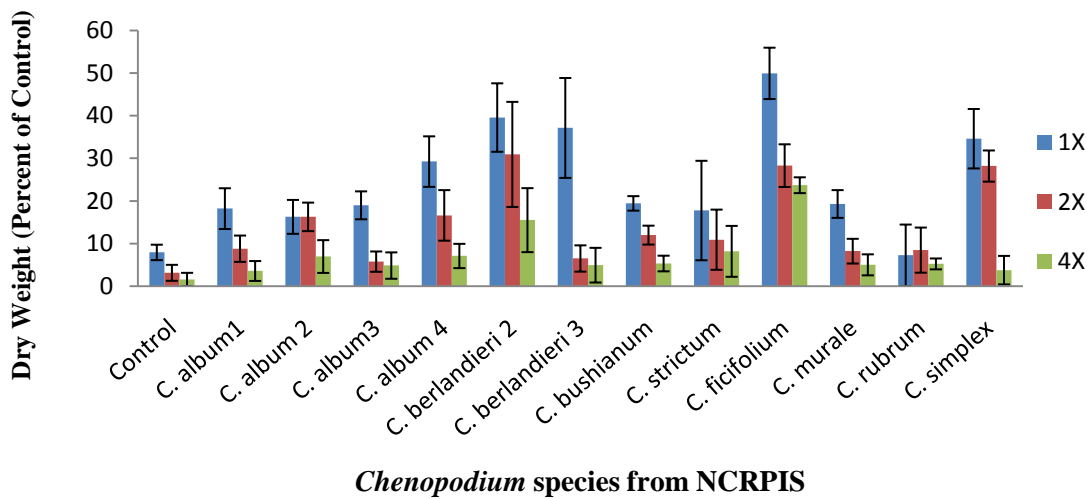


**Figure 2.1.** Mean dry weight of *Chenopodium* averaged across 800, 1600 and 3200 g a.e. ha<sup>-1</sup> of glyphosate. Bars followed by same letter are not different according to Fischer's LSD test ( $p < 0.05$ ).

As shown in Figure 2.1, when we average the herbicide dose and look at the variability in response to glyphosate, we found *C. berlandieri 2* and *C. ficifolium* had significantly more tolerance than other accessions, indicating variability to glyphosate within the species. The more

detailed Figure 2.2 shows the responses of various *Chenopodium* species to individual doses of glyphosate.

At the 1X dose of glyphosate (800 g a.e ha<sup>-1</sup>), there was a difference in tolerance to glyphosate within and among *Chenopodium* species. Differences were observed between *C. album* biotypes (different accessions of *C. album*) and morphologically similar species such as *C. berlandieri*, *C. strictum* and *C. ficifolium*. Compared to untreated control plants, at 800 gm a.e ha<sup>-1</sup> dose of glyphosate, the percent reduction in dry weight of *C. album* ranged from 70 to 90 percent of the untreated control. In contrast, dry weights of accessions of *C. berlandieri* ranged from 37.13 to 39.57 percent of the untreated control.



**Figure 2.2.** Glyphosate responses of different *Chenopodium* spp. from North Central Regional Plant Introduction Station.

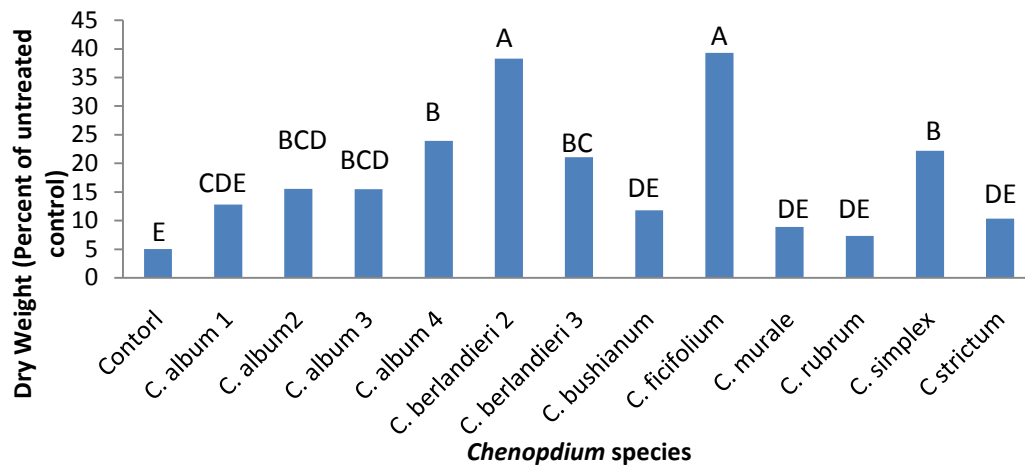
When we compared accessions of *C. album* and *C. berlandieri* to *C. ficifolium*, which is another weedy species in the *Chenopodium* complex, the reduction in dry weight in *C. ficifolium* was only 50 percent as compared to untreated control, indicating that there is more tolerance to glyphosate than other species with similar morphology (i.e. *C. album*, *C. berlandieri*).

Comparing other weedy species such as *C. strictum*, *C. murale*, *C. rubrum* and *C. simplex* to the susceptible control, reduction in dry weight indicates there was not a significant difference in tolerance to glyphosate among these species. When the plants of *C. album* 4, *C. berlandieri* 1, *C. ficifolium* and *C. simplex* were treated with 4X dose (3200 g a.e/ha) of glyphosate, some of the plants survived and were healthy enough to complete reproductive growth.

The data were also analyzed by excluding the highest dose (3200 g a.e ha<sup>-1</sup>) and averaging the results of 1X and 2X doses of glyphosate on various *Chenopodium* species (Fig 2.3). The highest dose was excluded because the dry weight expressed as percent control values at 4X dose were close to 0. So, by including only 800 (1X) and 1600 g a.e ha<sup>-1</sup> (2X), we obtained a better representation of variability in plants when they were treated with glyphosate at rates equivalent to field use rates. The results from Fig 2.3 indicated that *C. ficifolium* and *C. berlandieri* 2 were significantly more tolerant to glyphosate than other *Chenopodium* species and the susceptible biotype.

Variability in response to glyphosate was also observed among the species (Figure 2.2 and 2.3). Comparison between *C. album* 1, 2, 3 and 4 showed that *C. album* 4 was more tolerant to glyphosate than other *C. album* accessions. Similarly, among different *C. berlandieri* accessions, plants from *C. berlandieri* 2 showed more tolerance to glyphosate than *C. berlandieri* 3 or *C. bushianum*, which is often regarded as subspecies of *C. berlandieri*. This variability in response among different species is important because under herbicide selection, the species composition can change to more tolerant species in the ecosystem (McNeill, 1976).





**Figure 2.3.** Response of *Chenopodium* spp. averaged across 800 and 1600 g a.e. ha<sup>-1</sup> of glyphosate. Bars followed by same letter are not different according to Fischer's LSD test ( $p < 0.05$ ).

**Table 2.3.** Mean values for dry weight (percent of untreated control) of *Chenopodium* spp. and populations from North Central states averaged over 800, 1600 and 3200 g a.e. ha<sup>-1</sup> of glyphosate.

| Species                      | Mean Dry Weight<br>(Percent of untreated<br>control) | Populations of<br>common<br>lambsquarters | Mean Dry Weight<br>(% of untreated<br>control) |
|------------------------------|--|---|--|
| <i>C. album</i> 1 (38)       | 9.52 BCD   | IA20                                      | 19.56 CD                                       |
| <i>C. album</i> 2 (40)       | 11.89 BCD  | IA21                                      | 31.84 B  |
| <i>C. album</i> 3 (40)       | 11.95 BCD  | IA22                                      | 25.21 BC                                       |
| <i>C. album</i> 4 (39)       | 18.39 B  | IA23                                      | 29.02 B  |
| <i>C. berlandieri</i> 2 (40) | 30.85 A  | IA24                                      | 20.32 CD                                       |
| <i>C. berlandieri</i> 3 (39) | 15.19 BC   | IN11                                      | 14.97 DEFGHI                                   |
| <i>C. bushianum</i> (40)     | 9.80 CDE   | IN2                                       | 15.39 DEFGH                                    |
| <i>C. ficifolium</i> (40)    | 50.46 A  | IN3                                       | 7.77 JKL                                       |
| <i>C. murale</i> (40)        | 6.40 DE  | IN4                                       | 11.7 EFGHIJK                                   |
| <i>C. rubrum</i> 1 (40)      | 6.55 DE  | IN5                                       | 7.48 JKL                                       |
| <i>C. simplex</i> (40)       | 15.02 BC   | IN6                                       | 6.66 JKL                                       |
| <i>C. strictum</i> 1 (40)    | 9.25 CDE   | IN9                                       | 28.07 B  |
| <b>Control</b> (39)          | <b>3.82 DE</b>                                       | KS20                                      | 5.05 KL  |
|                              |  | KS21                                      | 32.0 B   |
|                              |  | KS22                                      | 9.12 GHIJKL                                    |
|                              |  | MO10                                      | 10.14 GHIJKL                                   |
|                              |  | MO6                                       | 10.44 FGHIJKL                                  |
|                              |  | ND14                                      | 9.47 GHIJKL                                    |
|                              |  | ND2                                       | 17.15 DEF                                      |
|                              |  | ND3                                       | 4.86 KL  |
|                              |  | ND7                                       | 4.51 L   |
|                              |  | ND9                                       | 52.52 A  |
|                              |  | IL1                                       | 4.464 L  |
|                              |  | IL2                                       | 9.52 GHIJKL                                    |
|                              |  | IL3                                       | 17.77 DE                                       |
|                              |  | IL4                                       | 8.49 IJKL                                      |
|                              |  | IL5                                       | 8.68 HIJKL                                     |
|                              |  | IL6                                       | 12.17 EFGHIJK                                  |
|                              |  | IL7                                       | 20.45 CD                                       |
|                              |  | IL8                                       | 15.74 DEFG                                     |
|                              |  | IL9                                       | 10.15 GHIJKL                                   |
|                              |  | IL10                                      | 6.5 JKL  |
|                              |  | <b>Control</b>                            | <b>4.75 L</b>                                  |
| <b>LSD (0.05)</b>            | <b>7.38</b>  |   | <b>6.88</b>                                    |

IA- Iowa                      KS-Kansas                      ND-North Dakota

IN-Indiana                      MO- Missouri                      IL- Illinois

<sup>a</sup> Number in parentheses refer to the number of plants screened with glyphosate

<sup>b</sup> Means followed by same letter are not different according to Fischer's LSD test (p<0.05)

**Table 2.4.** Mean values for dry weight (percent of untreated control) of populations from North Central states averaged over 800, 1600 and 3200 g a.e. ha<sup>-1</sup> of glyphosate.

| State              | Dry Weight (% of untreated control) |
|--------------------|-------------------------------------|
| Iowa               | 25.19 A                             |
| Kansas             | 19.54 B                             |
| North Dakota       | 18.38 BC                            |
| Indiana            | 14.86 CD                            |
| Illinois           | 11.49 DE                            |
| Missouri           | 10.29 E                             |
| <b>LSD ( 0.05)</b> | <b>4.34</b>                         |

<sup>a</sup> Means followed by same letter are not different according to Fischer's LSD test (p<0.05)

Our finding of variability in glyphosate response is not unique and several researchers have documented such patterns in *Amaranthus*, *Setaria* and *Chenopodium* spp. Differential response within species can be attributed to differences in plant height and herbicide rates (Coetzer et al. 2002), differences in resistance mechanism (Wang and Deker, 1995), competitive advantage as in the case of *C. album* when compared to *C. strictum*, phenotypic plasticity and spatial heterogeneity resulting in variability in adaptive traits such as herbicide resistance (Warwick and Black, 1981).

### **Variability Among Common Lambsquarters Populations from North Central States**

Variability in populations of common lambsquarters from the North Central states was studied and reflected in Table 2.3 and Fig 2.4. Without taxonomic study, we could not definitively classify them as *C. album*, so we referred to all the populations as common lambsquarters.

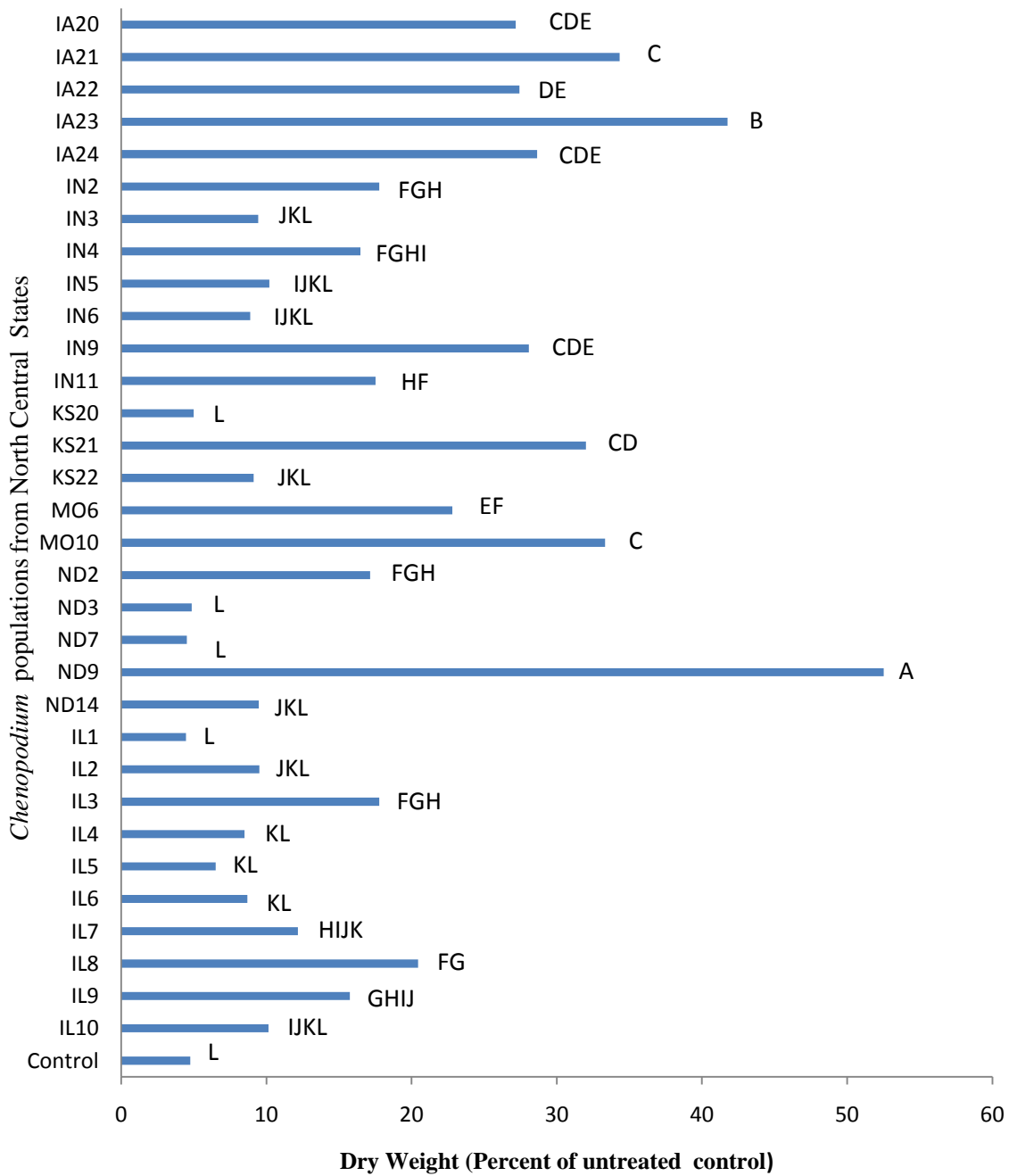
Thirty three populations were tested for glyphosate response using 4 doses (0, 800, 1600 and 3200 g a.e ha<sup>-1</sup>) of glyphosate. The samples screened included 5, 6, 3, 2, 5 and 10 populations from Iowa, Indiana, Kansas, North Dakota and Illinois fields, respectively.

The independent runs of the experiment were pooled and the combined analysis was done to evaluate the variability in responses of common lambsquarters populations to glyphosate across the North Central states (Table 2.3). The results from Table 2.3 showed that there was difference in responses within and among the *Chenopodium* populations across the North Central states. There was substantial variability within populations from different states. The average dry weight (percent of untreated control) ranged from 52.52 (ND9) to 4.46 (IL1). The populations from Iowa were, in general, more tolerant to glyphosate than populations from other states, however few populations from other states (Indiana, Illinois, North Dakota, Kansas) were also tolerant to glyphosate (Table 2.4).

When the results of 1X and 2X doses were averaged across the *Chenopodium* populations, the results showed that ND9 (Figure 2.4) had a significantly higher level of tolerance with less than 50 percent reduction in dry weight compared to untreated control, followed by IA23, IA21, IN9 and MO10, which also had considerably high tolerance levels. Previous reports from North Central states have also shown increase in tolerance to glyphosate in common lambsquarters populations (Westhoven et al. 2008a; Curran, 2005; Harder et al. 2007; Loux and Stachler, 2003; Kniss et al. 2006; Schuster et al. 2007). Some populations were not so different than the sensitive control, as indicated by the fact that there are populations where there was more than 90 percent reduction in dry weight of plants from Indiana (IN3, IN5, IN6), Kansas (KS20, KS22), North Dakota (ND3, ND7, ND14), and Illinois (IL1, IL2, IL4, IL5, IL6 and IL10).

Variability is also observed among populations within a state. For instance, most populations from North Dakota were sensitive to glyphosate with the exception of ND9. Similar results were also observed among populations from other states (Figure 2.4 and Table 2.2).

Previous research on populations of *Avena barbata*, *Avena fatua*, and *Clarkia williamsonii* also showed significant genetic variation in herbicide responses (Price et al. 1985). Such variability is not uncommon and has been reported previously in *Chenopodium* (Hite et al. 2008, Westhoven 2008b) as well as other important weed species (Werner and Putnam, 1980; Patzoldt et al. 2002). One reason for this variability might be repeated exposure of weeds to certain persistent herbicides over long periods of time (Westhoven et al. 2008b).



**Figure 2.4.** Dry weight (percent of untreated control) based on the average of mean values for two doses (800 and 1600 g a.e ha<sup>-1</sup>) of glyphosate. Bars followed by same letter are not different according to Fischer's LSD test (p<0.05).

## 2.5 Conclusions

The major finding from this experiment is that there is considerable variation in tolerance levels to glyphosate among and also within the different *Chenopodium* spp. This can be attributed to the fact that different species respond differently to herbicides, which can be mainly due to different levels of herbicide absorption, translocation or metabolism, and needs to be further tested. Our results indicate that if there are mixed populations of these species in a field, the control of these weeds will be difficult due to the differential tolerance of the species to herbicide. As glyphosate is the most commonly used herbicide for controlling weeds, it is possible that there can be a shift in weed species under selection pressure to more glyphosate tolerant *Chenopodium* species. As some populations and species even survived 4 times the recommended rate for glyphosate, our result raises the concern of tolerance in these *Chenopodium* species and populations to glyphosate. It also suggests that there is high potential for a decrease in glyphosate sensitivity within common lambsquarters populations to a point where they would not be controlled by a normal field use rate of glyphosate.

Our next step is to use DNA sequence data and classify the common lambsquarters populations from North Central states into different species and investigate if there are more than one *Chenopodium* species present in agricultural fields. Inter- and intra-specific variation in herbicide tolerance among weedy *Chenopodium* species can have important implications for the spread of herbicide resistant biotypes and genetic structure, and can help us in understanding the adaptations of weedy *Chenopodium* complex.

## 2.6 Sources of Materials

<sup>1</sup>LC1 Professional Growing Mix. Sun Gro Horticulture Canada Ltd.

<sup>2</sup> WEATHERMAX Roundup, Monsanto Company, St. Louis, MO.

<sup>3</sup> 80° flat fan nozzle, TeeJet, Spraying Systems Co., P.O. Box 7900 Wheaton, IL 60187.

<sup>4</sup>[SAS] Statistical Analysis Systems Institute, Inc., Cary, NC 27512-8000.

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**CHAPTER 3**  
**MULTI-FACETED APPROACH TO UNDERSTAND THE WEEDY**  
***CHENOPODIUM* COMPLEX**

**3.1 Abstract**

The *Chenopodium* genus is not a well-understood complex and taxonomy of the genus has been a point of controversy. In agricultural fields, most of the weedy *Chenopodium* species are referred to as common lambsquarters. Research was conducted to find informative markers that can help in species identification. Both morphological and DNA sequence data were explored. Among the morphological characters, the seed pericarp, calyx shape (keeled or not), and DNA content of the species proved to be informative characters. DNA content (2C) values proved to be informative in differentiating *C. album* (3.6 pg), *C. berlandieri* (2.7 pg), *C. strictum* (2.0 pg) and *C. ficifolium* (1.9 pg). Sequence data from 3 loci, ITS (nuclear), *matK* and *trnD-trnT* (chloroplast) were explored. DNA sequences from all three regions gave similar tree topologies, but ITS sequences had more polymorphic sites. A well supported ITS phylogenetic tree resolved the *Chenopodium* species into four main clusters. The *C. album* group was separated from the *C. berlandieri* with good bootstrap support from parsimony analysis and high posterior probabilities from Bayesian analysis. The chloroplast sequences also showed similar results. Closely related species such as *C. strictum*, *C. berlandieri* and *C. album* share highly similar sequences among homologous genes, but they can be differentiated based on genome size. ITS region of *Chenopodium* samples from agricultural fields in the North Central states were amplified, and the phylogenetic tree showed that all the field samples clustered with *Chenopodium album*. Our

conclusion is that only a multi-faceted approach using ITS sequence, cytogenetic, morphological data can differentiate the *Chenopodium* species.

### 3.2 Introduction

The genus *Chenopodium* L. consists of about 250 species (Giusti, 1970; Kadereit et al. 2005) and belongs to family Amaranthaceae, syn. Chenopodiaceae (APG II, 2003). The genus includes mostly annual and perennial herbs, and is distributed throughout Asia, America and Europe (Ruas et al. 1999). With the exception of *C. quinoa* and *C. berlandieri* subsp. *nuttalliae*, most of the *Chenopodium* spp. are considered annual weeds (Cole, 1961) that can compete with economically important crops and thereby affect yield.

The designation *Chenopodium album* has often functioned as a convenient taxonomic receptacle, loosely circumscribed to include material not readily assigned to other species of this difficult genus (Wilson, 1980). Species of *Chenopodium* are not easily defined because of lack of distinctive macroscopic morphological characters in the genus (Bassett and Crompton, 1982). The taxonomy of the genus *Chenopodium* has been a major point of controversy arising mainly due to phenotypic plasticity (Kurashige and Agarwal, 2005; Zhou et al. 2005), parallel evolution (Duke and Crawford, 1979), hybridization (Cole 1961; Rahiminejad and Gornall, 2004) and cytogenetic diversity as some species of *Chenopodium* can be found as diploid, tetraploid and hexaploid (Allen 1929). While studying the *Chenopodium* genus Wahl (1954) wrote that “No group of comparable size and wide distribution known to the writer has suffered the lack of understanding of the taxa involved as has the genus *Chenopodium*, especially those members of its Section *Chenopodium* that are closely related to *C. album* and *C. berlandieri*.”

The taxonomic classification of *Chenopodium* species has always proved to be very difficult and many researchers have used one or more morphological characters in combination to clear the confusion among the taxa. For instance, Allen and Just (1943) and Wahl (1954) made subsectional distinctions based on inflorescence and pericarp characters. Similarly, Cole (1961) subdivided *Chenopodium* into 4 subsections based on seed coat markings. Iljin (1936) also differentiated taxa based on pericarp structures and keeling of the calyx lobes. Some researchers have also used leaf color as an identifying character (Moquin-Tandon, 1840). Bassett and Crompton (1982) worked on clarification of taxonomic circumscriptions, nomenclature and distribution of taxa in *Chenopodium* across Canada; mainly based on the seed characteristics and information related to the chromosome count. Crawford and Reynolds (1974) studied narrow leaved *Chenopodium* species, including *C. atrovirens* Rybd., *C. desiccatum* A. Nelson var. *desiccatum*, *C. desiccatum* A. Nelson var *leptophylloides* (Murr.) Wahl, *C. leptophyllum* Nutt. Ex Moq., and *C. subglabrum* (S. Wats) A. Nelson, using numerical techniques in which several seed, leaf, and inflorescence characters were studied. Crawford and Reynolds (1974) were successful in elucidating the relationship of closely related *Chenopodium* species and found certain affinities that were contrary to the literature of that time. Such studies have been successfully conducted in other plant groups to gain insight into variation patterns within the plant species (Crovello, 1968; Gilmartin, 1969; Ornduff and Crevello, 1968; Heiser et al. 1965).

The use of DNA sequence data has proved to be a powerful approach to investigate relationships within morphologically complex groups like Ericaceae (Kron et al 2002) and Asteraceae (Kim and Jansen, 1995). Such studies have also been done in *Chenopodium* using random amplified polymorphic DNA (RAPD) (Anderson, 1999; Gangopadhay et al. 2002), direct amplification of minisatellite DNA (DAMDA) (Rana et al. 2010), microsatellite markers



(Mason et al. 2005), ribosomal DNA (Maughan et al. 2006; Soliai et al. 2009) and plastid genes (Downie et al. 1997; Soliai et al. 2009).

There are about 20 species of *Chenopodium* that are present in Illinois (USDA, NRCS. 2008), and some of them (*C. album*, *C. berlandieri*, *C. strictum*, *C. murale* and *C. hybridum*) are recognized as agricultural weeds. Some of these weeds are resistant to one or more class of herbicides (Heap, 2010). Much of the research on the genetic diversity and phylogeny within *Chenopodium* mainly emphasized domesticated species like *C. quinoa* and *C. berlandieri*. There are very few studies which include the important weed species like *C. album*, *C. berlandieri*, *C. strictum*, *C. ficifolium*, *C. murale* and *C. glaucum*, and there clearly is a lack of attempts at understanding among these species using both morphological and molecular data.

The aim of this study was to: 1) study morphological characters in the *Chenopodium* complex in order to identify those that can be used to differentiate the species; 2) develop markers based on DNA sequences data which can aid in species identification, and can be used as additional tools to morphological data in understanding the *Chenopodium* complex; and 3) determine which *Chenopodium* species predominate across the North Central states using DNA sequences.

This study was not done to revise the phylogeny of the *Chenopodium* genus, but to increase the precision of classification in the *Chenopodium* genus with a multi-faceted approach.

### **3.3 Material and Methods**

#### **Plant Materials**

The *Chenopodium* species for this study were selected based on the prevalence of the species in North Central states. There are about 20 species of *Chenopodium* that are found

commonly in the North Central US, and depending on seed availability, 28 accessions representing 12 species were selected (Table 3.1). Five additional ITS sequences of *C. album* were downloaded from the National Center for Biotechnology Information (NCBI) for sequence comparison. Most seed for different *Chenopodium* species were provided by North Central Regional Plant Introduction Station (NCRPIS) and some by other researchers. All the *Chenopodium* samples were sequenced for ITS region, but only a representative subset of *Chenopodium* species were sequenced for chloroplast markers. Information related to the accessions used and loci examined is provided in Table 3.1.

A subset of *Chenopodium* samples from North Central states that were earlier screened with glyphosate (Chapter 2), were also sequenced to amplify the ITS region (details for choosing this marker is discussed later in the chapter). As stated in the objectives, the aim of this experiment was to use molecular marker(s) to determine if the samples collected from North Central states were genetically more close to *C. album* or other weedy *Chenopodium* species.

Seeds from all the populations that were used were stratified and then sown in a greenhouse. At maturity, seeds were harvested from single parents and again stratified and sown in the greenhouse for morphological, cytogenetic and molecular data analyses.

### **Morphological Analysis**

Based on the literature available, we selected all the characters that were used by researchers to identify the *Chenopodium* species. The qualitative characters studied included plant height, seed length and width, stem diameter and length to width ratio of primary, secondary and tertiary leaves (description given by Utolia, 1978). The mean values were

calculated for each character and compared using the LSD values obtained by the PROC GLM procedure in SAS 9.2 (Table 3.2).

**Table 3.1.** List of accessions used for molecular analysis. Loci examined indicate the species that have been tested with corresponding marker.

| S. No | Genus  | PI         | Origin  | Loci examined |      |           |
|-------|--|------------|---------|---------------|------|-----------|
| 1     | <i>C. album 1</i>                                | Ames 23855 | Poland  | ITS           | matK | trnD-trnT |
| 2     | <i>C. album 2</i>                                | PI 605700  | MI, USA | ITS           | matK | trnD-trnT |
| 3     | <i>C. album 3</i>                                | PI 262168  | France  | ITS           | matK | trnD-trnT |
| 4     | <i>C. album 4</i>                                | PI 605701  | CO, USA | ITS           | -    | -         |
| 5     | <i>C. album 5</i>                                | ?          | Germany | ITS           | -    | -         |
| 6     | <i>C. album 6</i>                                | FN561545   | NCBI    | -             | -    | -         |
| 7     | <i>C. album 7</i>                                | FN561549   | NCBI    | -             | -    | -         |
| 8     | <i>C. album 8</i>                                | FN561547   | NCBI    | -             | -    | -         |
| 9     | <i>C. album 9</i>                                | FN561552   | NCBI    | -             | -    | -         |
| 10    | <i>C. album 10</i>                               | FN561550   | NCBI    | -             | -    | -         |
| 11    | <i>C. bushianum</i>                              | PI 608030  | IL, USA | ITS           | matK | trnD-trnT |
| 12    | <i>C. berlandieri 1</i>                          | PI 595315  | IA, USA | ITS           | matK | trnD-trnT |
| 13    | <i>C. berlandieri 2</i>                          | PI 595316  | IA, USA | ITS           | matK | trnD-trnT |
| 14    | <i>C. berlandieri 3</i>                          | PI 612858  | UT, USA | ITS           | matK | trnD-trnT |
| 15    | <i>C. berlandieri</i><br><i>var. zschackei 4</i> | ?          | IN, USA | ITS           | -    | -         |
| 16    | <i>C. berlandieri</i><br><i>macrocalycium 5</i>  | BYU-803    | ME, USA | ITS           | -    | -         |
| 17    | <i>C. berlandieri</i><br><i>sinuatum 6</i>       | BYU 865    | AZ, USA | ITS           | -    | -         |

**Table 3.1 Cont'd**

|    |                           |            |             |     |      |           |
|----|---------------------------|------------|-------------|-----|------|-----------|
| 18 | <i>C. capitatum 1</i>     | NSL 90209  | CO, USA     | ITS | matK | trnD-trnT |
| 19 | <i>C. capitatum 2</i>     | PI 658745  | MI, USA     | ITS |      |           |
| 20 | <i>C. bonus-henricus</i>  | Ames 23109 | Germany     | ITS | matK | trnD-trnT |
| 21 | <i>C. ficifolium</i>      | Ames 25246 | Switzerland | ITS | matK | trnD-trnT |
| 22 | <i>C. glaucum</i>         | PI 612859  | IA, USA     | ITS | matK | trnD-trnT |
| 23 | <i>C. glaucum salinum</i> | BYU-534    | UT, USA     | ITS | -    | -         |
| 24 | <i>C. murale</i>          | PI 614895  | Portugal    | ITS | matK | trnD-trnT |
| 25 | <i>C. murale</i>          | Ames 26140 | CA, USA     | ITS | -    | -         |
| 26 | <i>C. quinoa 1</i>        | PI 614880  | Chile       | ITS | -    | -         |
| 27 | <i>C. quinoa 2</i>        | PI 614881  | Argentina   | ITS |      |           |
| 28 | <i>C. rubrum1</i>         | Ames 23860 | Poland      | ITS | matK | trnD-trnT |
| 29 | <i>C. rubrum2</i>         | BYU 665    | UT, USA     | ITS | -    | -         |
| 30 | <i>C. simplex</i>         | Ames 21981 | MI, USA     | ITS | matK | trnD-trnT |
| 31 | <i>C. strictum 1</i>      | Ames 23893 | Germany     | ITS | matK | trnD-trnT |
| 32 | <i>C. strictum 2</i>      | BYU-436    | CA, USA     | ITS | -    | -         |
| 33 | <i>C. vulvaria</i>        | PI614896   | Portugal    | ITS | matK | trnD-trnT |

- Not tested, (\*) used from gene bank (NCBI) for phylogenetic analysis

? - accession no. not provided

Apart from the quantitative characters, some important morphological characters were also studied. Several researchers have mainly classified the species based primarily on seed characters, inflorescence studies and vegetative characters like leaf shape and pubescence. In this

study, we evaluated plants for growth habit of stem, branching pattern, base branching presence, pubescence of the leaves, perianth structure, fruit covering at maturity, and seed pericarp.

Analysis of the morphological data was conducted using Mesquite v 2.72 (Madison and Madison 2009). Morphological data were converted into a similarity matrix and the nexus file generated from Mesquite was used to derive maximum parsimony tree and presented as phylogram. The characters used and their codes are provided below:

1) Growth habit of main stem:

1- erect; 2- prostrate; 3: ascending

2) Perianth Keeled or not

1-not keeled; 2- slightly keeled; 3-moderately keeled; 4-sharply keeled

3) Branching

0-Simple; 1-less branched; 2-medium branched; 3-highly branched

4) Fruits covering at maturity:

1-exposed; 2- slightly exposed; 3-completely covered

5) Branching at the base of plant

1-present; 2-absent

6) Seed Pericarp:

1-Smooth to reticulate; 2-honeycomb pitted

7) Farinose (adaxial surface of leaf)

1-medium to highly farinose; 2-low to medium; 3- slightly to glabrous

8) Farinose (abaxial surface of leaf)

**Table 3.2.** Binary codes for selected morphological characters.

| Species                | Stem | Branching | Base<br>branching | Keeled | Fruit at<br>maturity | Pericarp | Farinose<br>Adaxial | Farinose<br>Abaxial |
|------------------------|------|-----------|-------------------|--------|----------------------|----------|---------------------|---------------------|
| <i>C album</i> 1       | 1    | 3         | 2                 | 4      | 3                    | 1        | 3                   | 2                   |
| <i>C album</i> 2       | 1    | 0         | 2                 | 3      | 1                    | 1        | 3                   | 1                   |
| <i>C album</i> 3       | 1    | 1         | 2                 | 3      | 1                    | 1        | 3                   | 1                   |
| <i>C album</i> 4       | 1    | 3         | 2                 | 4      | 3                    | 1        | 3                   | 2                   |
| <i>C berlandieri</i> 1 | 1    | 0         | 2                 | 3      | 3                    | 1        | 1                   | 3                   |
| <i>C berlandieri</i> 2 | 1    | 0         | 2                 | 3      | 1                    | 2        | 3                   | 2                   |
| <i>C berlandieri</i> 3 | 1    | 1         | 2                 | 3      | 1                    | 2        | 3                   | 2                   |
| <i>C berlandieri</i> 5 | 1    | 2         | 2                 | 2      | 1                    | 2        | 2                   | 1                   |
| <i>C berlandieri</i> 6 | 1    | 0         | 2                 | 2      | 1                    | 2        | 3                   | 2                   |
| <i>C bushianum</i>     | 1    | 0         | 2                 | 2      | 1                    | 1        | 3                   | 2                   |
| <i>C ficifolium</i>    | 1    | 0         | 2                 | 2      | 3                    | 2        | 3                   | 2                   |
| <i>C glaucum</i> 2     | 2    | 3         | 1                 | 1      | 3                    | 1        | 3                   | 1                   |
| <i>C murale</i> 1      | 1    | 2         | 2                 | 1      | 3                    | 1        | 3                   | 3                   |
| <i>C rubrum</i> 1      | 1    | 3         | 2                 | 1      | 3                    | 1        | 3                   | 3                   |
| <i>C strictum</i> 1    | 1    | 2         | 2                 | 3      | 1                    | 1        | 3                   | 3                   |
| <i>C strictum</i> 2    | 1    | 2         | 2                 | 3      | 1                    | 1        | 3                   | 2                   |
| <i>C vulvaria</i>      | 3    | 3         | 1                 | 1      | 3                    | 1        | 2                   | 1                   |

## DNA Content Analysis

*Chenopodium* samples were prepared for flow cytometric analysis using the protocols of Rayburn et al. (1989). Briefly, leaf tissues ( $\sim 1 \text{ cm}^2$  of each) from *Chenopodium* species and maize inbred line W22 (Biridar and Rayburn, 1993), used as an internal control, were co-chopped and placed in a small (15 mL) beaker containing 10 mL extraction buffer (13% (v/v) hexylene glycol, 10 mmol Tris-HCl  $\text{L}^{-1}$  (pH 8.0), and 10 mmol  $\text{MgCl}_2 \text{L}^{-1}$ ) and 200  $\mu\text{L}$  25% Triton®-X . The nuclear DNA content of maize used as internal control was 5.35 pg/2C. The tissue was homogenized using a tissue grinder for 25-30 s at  $4500 \times g$ , and samples were filtered through 250- and 53- $\mu\text{m}$  nylon meshes into a labeled test tube kept on ice. The samples were then centrifuged for 15 min at  $11000 \times g$  at  $4^\circ\text{C}$ . The supernatant was removed, and nuclei were re-suspended in 300  $\mu\text{L}$  of propidium iodide stain (Bashir et al. 1995). The solution was then transferred to a 1.5 mL microcentrifuge tube and incubated for 20 min at  $37^\circ\text{C}$ . Following incubation, 300  $\mu\text{L}$  of propidium iodide salt was added to each sample. Samples were then lightly vortexed and stored at  $4^\circ\text{C}$  for 2 hours.

For each sample, 10000 nuclei were analysed using a laser Flow Cytometer-Cell Sorter Epics XL-MCL (Coulter Electronics, Hialeah, Florida, US). Mean fluorescence of sample G1 peak was divided by the fluorescence reading of the internal control, multiplied by 5.35 pg/2C, and expressed in pg/2C nucleus. For each genotype, 2 samples (1 leaf per replication) were analyzed. Readings on nuclear DNA content were used for GLM analysis, and LSD tests were conducted using SAS v 9.2<sup>1</sup>.

## **Scanning Electron Microscope Analysis**

Several researchers found that fruit and seed characters are useful in the identification and classification of plant taxa, and in establishing phylogenetic and evolutionary relationships among taxa. Mature seeds from *Chenopodium* plants were used for examining the pericarp using Scanning electron microscopy (SEM). Briefly, the seeds were mounted onto a metal plate and were then sputter coated in Denton Desk II TSC turbo-pump. The samples were then viewed under Field-Emission Environmental Scanning Electron Microscope (ESEM-FEG) with Energy-Dispersive Spectroscopy (EDS) at the Beckman Institute<sup>2</sup> for Advanced Science and Technology, University of Illinois. The pictures were taken at 120X and the exposure was manually adjusted.

## **Molecular Analysis**

### **DNA Extraction, Amplification and Sequencing**

Total genomic DNA was extracted following the procedure of Doyle and Doyle (1987) with slight modifications. Briefly, 100 mg of fresh tissue was placed individually in 1.5 ml sterile microcentrifuge tubes. The tubes were then dipped in liquid nitrogen and samples were finely grounded. Then 600  $\mu$ L of CTAB extraction buffer was added and the tubes were incubated at 60°C for 30 min, during which the tubes were inverted several times to mix the contents. The samples were then extracted with 500  $\mu$ L of chloroform by vigorously inverting the tubes for 2 mins. The samples were centrifuged for 5 min at 10000 x g in a micro-centrifuge. The upper phase was transferred to a fresh tube. DNA was precipitated with 400  $\mu$ L of absolute ethanol and the DNA was allowed to precipitate at -20°C for 30 min. The precipitated DNA was collected by centrifugation at > 12000 x g for 10 min. The ethanol was decanted and DNA was washed with



250  $\mu\text{L}$  of 70 % ethanol, followed by another wash with 250  $\mu\text{L}$  90 % ethanol and then samples were centrifuged. The ethanol was decanted and DNA pellets were dried in a vacuum evaporator centrifuge for 1 to 2 mins. Finally, the DNA pellet was dissolved in 50  $\mu\text{L}$  of TE buffer for use in polymerase chain reaction amplification.

## **Loci Examined**

### **Nuclear Internal Transcribed Spacer (ITS) Region**

The entire ITS region (ITS1, 5.8S, and ITS2) was amplified using the ITS 5 and ITS 4 primers (Table 3.5). These are universal primers and the ITS region was amplified following protocol of Wetzel (1999) with slight modifications. The following were combined to a final PCR volume of 25  $\mu\text{L}$ : 1  $\mu\text{L}$  of approximately 100 ng DNA, 2 units of Taq polymerase (Promega<sup>3</sup>), 1X reaction buffer (Bioline), 2.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTPs, and 0.2  $\mu\text{M}$  of forward and reverse primer. Reactions were incubated for 2 min at 95° C, followed by 36 cycles of 1 min at 95° C, 1 min at 50°C, 2 min at 72°C, followed by final extension at 72°C for 5 min.

Five micro liters of each double-stranded DNA PCR product was resolved by electrophoresis in a 1 % agarose gel using 1X TBE as the gel buffer. Agarose gels were stained with 0.5  $\mu\text{g ml}^{-1}$  ethidium bromide and visualized under ultraviolet light to observe the DNA fragments. Subsets of amplified fragments were selected for DNA sequencing. Fragments were isolated from the gel using QIAGEN<sup>4</sup> gel purification kit following the manufacturer's instructions. The purified DNA product was then sequenced using AB BigDye Terminator v3.1 Cycle Sequencing Kit<sup>5</sup> and the sequence data were analyzed using Sequencher 4.7 software<sup>6</sup>. Sequencing reactions consisted of 0.25  $\mu\text{L}$  of primer, 4  $\mu\text{L}$  of purified PCR product and 4  $\mu\text{L}$  of ddH<sub>2</sub>O. Cycle sequencing conditions were 96°C for 1 min of denaturing, followed by 30 cycles

of 15 sec at 95°C, 5 sec at 45°C and 4 min at 60°C. Sequencing was carried out at the W. M. Keck Center<sup>7</sup> for Comparative and Functional Genomics at the University of Illinois.

In *C. album* 3, polymorphic sequences were observed at single loci, which indicated the possibility of heterogeneous ITS repeat, but as they were localized to one nucleotide position with double peaks, this position was not included in sequence analysis.

### **Chloroplast DNA**

The chloroplast genome is smaller than the nuclear genome and is found in large numbers which makes it easy to isolate. Apart from the nuclear markers, chloroplast markers are also among the most commonly used markers for phylogenetic studies in plants. The chloroplast DNA is useful because it is structurally stable, non recombinant, mostly uniparentally inherited and highly conserved in gene content in closely related species (Downie and Palmer 1991). Nuclear substitution rates are slower than those of the nuclear genome, making it a good marker for high level taxonomic studies.

**Table: 3.3.** List of primers used for amplifying nuclear and chloroplast loci.

| Loci                   | Primer | Primer sequence (5'-3') | Annealing temp (°C) |
|------------------------|--------|-------------------------|---------------------|
| <b>NUCLEAR</b>         |        |                         |                     |
| ITS                    | ITS5   | GGAAGTAAAAGTCGTAACAAGG  | 50                  |
|                        | ITS4   | TCCTCCGCTTATTGATATGC    |                     |
| <b>CHLOROPLAST</b>     |        |                         |                     |
| matK<br>(partial gene) | 390F   | CGATCTATTCATTCAATATTTC  | 48                  |
|                        | 1326R  | TCTAGCACACGAAAGTCGAAGT  |                     |
| trnD-trnT              | trnD   | ACCAATTGAACTACAATCCC    | 53                  |
|                        | trnT   | CTACCACTGAGTTAAAAGGG    |                     |

The chloroplast regions *matK* and *trnD-trnT* were PCR amplified using the primers listed in Table 3.3. Due to difficulty in amplifying complete regions because of background noise, only partial sequences were used. Both the chloroplast regions were amplified in 25 µL reaction with the same concentrations of reagents as used for amplifying ITS regions. PCR protocol included initial denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 30 sec, annealing at 52°C for 45 sec and extension at 72°C for 2 min followed by final extension of 72°C for 5 min.

### Outgroup Selection

The choice of *Amaranthus* for outgroup was based on several factors. The molecular systematic studies of the Caryophyllales in which more than two taxa were studied from Amaranthaceae and Chenopodiaceae, and several researchers such as Giannasi et al. (1992), Rettig et al. (1992), Downie and Palmer (1994), all treated Amaranthaceae and Chenopodiaceae

as sister families. In similar studies by Rodman (1990), he concluded that the two families are sisters to each other. Based on such facts, Judd and Ferguson (1999) also suggested the inclusion of Chenopodiaceae into Amaranthaceae in the APGII system of classification. In a recent study on *Chenopodium*, Rana et al. (2010) have also used *Amaranthus* species as outgroup to the *Chenopodium* species.

### **Assessing Homology**

Hypothesis of homology is the basis of the phylogenetic analysis (Phillips, 2006). The term homology was first introduced by Owen (1843) to express similarities in basic structure found between organs of animals that he considered to be more fundamentally similar than others. In other words, homology is the similarity that is the result of inheritance from a common ancestor. Homology can be difficult to identify; this may be attributed in part to the presence of large number of character states and rates of change sufficient to yield independent expressions of the same state. We assigned the putative homology to molecular data using multiple sequence alignments. The best alignment in context with phylogeny is the one that generates the most parsimonious tree when analyzed in conjunction with all relevant data. We therefore were very careful when aligning the sequences and checked visually for shifts in sequences and manually edited the sequences if required.

### **Sequence Alignment**

Sequence alignment is the first step in identification of homologous sites and phylogenetic reconstruction. It is the starting point of any kind of analysis that involves the comparison of molecular data (Mullan, 2002). When we align ITS sequences, it is very common

to have indels due to alignment. This makes aligning of unrelated species difficult and leads to incorrect homology assessment.

The most common multiple sequence alignment is CLUSTAL W (Thompson et al. 1994). When sequences are aligned using CLUSTAL W, first the sequences are compared to each other (pairwise alignment), then a dendrogram is constructed which describes the approximate groupings of the sequences by similarity and finally the multiple alignment is carried out using the dendrogram as a guide. When the numbers of sequences are small, then CLUSTAL or any other method of sequence alignment like Multiple sequence comparison by log-expectation (MUSLCE) (Edgar 2004), MAFFT (Katoh and Toh 2008) and T-COFFEE (Notredame et al. 2000) all give similar results (Edgar and Batzoglou 2006). However, in some cases, MUSCLE proved to be slightly better in assessing homology. MUSCLE (Edgar 2004) proceeds in three stages - draft progressive using kmer counting, improved progressive using a revised guide-tree from the previous iteration, and refinement by sequential deletion of each tree and new multiple alignment is produced by realignment of the two profiles (Edgar and Batzoglou 2006).

For this study, the forward and reverse sequences were examined in Sequencher v4.7 and assembled into contiguous sequences. The sequences from all the taxa were then aligned using MUSCLE v 3.6 and Clustal W. The aligned sequences were opened in MEGA v3.1 (Tamura et al. 2007). Alignment of contigs was done by inserting gaps if required to minimize nucleotide mismatches. The taxa were aligned to already published sequences of *Chenopodium* (Rana et al. 2010) deposited in GenBank. The length of the ITS region was 643 bp and included gaps in ITS1 and ITS2, but the 5.8S region was well conserved. Chloroplast DNA regions were aligned in

ClustalW and the sequences were compared to *Amaranthus* sequences. A few ambiguously aligned nucleotides in the 5' or at the 3' end were excluded from the analysis.

### **Treatment of the Gaps**

Multiple sequence alignment converts the unequal length sequences to equal length sequences by introduction of gaps that represent the insertion or deletion (indel) event. The position of indels in molecular data can be useful phylogenetic information (Simmons and Orchoferena, 2000; Egan and Crandall, 2008). The pitfall of using gap characters is that they are the product of sequence alignment, and contrary to nucleotides, gaps are not actually present in the organism and they do not have anything to compare with other sequences at the loci where gap occurs (Simmons et al. 2001). Gaps can be treated in different ways. Simmons and Orchoferena (2000) provided a detailed discussion of how to treat gaps. In general, there are three common ways of treating gaps: 1) gap positions are excluded from the analysis altogether and the advantage of excluding gaps is that the missing data are eliminated; 2) treat gaps as 5<sup>th</sup> character base in the DNA based alignment (Eernisse and Kluge. 1993); 3) simple indel coding in which gaps are coded as presence or absence of characters (Simmons and Orchoferena, 2000). The advantages and the concerns of using the above three methods for treating gaps has been reviewed by several researchers (Simmons and Orchoferena, 2000; Muller 2006; Ogden and Rosenberg 2007; Dwivedi and Gadagkar, 2009). In our analysis, gaps were treated as absence or presence of characters. The gaps were binary coded as 0 for absence and 1 for presence of character with the help of Gapcoder (Young and Healy, 2003)

## **Phylogenetic Analysis**

The multiple sequence alignments were subjected to phylogenetic analyses, including maximum parsimony (MP) and model-based Bayesian Inference methods. For all the regions investigated, *Amaranthus* species were used as the outgroup, and trees were rooted to them. Review on the methods for phylogenetic analyses is provided by Swafford et al. (1996) and more recently by Holder and Lewis (2003), who discussed the advantages and limitations of various estimation methods.

### **Maximum Parsimony**

MP analyses were conducted using PAUP v4.0b (Swofford 2003). Characters were treated as unordered and equally weighted. Heuristic searches were carried out with MulTree ON, tree-bisection-reconnection branch swapping, starting tree obtained via random stepwise addition with 10 replications. Clade support was assessed using bootstrap estimation (Felsenstein 1985). One thousand bootstrap replicates were analyzed in PAUP and only those values compatible with the strict consensus tree were recorded.

### **Bayesian Analysis**

Bayesian analysis has a computational advantage over maximum likelihood (ML) approach. The Bayesian analysis was performed using MrBayes v 3.1.2 (Ronquist and Huelsenbeck, 2003). Starting trees were chosen at random and 2 million generations were run with sampling occurring every 100 generations. For ITS and trnD-trnT, the best model was general time reversible with invariable gamma (GTR +I+J) model, and for *matK* and combined analysis of DNA data, the model selected was TMI+I+J. The models were selected based on the

AIC criteria using the Modeltest v 3.7 (Pasoda and Crandal 1998). The “burn in” was determined and those trees prior to stationarity (2000-2500) were discarded before a 50% majority-rule consensus tree was calculated from the remaining trees along with the posterior probability values.

### **3.4 Results and Discussion**

#### **Importance of Morphological Characters in Identifying the *Chenopodium* Species**

For any study that deals with the identification or classification of plants, the first and foremost step is to use morphological characters as a tool for classification. Key morphological characters can be compared among plants to determine the differences or similarities in plant taxa. These key characters can be either quantitative characters that can be counted or measured or qualitative characters such as flower color, leaf shapes, pubescence or seed color.

In the present study, both quantitative and qualitative characters were measured. The key characters were identified based on the previously published monographs of *Chenopodium* species (Moyaskin and Clements, 1996). Quantitative characters studied are presented in Table 3.4. It shows the mean values of measurements from 10 plants for studied characters of each *Chenopodium* accessions. The results indicate that there is variability within and among the *Chenopodium* species. For instance, when we studied plant height, the plants ranged from 52.6 cm (*C. album* 1) to 141.4 cm (*C. album* 4). Similar trends were also observed in accessions of *C. berlandieri* (94-119.6 cm). *Chenopodium album* and *C. berlandieri* species were significantly taller than other *Chenopodium* species investigated. Stem diameter values overlapped within the *Chenopodium* species and no grouping was observed in the species.



The leaf length/width approximates the shape of the leaves. Measurements were taken from 10 plants and from each plant, three stems (near base, middle and terminal area of main stem) were selected. From each stem, primary, secondary and tertiary leaves were measured for length and width and we present the data as mean values for ratio of length to width of the leaves. The primary leaf was the oldest leaf, secondary is the middle leaf, and the youngest leaf was the tertiary leaf. This measuring scheme was used to capture the maximum variability in the leaf size. The results indicated that for primary leaves, the species that have length/width ratios greater than 2, such as *C. ficifolium* and *C. glaucum*, were different from the other *Chenopodium* species. The differences were non-significant between the accessions of *C. album* and *C. berlandieri*. For secondary and tertiary leaves, there was not a clear distinction and these cannot be confidently used as important characters. Another character studied was the seed length and width. The results indicated that length and width of seeds of *C. berlandieri* accessions were significantly different than accessions of *C. album*, with an exception of *C. berlandieri* 1 and *C. bushianum*.

The results indicated that although some characters are important in differentiating some species from each other, due to overlap in values, the same character cannot be used to differentiate the other species. Among the characters measured, seed characters were the most important and they have been studied extensively in various phylogenetic studies (Bassett and Crompton, 1982). One interesting finding from this experiment was that when we measured seed length and width, the *C. berlandieri* 1 and *C. bushianum* seeds had values that were more similar to the *C. album* group and were significantly different than other accessions of *C. berlandieri*.

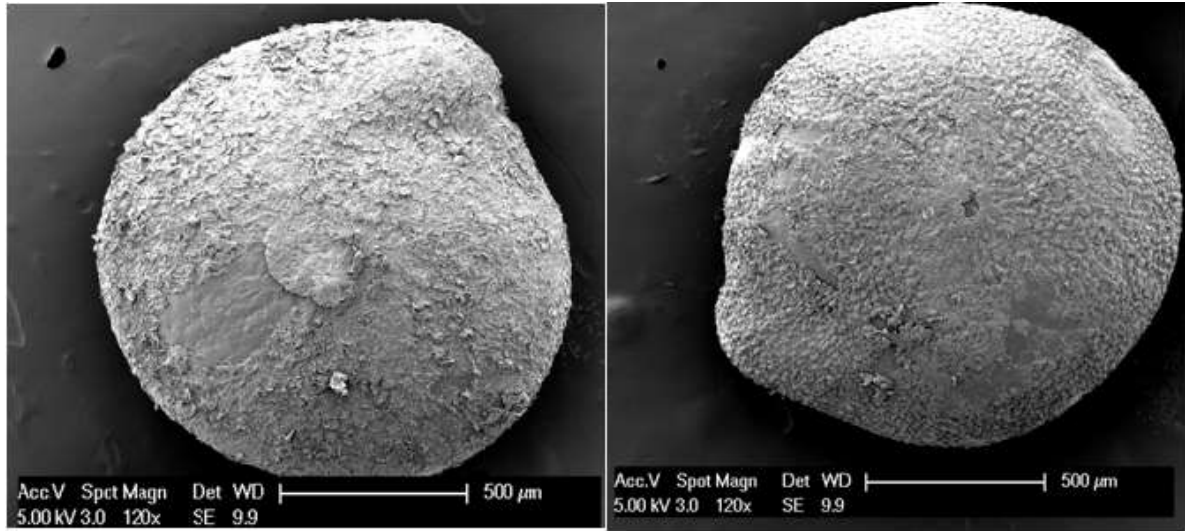
**Table 3.4.** Mean values of quantitative characters studied in differentiating *Chenopodium* spp.

| Species                                 | Plant Height (cm) | Stem Diameter (mm) | Leaf (Length/Width) 1° Leaf | Leaf (length/Width) 2° Leaf | Leaf (Length/Width) 3° Leaf | Seed Length (mm) | Seed Width (mm) |
|---|-------------------|--------------------|-----------------------------|-----------------------------|-----------------------------|------------------|-----------------|
| <i>C. album 1</i>                       | 52.65 H           | 3.66 E             | 1.50 CD                     | 1.68 DE                     | 1.89 CDE                    | 1.38 C           | 1.28 CD         |
| <i>C. album 2</i>                       | 116.51BC          | 4.66 AB            | 1.08 F                      | 1.69 DE                     | 1.67 E                      | 1.28 D           | 1.17 E          |
| <i>C. album 3</i>                       | 141.47 A          | 4.46 AB            | 1.29 DEF                    | 1.71 DE                     | 2.42 BC                     | 1.27 D           | 1.21 DE         |
| <i>C. album 4</i>                       | 60.79 G           | 3.69 E             | 1.47 CDE                    | 1.87 CDE                    | 2.34 BCD                    | 1.40 C           | 1.27 CD         |
| <i>C berlandieri 1</i>                  | 95.59 E           | 4.28 CD            | 1.11 F                      | 1.70 DE                     | 1.99 CDE                    | 1.18 E           | 1.08 F          |
| <i>C berlandieri 2</i>                  | 112.05CD          | 4.02 D             | 1.23 EF                     | 1.75 DE                     | 1.95 CDE                    | 1.49 B           | 1.41 B          |
| <i>C berlandieri 5</i>                  | 94.46 E           | 4.06 D             | 1.61 C                      | 1.84 CDE                    | 2.55 AB                     | 1.86 A           | 1.72 A          |
| <i>C berlandieri 6</i>                  | 119.61 B          | 4.47 BC            | 1.31 DEF                    | 1.79 DE                     | 2.34 BCD                    | 1.50 B           | 1.41 B          |
| <i>C. bushianum</i>                     | 107.79 D          | 4.80 A             | 1.39 DEF                    | 2.01 CD                     | 2.57 AB                     | 1.24 DE          | 1.19 E          |
| <i>C.ficifolium</i>                     | 85.17 F           | 4.47 BC            | 2.20B                       | 3.03 A                      | 3.08 A                      | 1.10 G           | 0.98 GH         |
| <i>C glaucum salinum</i>                | 31.32 J           | 3.70 E             | 2.75 A                      | 2.77 AB                     | 2.71 AB                     | 0.74 H           | 0.68 I          |
| <i>C murale 1</i>                       | 31.66 J           | 2.87 F             | 1.11 F                      | 1.37 E                      | 1.72 E                      | 1.39 C           | 1.32 C          |
| <i>C strictum 1</i>                     | 44.36 I           | 4.06 D             | 1.61 C                      | 1.67 DE                     | 1.85 DE                     | 1.10 F           | 1.01 G          |
| <i>C strictum 2</i>                     | 64.83 G           | 4.06 D             | 1.30 DEF                    | 1.53 DE                     | 1.91 CDE                    | 1.18 E           | 1.08 F          |
| <i>C vulvaria</i>                       | 32.71 J           | 2.30 G             | 1.28 DEF                    | 1.55 DE                     | 1.60 E                      | 1.01 G           | 0.92 H          |
| <i>C. rubrum1</i>                       | 32.38J            | 4.23 CD            | 1.37 CDE                    | 2.35 BC                     | 2.37 BCD                    | 0.68 H           | 0.65 I          |
| <b>LSD (<math>\alpha = 0.05</math>)</b> | <b>7.27</b>       | <b>0.27</b>        | <b>0.24</b>                 | <b>0.52</b>                 | <b>0.54</b>                 | <b>0.068</b>     | <b>0.062</b>    |

<sup>a</sup> Means followed by same letter are not different according to Fischer's LSD test ( $p < 0.05$ ).

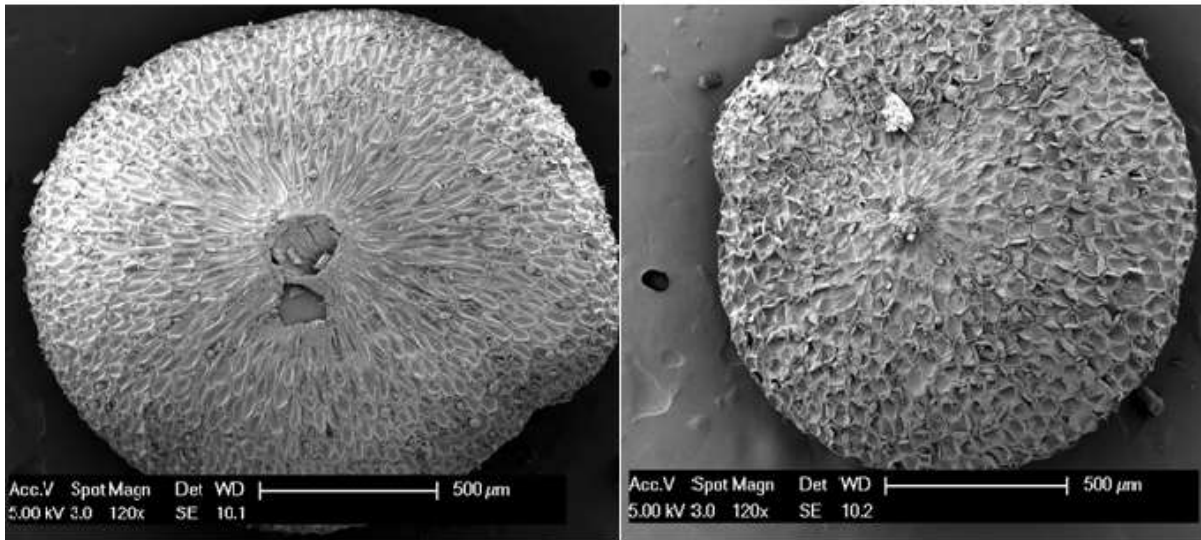
Along with the quantitative characters, a parallel study was also initiated to study qualitative characters which include stem growth habit, branching, calyx keeled or not, whether or not the fruit is covered with perianth at maturity, pubescence of leaves and surface features of the pericarp. Most of these characters have been used as key characters in writing monographs for these species (Aellen 1929, Mosyakin and Clemants, 1996; Clemants and Mosyakin 2004).

Morphological characters, including pericarp structure, calyx shape (keeled or not) and DNA content values have proved to be the “key” characters in differentiating various *Chenopodium* species (Bassett and Crompton 1982; Cole 1961; Aellen and Just 1943; Wahl 1954; Bhargava et al. 2006; Wang et al. 1993; Sederberg et al. 2009). For studying the pericarp structure, seeds were selected from single plant and 2-3 seeds were examined under Environmental scanning electron microscope (ESEM). The results of the surface pattern were similar to the findings of Cole (1961), Uotila (1978) and Karcz (2005). Based on the pericarp structure (Figure 3.1, 3.2), we can easily separate the accessions of *Chenopodium berlandieri*, *C. ficifolium* and *C. quinoa* from *C. album* and other species of *Chenopodium* (Figure 3.1 and 3.2). The *C. berlandieri* and *C. ficifolium* have a distinct honeycomb pitted pericarp that is very different from the smooth to reticulated pericarp of *C. album*, *C. strictum* and *C. glaucum*. Similar to our previous findings using quantitative characters, *C. berlandieri* 1 and *C. bushianum* resembled *C. album* more than accessions of *C. berlandieri* (Figure 3.2). When we studied the sepals for being keeled or not, the results were overlapping and similar results were shared with many species.



*C. album 1*

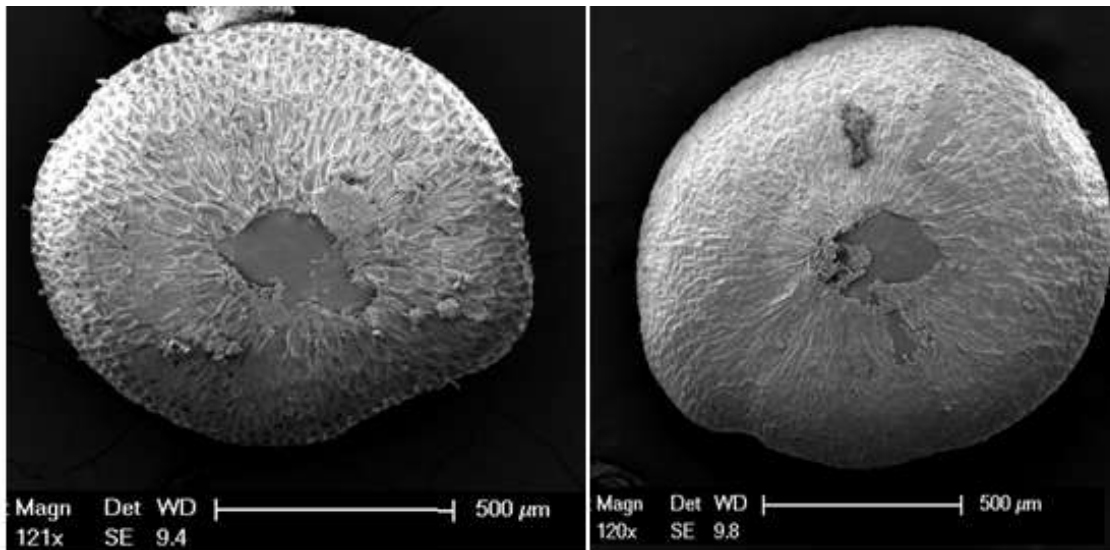
*C. album 2*



*C. berlandieri 2*

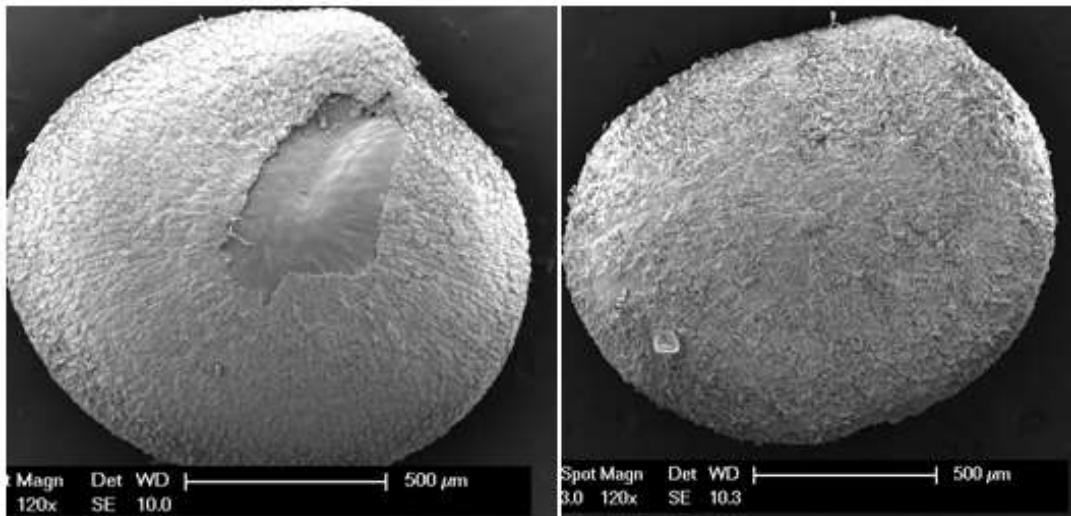
*C. berlandieri 3*

**Figure 3.1.** Seed pericarp features of *C. album* and *C. berlandieri* using scanning electron microscope.



*C. ficifolium*

*C. strictum*

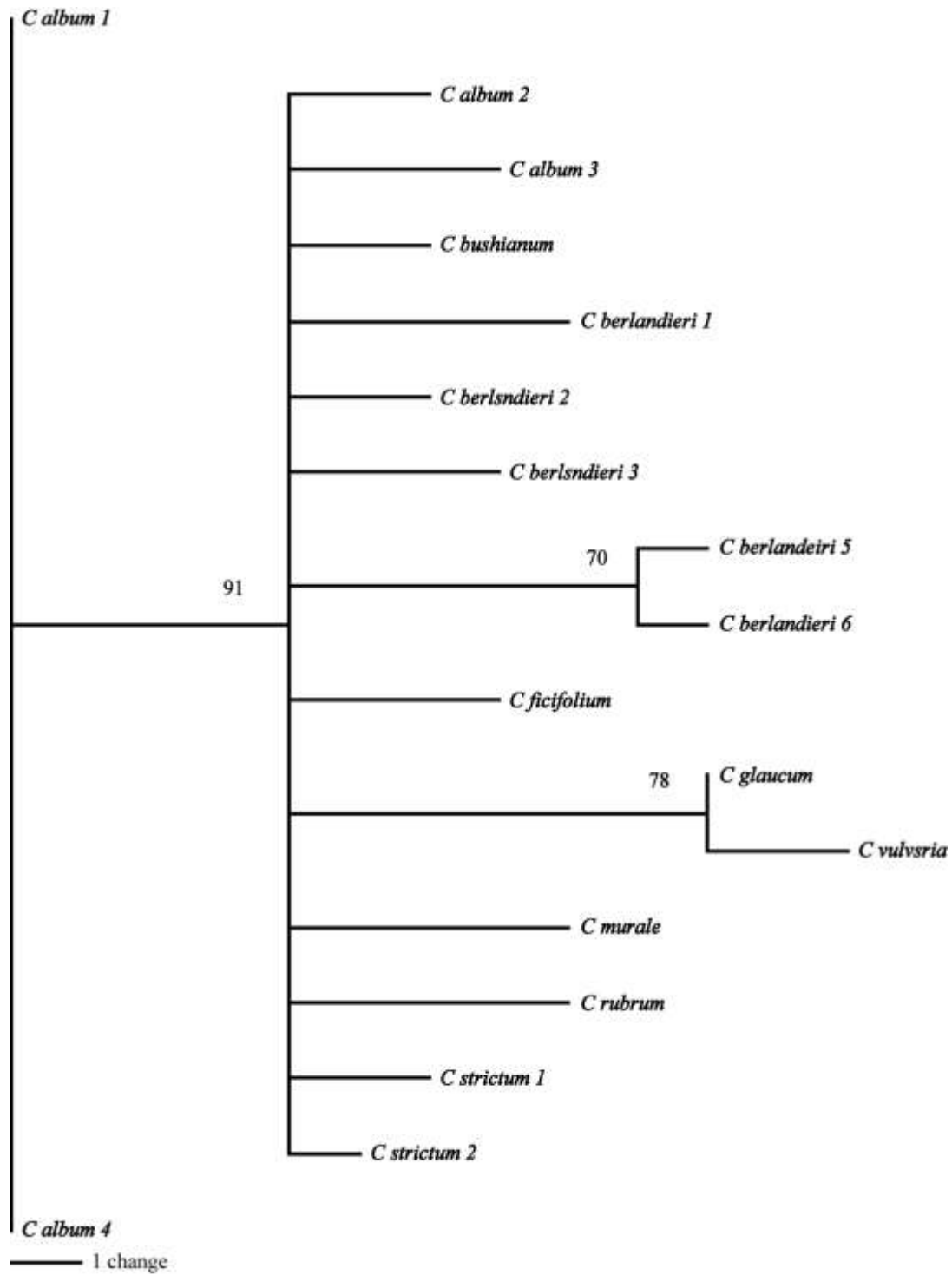


*C. berlandieri* 1

*C. bushianum*

**Figure 3.2.** Seed pericarp features of *C. ficifolium*, *C. strictum*, *C. berlandieri* 1 and *C. bushianum*.

We were not very successful in identifying key morphological character(s) that can distinguish the *Chenopodium* species. Our next approach was to combine the information from all the morphological characters and develop a phylogenetic tree based on maximum parsimony analysis. The tree (Figure 3.3) shows the clustering of the species based on eight morphological characters (coded as binary codes). The CI value was 0.39 and the homoplasy index (HI) was very high at 0.60. The phylogram shows that the tree was not resolved due to high homoplasy between the morphological characters, which is evident from high homoplasy index values. The reason for less reliability of the morphological characters is based on the fact that *Chenopodium* genus is a complex group. Several species in this genus have more than one ploidy level and they have remarkable phenotypic plasticity, which makes it difficult to use the morphological characters alone for identifying *Chenopodium* species. Seed characters such as size and pericarp are considered to be unmistakably distinct in *C. berlandieri* and *C. album* but they can also show polymorphism as they are partly a function of the environment (Cole, 1961). One reason for polymorphism is that pericarp surface patterns are influenced by temperature, and low temperature near maturity results in more reticulated seeds than when grown under warm conditions (Cole, 1961). So, although seed coat has a genetic basis, it is also influenced by environment; it is not advised to use the seed coat pattern alone to indicate even incipient speciation (Cole 1961).



**Figure 3.3.** Unrooted maximum parsimony tree based on the differences in morphological characters of 17 accessions of *Chenopodium*.

From our study using the morphological characters, we conclude that the morphological characters alone cannot be used for phylogenetic analysis as the characters overlap within different species. The fact that there are no key characters that can be used across the genus to identify the species explains the taxonomic difficulty of the genus *Chenopodium*. The morphological data need additional support from molecular or cytological data to resolve the phylogeny.

### **DNA Content Analysis of *Chenopodium* Species and Populations from North Central States**

Relative nuclear DNA content of nuclei isolated from *Chenopodium* species and selected populations of *Chenopodium* across North Central states along with the *Zea mays* (internal standard) using flow cytometry is shown in Table 3.5. The reason for measuring the genome size is that it is one character under strict genotypic control within defined limits (Benett et al. 2000). Nuclear DNA analysis has proved to be very effective in delimiting infrageneric division in a number of taxa (Ohri, 1998). Based on the range of 2C values (1.8 to 3.8 pg), *Chenopodium* species can be grouped into three categories. These categories resemble the ploidy level, but as we did not count the chromosomes, we used a general classification or grouping of *Chenopodium* species. Different accessions of *C. album* had a genome size between the range of 3.6 to 3.8 pg/2C, which was well outside the range of the closely related *C. strictum* (2.0 to 2.1 pg/2C) and *C. berlandieri* (2.7 to 2.9 pg/2C). *C. ficifolium*, which is closely related to *C. berlandieri*, had a smaller genome size of 1.85-1.9 pg/2C. The DNA content data were useful in differentiating *C. album* from *C. strictum* and *C. berlandieri* from *C. ficifolium*. There is a significant difference between the genome size of *C. album* and *C. berlandieri*. Although no study of the ploidy level was done, based on the DNA content values, the best estimate is that *C.*



*strictum* and *C. ficifolium* are diploid; *C. berlandieri* is a tetraploid and *C. album* accessions are hexaploid. The *C. berlandieri* 1 and *C. bushianum* also had 2C values that were close to that of *C. album*. The results from our experiments are consistent with those of Sederberg et al. (2009) who estimated ploidy levels for various *Chenopodium* species using fluorescent in situ hybridization. In fact, some of the accessions used in our study were from the same seed source. The ploidy levels they determined were very similar to our grouping based on the DNA content values.

**Table 3.5.** DNA content values (pg/2C) of some *Chenopodium* samples using flow cytometry.

| <b>Accessions examined</b> | <b>DNA content (pg/2C)</b> |
|----------------------------|----------------------------|
| <i>C. album</i> 1          | 3.6 ABCD                   |
| <i>C. album</i> 2          | 3.6 ABCD                   |
| <i>C. album</i> 3          | 3.7 ABCD                   |
| <i>C. bushianum</i>        | 3.7 ABCD                   |
| <i>C. berlandieri</i> 1    | 3.7 ABCD                   |
| <i>C. berlandieri</i> 2    | 2.7 E                      |
| <i>C. berlandieri</i> 3    | 2.7 E                      |
| <i>C. berlandieri</i> 5    | 2.8 E                      |
| <i>C. ficifolium</i>       | 1.8 G                      |
| <i>C. strictum</i> 1       | 2.0 FG                     |
| <i>C. strictum</i> 2       | 2.0 FG                     |
| IA 20                      | 3.6 ABCD                   |
| IA 21                      | 3.6 ABCD                   |
| IL 7                       | 3.5 CD                     |
| IL 6                       | 3.8 AB                     |
| IN5                        | 3.7 ABC                    |
| KS22                       | 3.5 D                      |
| KS8                        | 3.7 ABC                    |
| KS 9                       | 3.7 ABC                    |
| MO 6                       | 3.5 BCD                    |
| ND 7                       | 3.8 A                      |
| <b>LSD (alpha=0.05)</b>    | <b>0.30</b>                |

<sup>a</sup> Means followed by same letter are not different according to Fischer's LSD test (p<0.05).

The DNA content analysis of *Chenopodium* populations sampled from the North Central states were 3.5-3.9 pg/2C, and are not significantly different from the *C. album* samples, but are significantly different from the other species sampled (Table 3.5). These populations therefore all appear to be of *C. album*. The variability among the *Chenopodium* populations can be due to deletions, duplications or genome responses to environmental stress (Price 1976).

### **Molecular Data Analysis**

Towards the second research objective, DNA sequences from nuclear and chloroplast regions were explored to find a locus that has enough polymorphic positions that can be used either alone or in combination with morphological characters to resolve the *Chenopodium* phylogeny. Three loci, ITS, *matK* and *trnD-trnT* were investigated. In the following sections I will discuss the results from all three regions separately.

### **ITS Sequence Analysis**

Sequences were obtained for two or more plants from 33 accessions (including 5 samples of *C. album* from Genbank) representing 12 *Chenopodium* species. In addition to the *Chenopodium* species, sequences for ITS region were also obtained for *A. spinosus* (DQ005961.1) and *A. retroflexus* (AF210906.1) which were used as outgroup. The numbers of accessions were increased in closely related species like *C. album* and *C. berlandieri*, however due to limited number of accessions for other species, we could not increase the accessions of *C. strictum*, *C. bushianum* and *C. strictum*. Increasing the populations within the species increases the confidence in clustering and increases the reliability of our analyses.

To check if there is any difference in the alignment methods, the ITS sequences were aligned with Clustal W and MUSCLE alignment software. Alignment results from both the methods were almost identical (data not shown) and required manual editing by adding or deleting gaps. The number of parsimony informative (PI) sites obtained using Clustal W were 201 as compared to 199 from MUSCLE. This small difference can either be due to different algorithms used by the software, but more importantly, it can also be an artifact due to manual editing of the alignments to increase the homology. We used the Clustal W alignment algorithms for aligning all the genera used in this study.

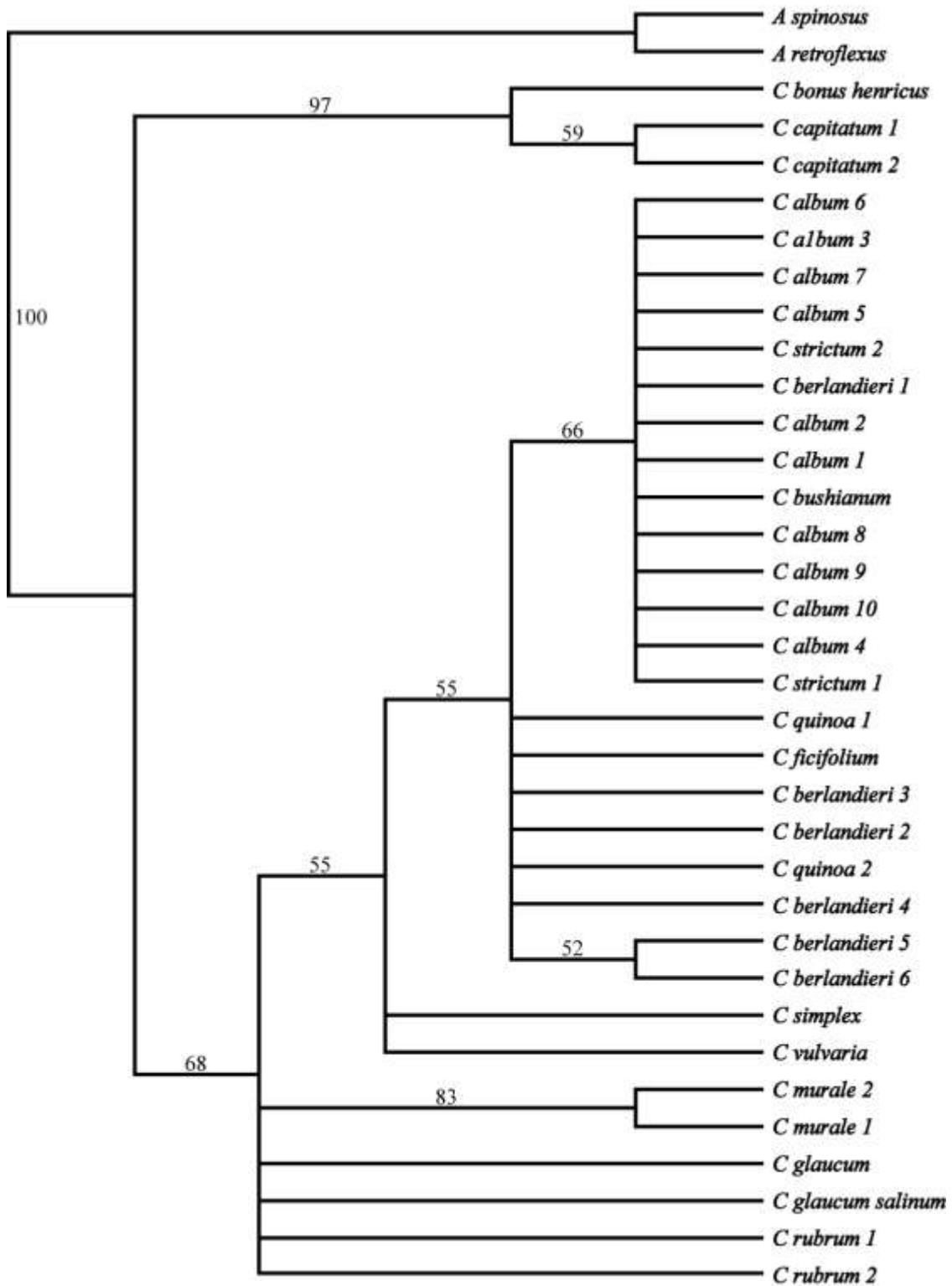
The ITS region was 643 bp long and it included 249 bp of ITS-1 spacer and 232 bp of ITS-2 spacer region (Table 3.6). One important character in phylogenetic studies is the number of parsimony informative (PI) sites. These sites are different than the variable sites by the fact that any variable site is parsimony informative only when there are at least two different kinds of nucleotides at the site, each of which is represented in at least two or more sequences under study. There were 201 parsimony informative sites, which represent 31.2% of the entire region. Among ITS-1 and ITS-2, the PI sites were more in ITS1 (45.7%) than ITS2 (34%).

**Table 3.6.** Sequence characteristics of the nuclear rDNA internal transcribed spacer region for 33 accessions of different *Chenopodium* species.

|  | ITS  | ITS-1 | ITS-2 | ITS-1 & ITS-2 |
|--|------|-------|-------|---------------|
| <b>No. of total characters</b>                 | 643  | 249   | 232   | 488           |
| <b>No. of conserved characters</b>             | 413  | 123   | 137   | 260           |
| <b>No. of variable sites</b>                   | 229  | 125   | 95    | 130           |
| <b>No. of Parsimony Informative characters</b> | 201  | 114   | 79    | 193           |
| <b>G+C content (%)</b>                         | 57.1 | 58.1  | 55.4  | 56.7          |

Alignment of ITS sequences resulted in gaps across the ITS-1 and ITS-2 regions. The gaps from the alignment were analyzed separately to check if the gaps are informative and if they can increase the overall resolution of the tree. Using Gapcoder (Young and Healy, 2003), the gaps were coded as presence or absence of character. Gapcoder program codes the gaps and puts these values at the end of the sequence. For phylogenetic analysis, additional phylogenetic information is obtained from the gaps.

The result from the analyzing the gaps is presented in Figure 3.4. Two measures used for estimating the relative amount of homoplasy are consistency index (CI) and retention index (RI). The CI is calculated as the number of steps expected given the number of character states in the data, divided by the actual number of steps multiplied by 100. The RI measures the amount of synapomorphy expected from a data set that is retained as synapomorphy on a cladogram. The uncorrected consistency index (CI) was 0.89 and the corrected CI, which excluded uninformative sites, was 0.880. The retention index (RI) was 0.95. The bootstrap values ranged from 55 to 100%. Using the gaps alone as characters, there was grouping among the *Chenopodium* species. Different accessions of *C. album* were grouped together with 66% bootstrap support values. The other major clade consisted of *C. berlandieri* along with other species in the subsect. Favosa. Despite the fact that bootstrap values are not large, these results suggest that gaps are phylogenetically informative, and it was decided to include the gaps as additional characters for maximum parsimony analysis of ITS and *trnD-trnT* spacer regions.

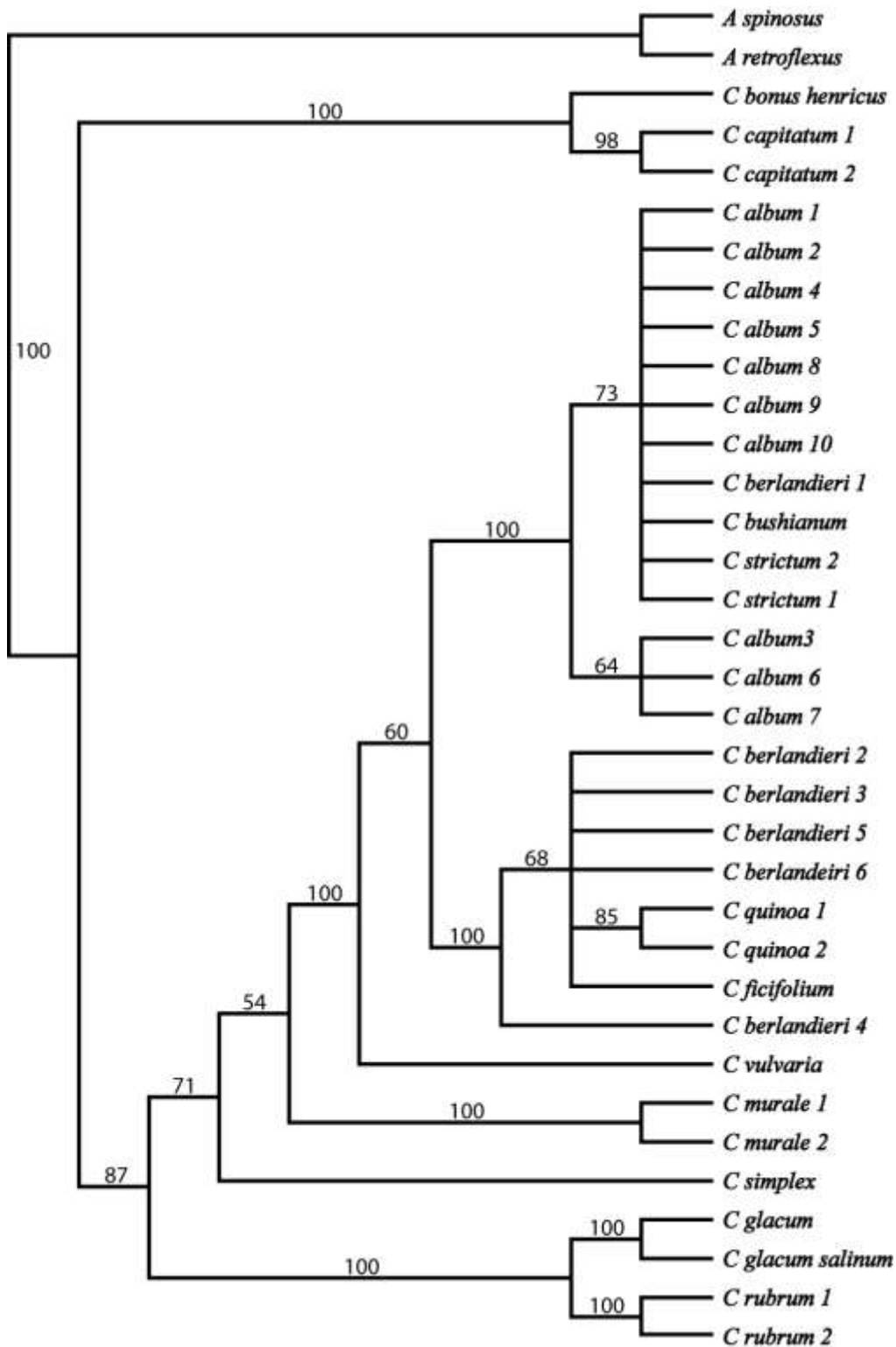


**Figure 3.4.** Strict consensus tree of gaps from ITS region coded as binary characters using maximum parsimony analysis. Bootstrap values are shown above the branches.

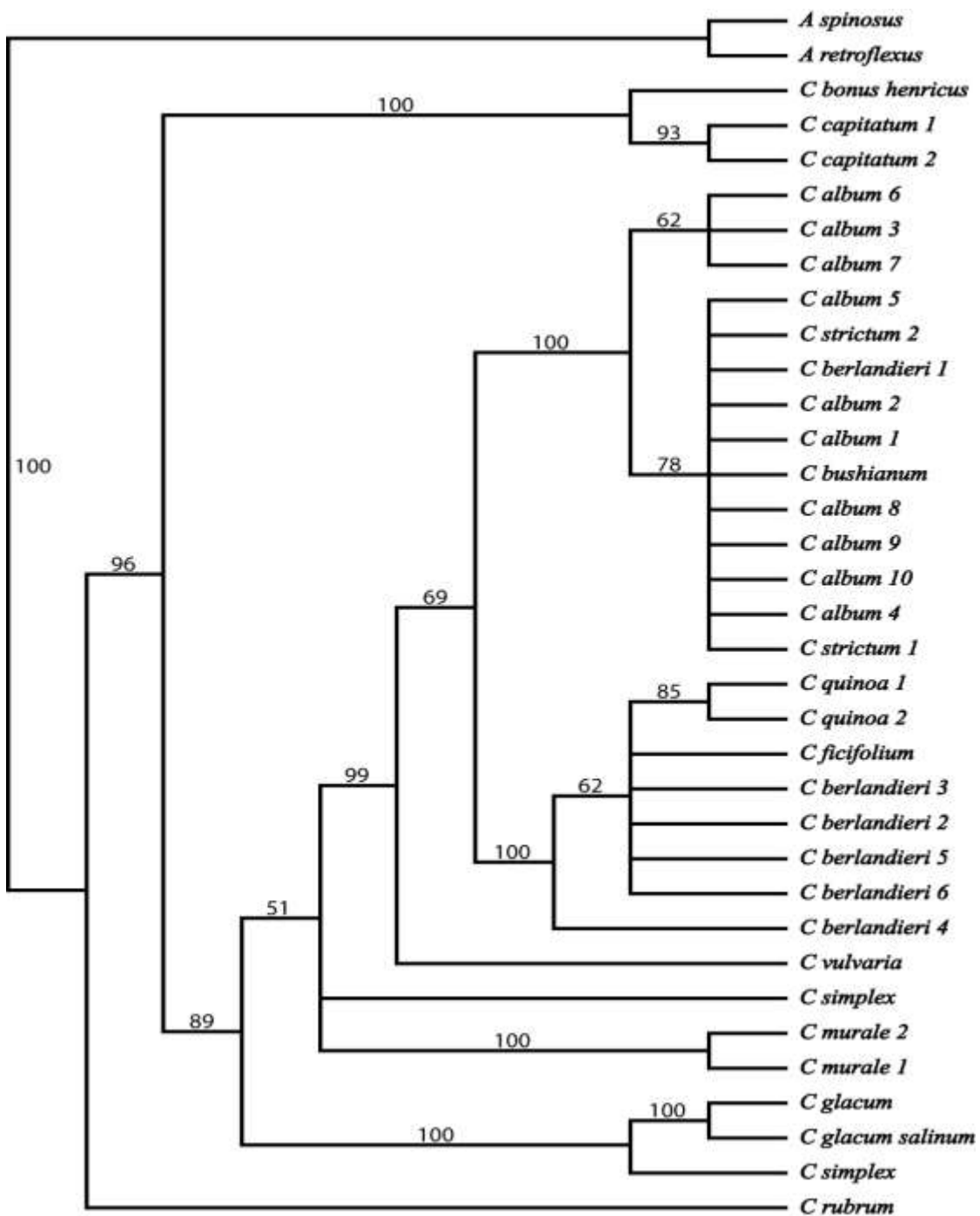
## Maximum Parsimony Analysis of Nuclear Regions

Maximum parsimony analysis of the ITS DNA matrix, with gaps treated as missing data is presented in Figure 3.5. The topology with the minimum tree length is known as the maximum parsimony tree. MP analysis of the entire ITS region resulted in 4 most parsimonious trees with 419 steps (steps refers to the tree length). The uncorrected CI was 0.89 and the corrected CI, which excluded uninformative sites, was 0.88. The RI for the current tree was 0.95. Both the *C. album* and *C. berlandieri* group were separated as separate clades with bootstrap of 100%. Some variability was visible among the accessions of *C. album* but all of them shared the same clade. Two species, *C. vulvaria* and *C. simplex* did not group with other *Chenopodium* species.

When we compared these results to the MP tree obtained by including gaps in the analysis and scoring them as binary characters (Fig 3.6), there was an increase in the bootstrap values. The length of the sequences increased from 643 to 671 bp after the inclusion of the gaps to the sequences. Even with including gaps to the sequences, four trees were retained with 237 steps. The uncorrected consistency index (CI) was 0.76 and the corrected CI, which excluded uninformative sites, was 0.75. The retention index was estimated to be 0.90. Irrespective of if the gaps are included or excluded, there were no major changes in the tree topology, but the bootstrap values did increase with inclusion of the gaps. A similar trend was also observed for bootstrap support for species within the clade comprising the *C. berlandieri* species. Outside the *C. album* and *C. berlandieri* groups, the bootstrap values decreased which may be due to homoplasy in gaps among more distantly related groups. This is consistent with lower CI and RI values.



**Figure 3.5.** Maximum parsimony strict consensus tree of ITS region with gaps treated as missing data. Bootstrap values are shown above the branches.



**Figure 3.6.** Strict consensus tree derived from maximum parsimony analysis of 35 nuclear ITS sequences. The values on top of the branch represent bootstrap values.



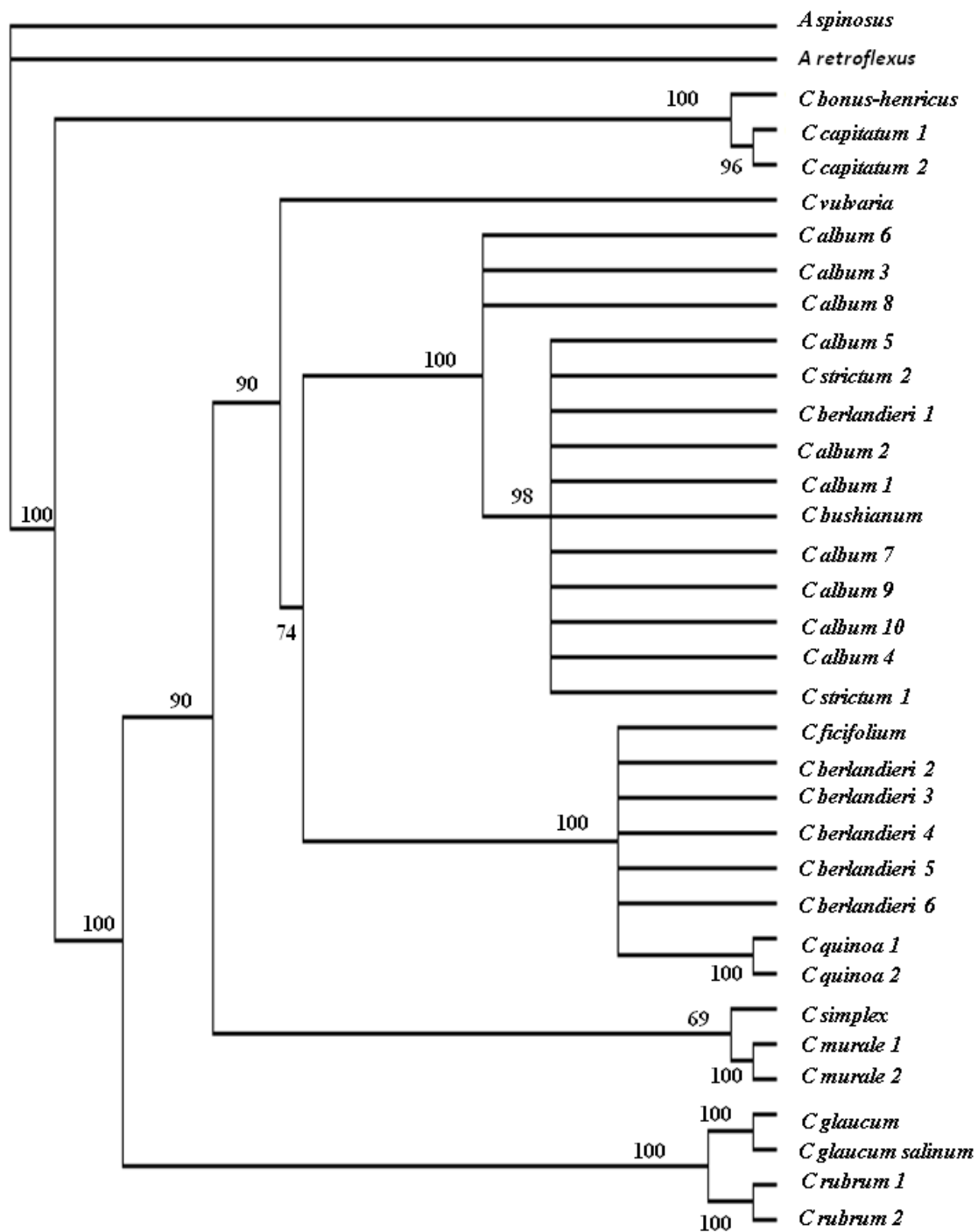
The ITS-1 and ITS-2 spacer regions were also individually tested (trees not shown), but the results were not different than the topology from the entire ITS region. The CI (0.73) and RI (0.90) were close to what we obtained by analyzing the entire ITS region.

Our conclusion from estimating the phylogeny based on ITS region is that the species studied can be mainly grouped into four main clades. All four clades are supported with high bootstrap values and there is no conflict between trees using ITS-1, ITS-1, ITS1-2 (excluding 5.8S), and ITS (with or without gaps as additional character).

### **Bayesian Analysis**

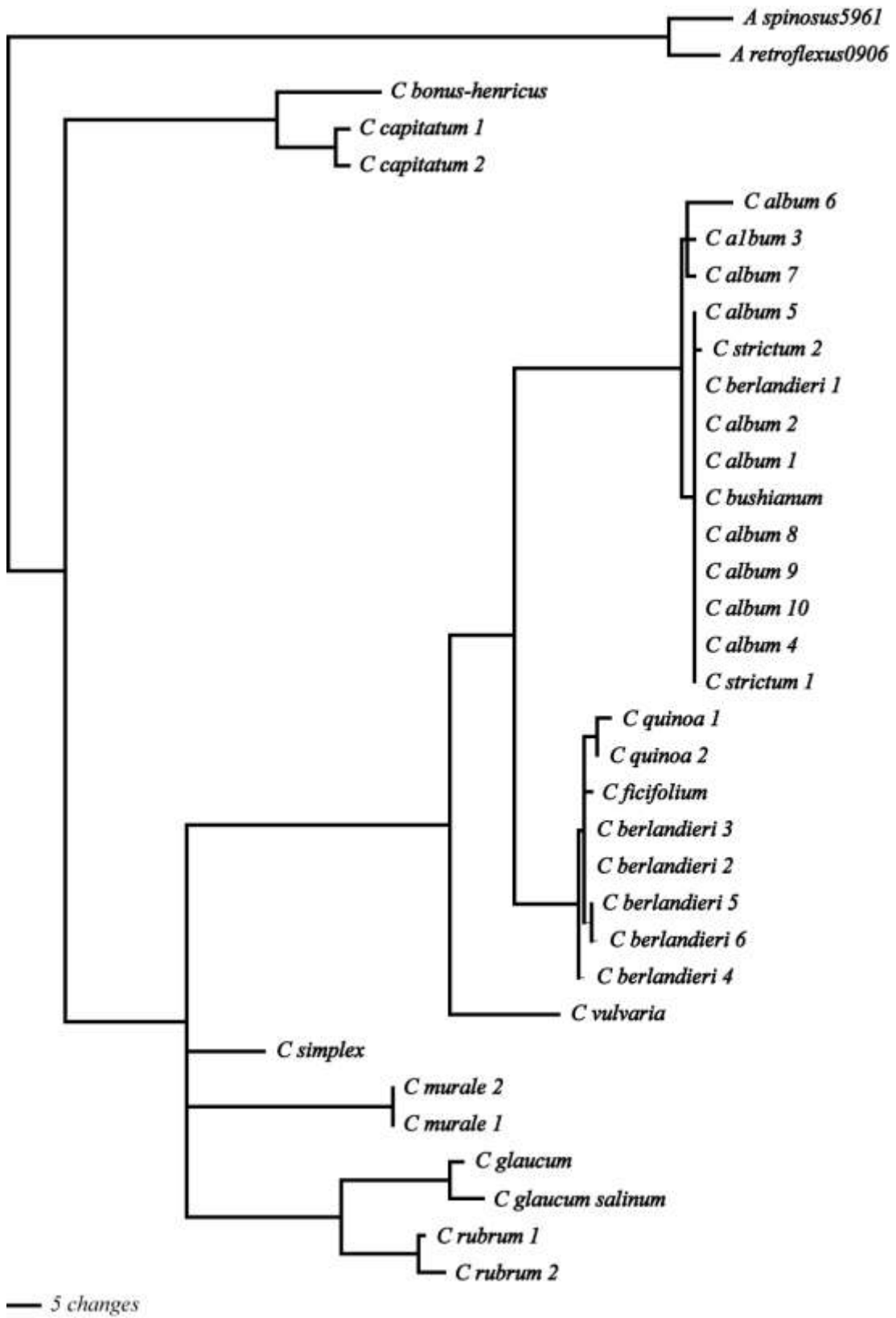
Based on the AIC estimator, Modeltest (Pasoda and Crandall, 1998) selected the GTR+I+G model as best fitting the ITS dataset. General time reversible (GTR) model is a parameter rich model and it considers base frequencies and substitution rates as unequal (GTR) with invariable sites (+I) and rate variation (+G) among sites. The base frequencies were as follows:  $f(A) = 0.2443$ ;  $f(C)=0.2931$ ;  $f(G)= 0.2717$ ;  $f(T)= 0.1908$ . The estimates of rates of substitution were:  $A \leftrightarrow C=1.3244$ ;  $A \leftrightarrow G=1.8458$ ;  $A \leftrightarrow T= 1.4470$ ;  $C \leftrightarrow G=0.3349$ ;  $C \leftrightarrow T= 4.5867$ ;  $G \leftrightarrow T=1.0000$ . The proportion of invariable sites (I) = 0.3961.

The 50% majority rule consensus tree from the Bayesian analysis is shown in Figure 3.7. The posterior probability values were higher than the parsimony bootstrap values but the resulting consensus tree from Bayesian analysis was very similar to the strict consensus tree obtained through MP analysis.



**Figure 3.7.** Fifty percent Majority rule tree of 35 sequences of ITS region using Bayesian analysis. The values on top of branch are posterior probabilities.

In Figure 3.8, a phylogram is presented based on the ITS sequences of *Chenopodium* species. It is a branching diagram that is assumed to be an estimate of a phylogeny. The branch lengths are proportional to the amount of inferred evolutionary change. These results show that there is little to no sequence divergence within species that are well separated from each other. The results from the analyses are consistent with the classification of Moyaskin and Clemants (2004). The evolutionary relationships between the *Chenopodium* species have not been investigated in detail, so our phylogram gives us an idea of the evolution of *Chenopodium* species over time.

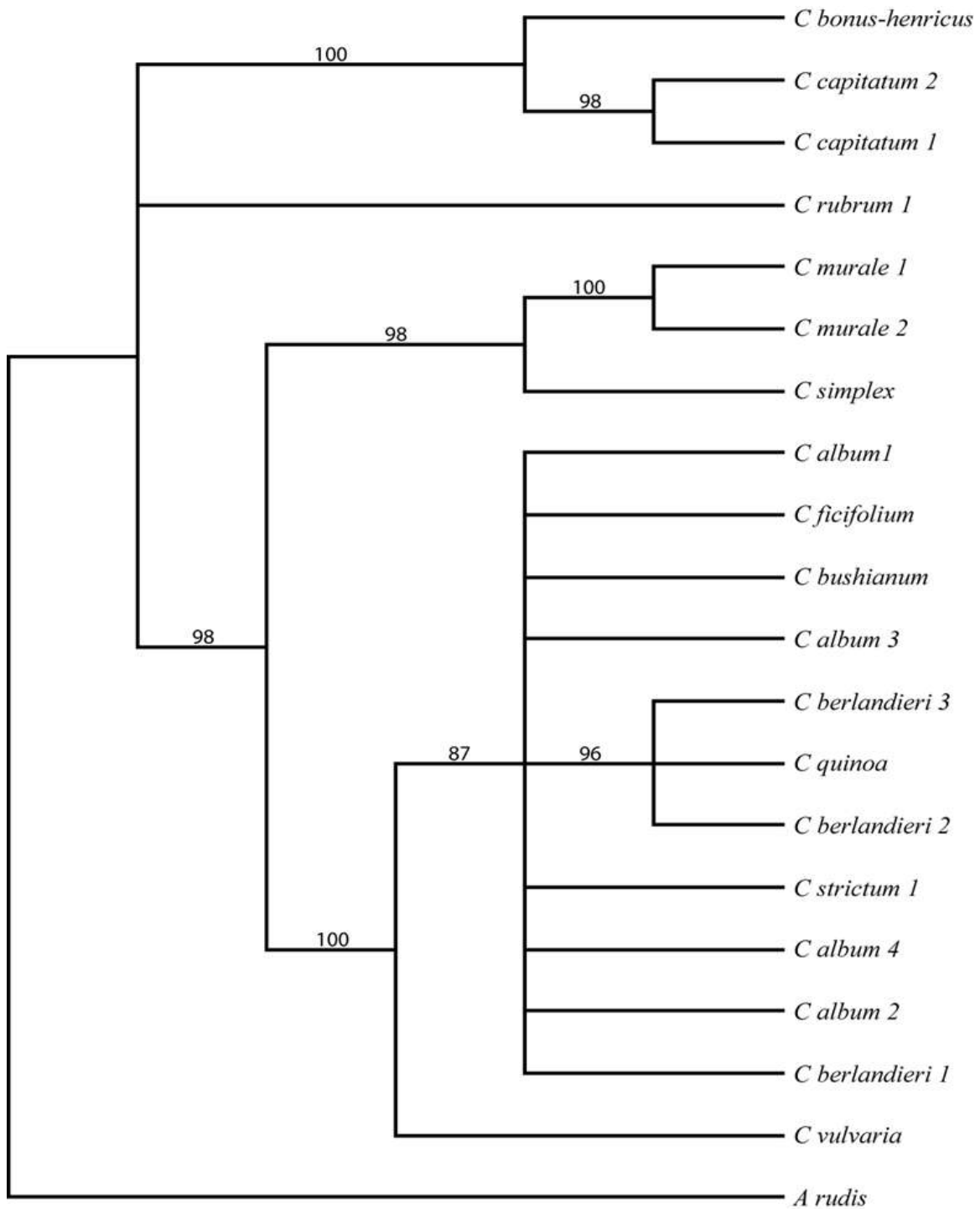


**Figure 3.8.** Phylogram derived with maximum parsimony analysis of entire ITS region.

## Chloroplast Markers

Two chloroplast markers, *matK* (gene) and *trnD-trnT* (spacer) were also investigated to check for robust markers that can aid in identifying the *Chenopodium* species. Both chloroplast regions were analyzed using MP and Bayesian analysis. For the MP analysis (Fig 3.10) of *matK* gene, 20 taxa were screened and aligned sequence length had 823 characters. Two parsimonious trees were retained with 198 steps. The uncorrected CI was 0.90 and the corrected CI, which excluded uninformative sites, was 0.82. The RI for the tree was estimated to be 0.92. Despite the fact that the bootstrap values for MP analysis were high, the number of parsimony informative characters were only 8.99 % (74 PI characters).

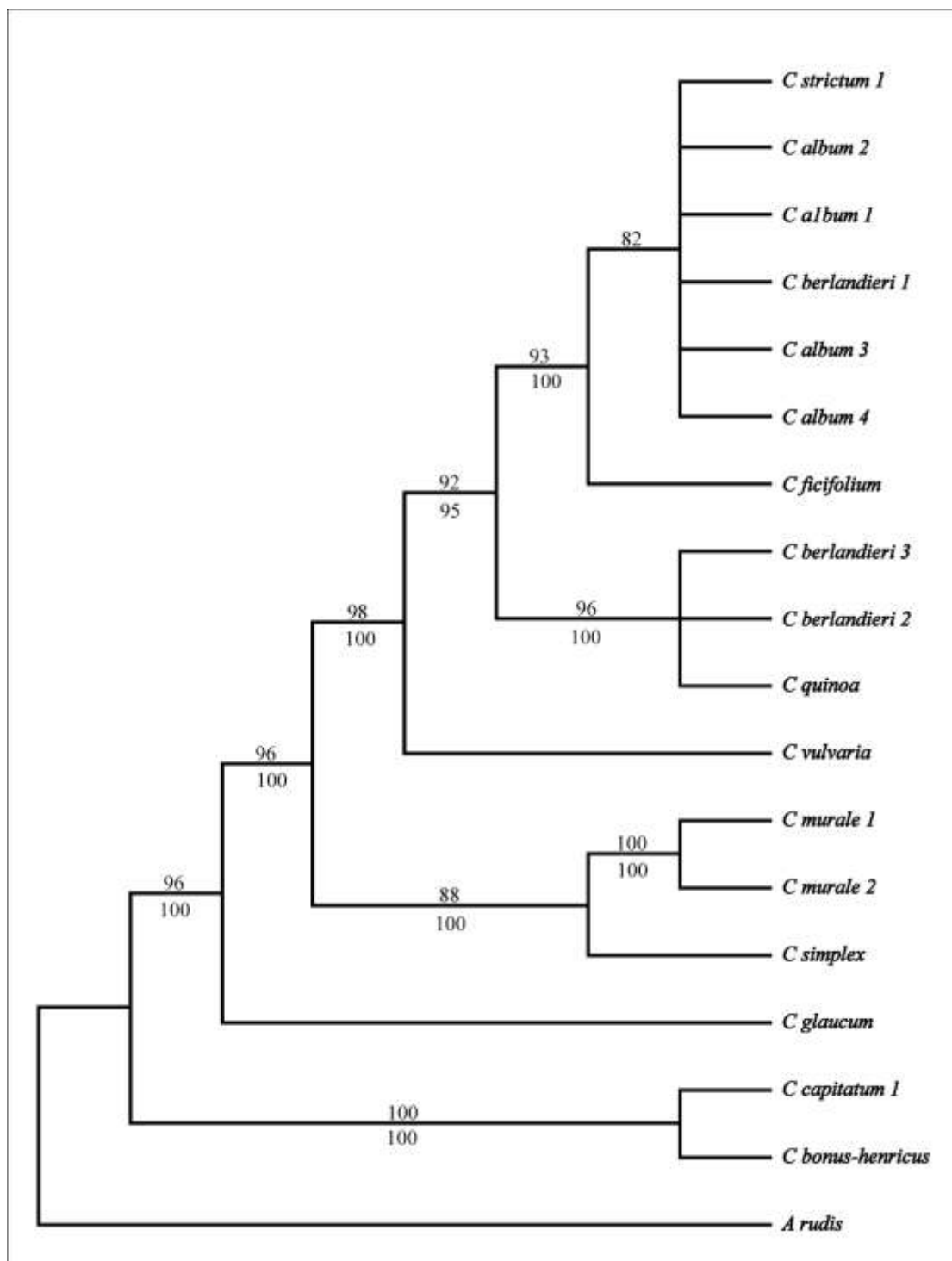
For the Bayesian analysis of the *matK* gene, the nucleotide substitution model selected for *matK* using Modeltest was TIM+G. The base frequencies were as follows:  $f(A) = 0.2852$ ;  $f(C) = 0.1762$ ;  $f(G) = 0.1699$ ;  $f(T) = 0.3687$ . The estimates of rates of substitution were:  $A \leftrightarrow C = 1.000$ ;  $A \leftrightarrow G = 0.9557$ ;  $A \leftrightarrow T = 0.2128$ ;  $C \leftrightarrow G = 0.2128$ ;  $C \leftrightarrow T = 1.6055$ ;  $G \leftrightarrow T = 1.0000$ . Four simultaneous analyses were run for 2 million generations, each with four Markov Chain Monte Carlo (MCMC) chains, and sampling every 100 generations. The resulting 50% majority rule tree was very similar to the MP consensus tree, and the posterior probability values were similar to bootstrap values (Figure 3.9). The trees for *matK* resulting from both analytic methods were less resolved than the trees based on the ITS data, particularly for the *C. album* and *C. berlandieri* groups. This reflects smaller number of informative characters in *matK* compared to ITS.



**Figure 3.9.** Strict consensus tree derived from maximum parsimony analysis of *matK* gene for 22 *Chenopodium* species. The values on top of branch are bootstrap values from maximum parsimony analysis and values under the branch are posterior probability values from Bayesian analysis.

The chloroplast spacer region (*trnD-trnT*) had an aligned length of 755 bp and included 102 parsimony informative characters (13.5% of total characters). When the data containing 18 taxa were subjected to MP analysis, only one parsimonious tree was retained with 233 steps. The uncorrected CI was 0.875 and the corrected CI, which excluded uninformative sites, was 0.801. The RI for the tree was estimated to be 0.884. The phylogenetic tree from MP analysis of *Chenopodium* species was well supported by the bootstrap values, ranging from 72-100 % (Figure 3.10).

The Bayesian analysis of the *trnD-trnT* spacer was conducted with the nucleotide substitution model, selected by Modeltest, GTR+I. The base frequencies were as follows:  $f(A) = 0.3413$ ;  $f(C) = 0.1845$ ;  $f(G) = 0.1615$ ;  $f(T) = 0.3127$ . The estimates of rates of substitution were:  $A \leftrightarrow C = 0.6125$ ;  $A \leftrightarrow G = 1.0832$ ;  $A \leftrightarrow T = 0.1701$ ;  $C \leftrightarrow G = 0.5993$ ;  $C \leftrightarrow T = 0.6638$ ;  $G \leftrightarrow T = 1.0000$  and the proportion of invariable site were 0.5605. Four simultaneous analyses were run for 1 million generations, each with four Markov Chain Monte Carlo (MCMC) chains, and sampling every 100 generations. The 50% majority rule tree was very similar to the MP consensus tree (Figure 3.10). Bayesian posterior probability values are shown in Figure 3.10. The topology of the *trnD-trnT* was similar to ITS gene topology, however, with *trnD-trnT* spacer, *C. ficifolium* did not share the clade with *C. berlandieri*, but instead was placed as sister to the *C. album* clade.



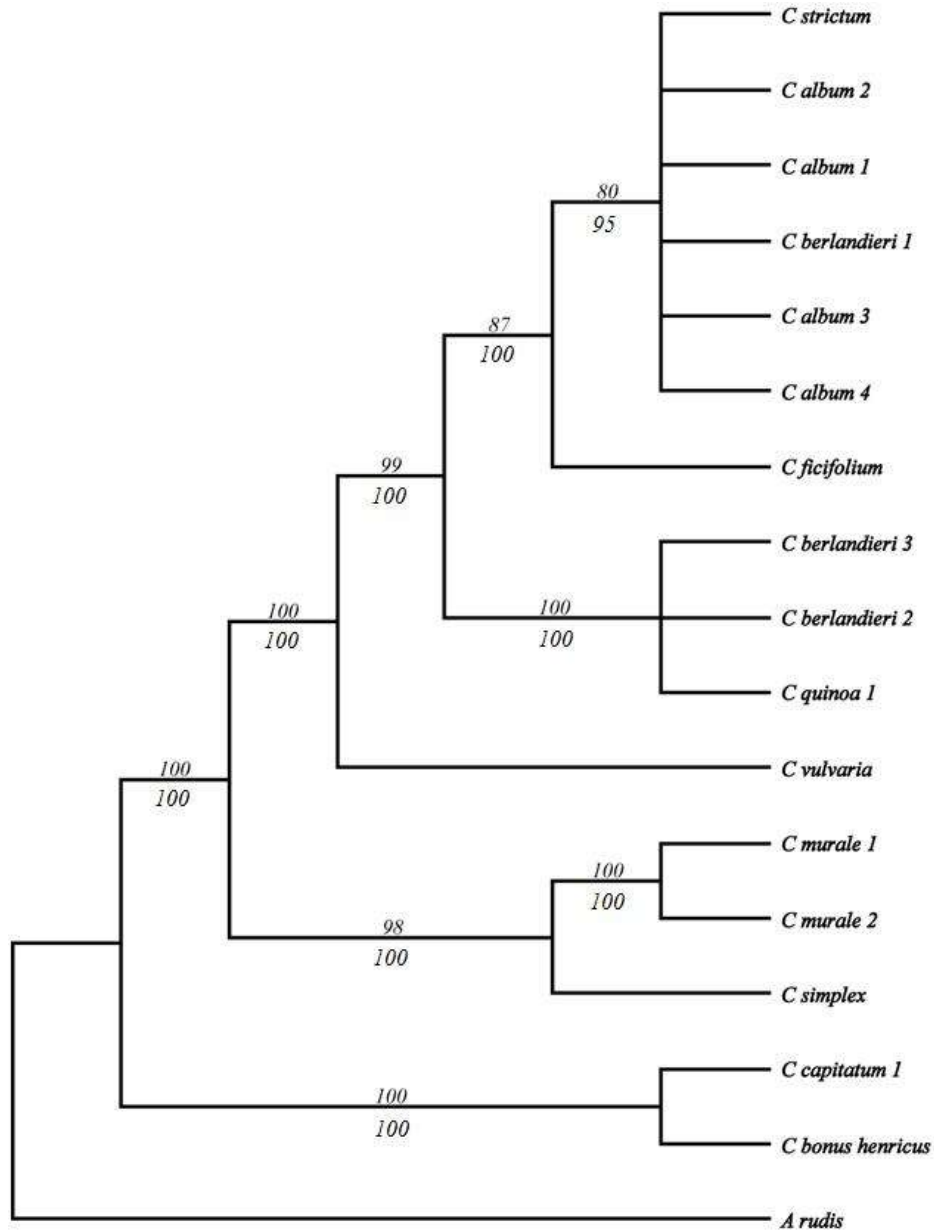
**Figure 3.10.** Strict consensus tree derived from maximum parsimony analysis of *trnD-trnT* region for 18 *Chenopodium* species. The values on top of branch are bootstrap values from maximum parsimony analysis and values under the branch are posterior probability values from Bayesian analysis.



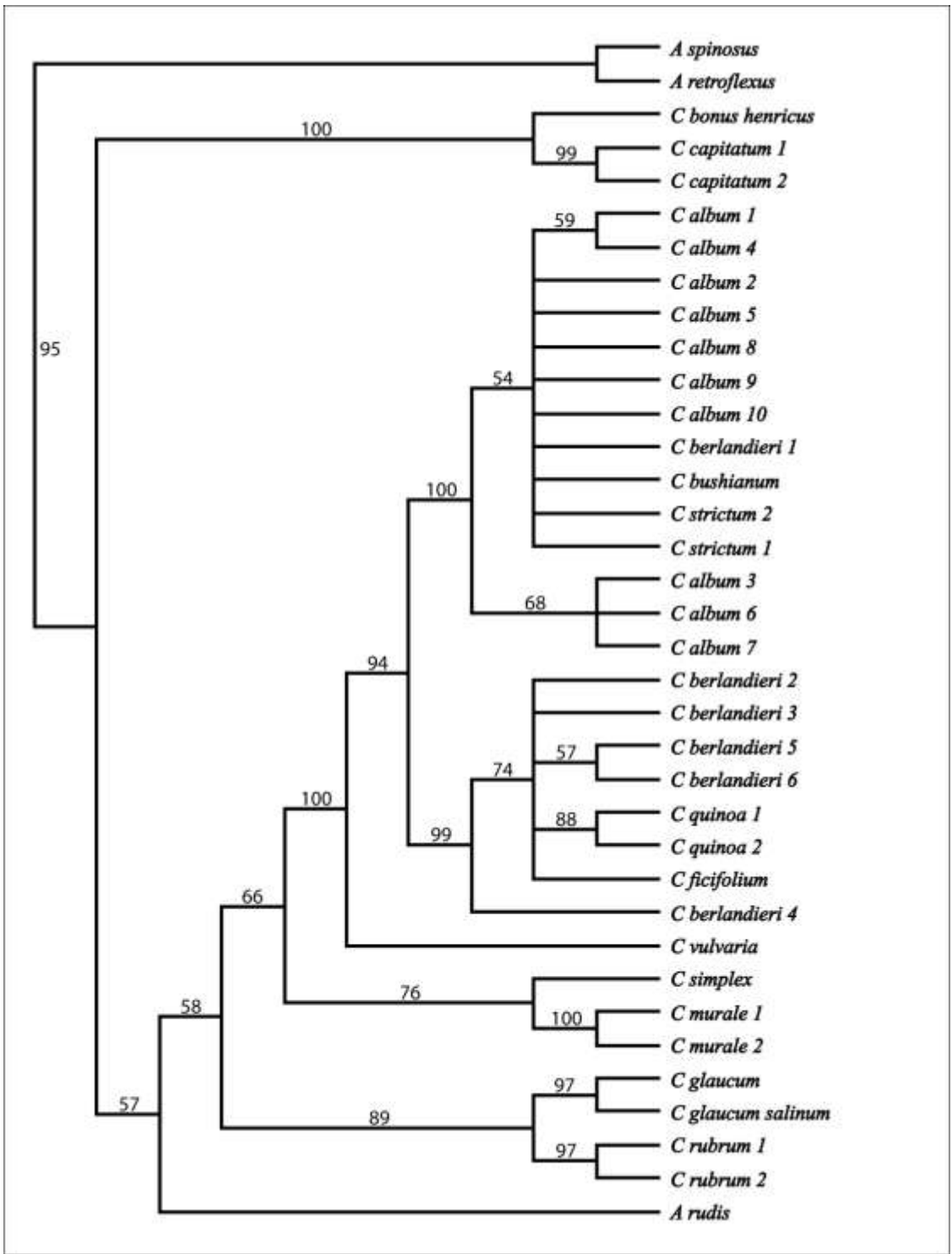
## Combined Analysis of Molecular Markers

Maximum parsimony and Bayesian analyses for the combined chloroplast region and combined nuclear and chloroplast regions were conducted to determine if they result in more resolved trees than the individual regions alone. The MP and Bayesian trees were almost identical and only the MP strict consensus tree is shown in Figure 3.11. The tree topology from the strict consensus tree was not different than the topology from the analysis of *trnD-trnT*. Compared to individual chloroplast markers, the bootstrap values increased for the combined analysis. The only significant difference between the ITS phylogeny and that from the chloroplast regions was that *C. ficifolium* grouped with *C. berlandieri* group based on ITS but was sister to the *C. album/C. strictum* clade based on the chloroplast regions.

Maximum parsimony analysis of the data set combining DNA sequences from the ITS, *matK*, *trnD-trnT* is presented in Figure 3.12. The bootstrap values ranged from 54 to 100%. The combined result showed similar tree topology to ITS alone with similar bootstrap values. The results indicated that *C. bonus-henricus* and *C. capitatum* are close to *C. glaucum*. Accessions of *C. murale* and *C. simplex* are sister to the clade consisting of the *C. berlandieri* and *C. album* group. *Chenopodium album* and *C. berlandieri* are very close to each other *C. strictum* is within the *C. album* clade, *C. ficifolium* and *C. quinoa* are nested in the *C. berlandieri* clade.



**Figure 3.11.** Strict consensus tree derived from maximum parsimony analysis of combined chloroplast DNA sequences from 18 sequences. The values on top of branch are bootstrap values from MP analysis and values under the branch are posterior probability values from Bayesian analysis.



**Figure 3.12.** Strict consensus tree derived from maximum parsimony analysis of 35 sequences using combined DNA sequence data of ITS, *matK*, *trnD-trnT*. Bootstrap values are shown above the branches.

## Phylogenetic Information from the Phylogenetic Study

The ITS region is a good marker and the tree is well resolved. Compared to the chloroplast markers, ITS markers generated more phylogenetic informative sites that had a direct effect on tree topology and the clades. Little resolution was gained by adding the chloroplast regions, although bootstrap support did increase in some cases. The few morphological characters added little phylogenetic information. We can broadly classify the sampled *Chenopodium* species into four clades or groups (Figure 3.6). The clade I comprises *C. capitatum* and *C. bonus-henricus* and they are basal to the remaining *Chenopodium* species. The position of these species is in accordance with the morphological classification in the Flora of North America (Clemants and Mosaykin 2004) which separates two species as *Chenopodium* subg. *Blitum* sect. *Agathophytum*. These species have the distinct characteristics of horizontal seeds (relative to the position of the flower) with smooth pericarps and flowers not covering the seed at maturity. Another sect. in the Subgenus *Blitum* is sect. *Pseudoblitum*, which contains *C. rubrum* and Sect. *Glauca* which contains *C. glaucum*. This grouping of *Chenopodium* subg. *Blitum* is also strongly supported by our results from molecular data analysis of nuclear and chloroplast regions. *Chenopodium simplex* belongs to subg. *Chenopodium* sect. *Grossefoveata* and is the only North American species classified under the section. The leaves of *C. simplex* are large in size and they resemble maple (*Acer* spp.) shaped leaves. *Chenopodium murale*, which belongs to subsect. *Undata*, forms a separate clade from *C. simplex* based on ITS, but *matK* and *trnD-trnT* sequence data support it forming a clade with *C. simplex*.

The phylogenetic tree was not well resolved for the subsect. *Favosa* (*C. berlandieri*, *C. ficifolium*, *C. quinoa*) and subsect. *Chenopodium* (*C. album*, *C. strictum*). Two accessions labeled as species within the subsect. *Favosa*, *C. berlandieri* 1 and *C. bushianum* were found to be more closely related to *C. album*. The morphological and molecular data supported the position of these two accessions in the *C. album* group. The best explanation is that these accessions are misidentified as both these species did not had the honeycomb pitted pericarp, and the DNA content values were also more close to *C. album* as compared to *C. berlandieri* (Table 3.3). Rana et al 2010 also used the same accession of *C. bushianum* and they also reported that *C. bushianum* is more related to *C. album* than *C. berlandieri* species.

Within the subsect. *Chenopodium*, we were not successful in finding any polymorphic sites between *C. album* and *C. strictum*. Phylogenetic analyses of ITS and *trnD-trnT* revealed similar results, but sequences from *matK* did not improve the resolution of phylogenetic tree. The morphological differentiating characters between *C. album* and *C. strictum* were the size of the seeds (small as compared to *C. album*), and *C. strictum* having a smaller 2C values than *C. album*. Although the chromosomes were not counted for *Chenopodium* species, but based on the 2C values, we expect that *C. strictum* to be a diploid and may be an ancestor to hexaploid *C. album*.

### ***Chenopodium* Diversity and Glyphosate-Based Species Shift in the North Central States**

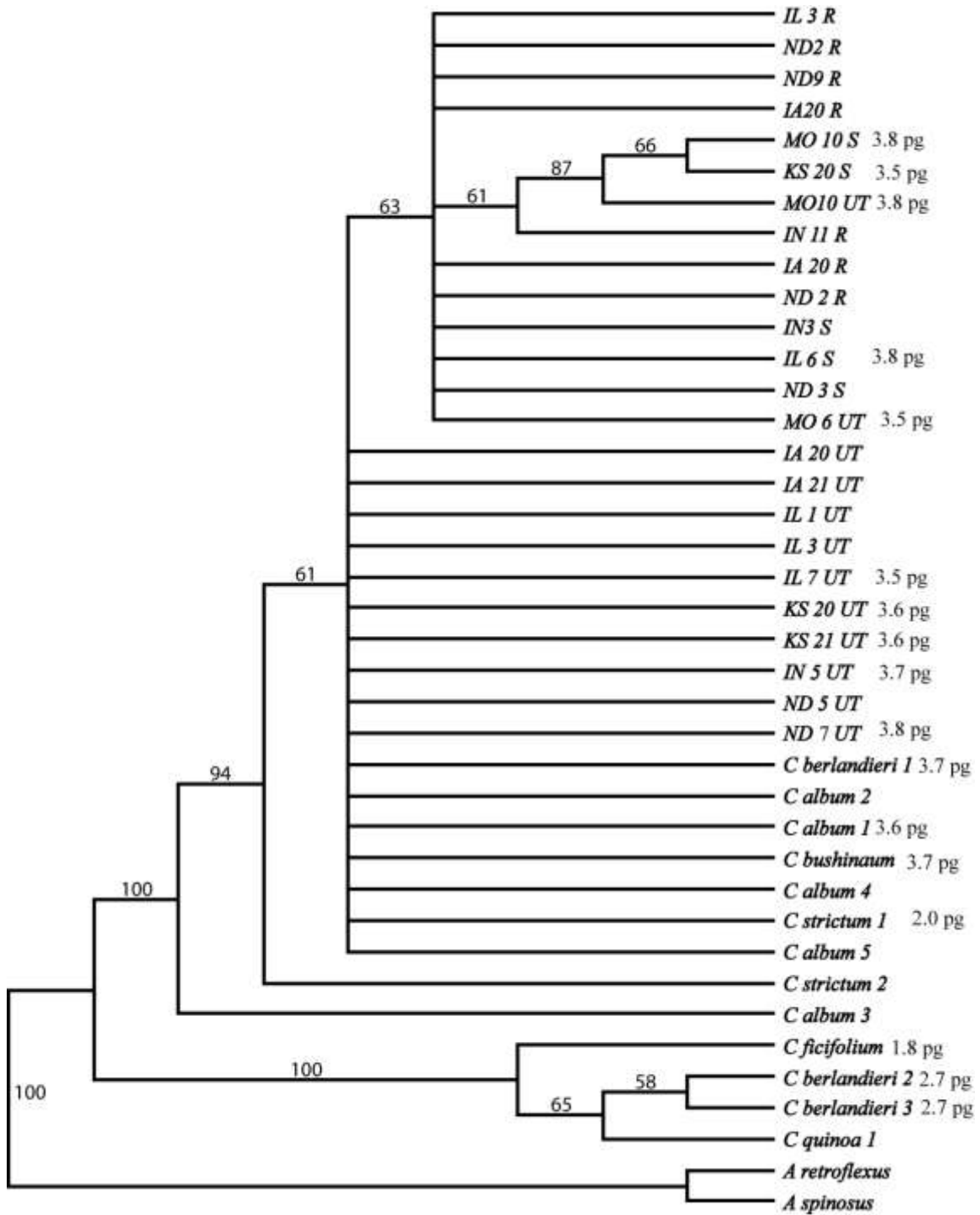
The third objective of our research was to use the informative sequence data and investigate if the samples from North Central states belong to *C. album* or if there is

diversity in *Chenopodium* species which might indicate a species shift. For the ease of amplifying the ITS region with universal primers and having more parsimony informative character (35%), ITS loci was sequences from *Chenopodium* populations. The molecular data from the ITS region was in accordance with the morphological data that is used by the taxonomist for classifying the *Chenopodium* genus (Clemants and Moysakin, 1996).

In chapter 2, we found that when we screened different populations of *Chenopodium* from the North Central states, there was variability in response to the application of glyphosate herbicide. In this section, using the ITS markers, first we investigated if there is any population structure across the North Central states and then we investigated if there is any population structure between glyphosate resistant (R) and sensitive (S) samples.

ITS region from 24 populations of *Chenopodium* populations across the North Central states (7R, 5S and 12 Untreated), were sequenced. The consensus sequences were then aligned with Clustal W and manually adjusted. Neighbor joining (NJ) method of tree construction was used for these *Chenopodium* populations and selected accessions from the *C. album* and *C. berlandieri* groups. Bootstrap values were calculated to estimate the reliability of the tree (Figure 3.13). The NJ tree revealed two results. First, all the *Chenopodium* populations from the North Central states grouped into the same cluster as *Chenopodium album*, indicating that all sampled populations are more closely related to *C. album* than to other weedy *Chenopodium* species. Furthermore, there was no separation based on the geographic location. With additional support from DNA content values and plant morphology (personal observations), we conclude that the samples

tested were different biotypes of *C. album*. The second result is that no apparent structure was observed for R and S samples. Had the tree separated the *Chenopodium* plants based on whether they were resistant or sensitive, it would have indicated that under glyphosate selection pressure, there might be shift in population to more tolerant species. In conclusion, the variability in responses to glyphosate among populations from different states cannot be attributed to variable response among the *Chenopodium* species. Further, the reported increase of glyphosate resistance in common lambsquarters are likely due to evolution of *C. album* rather than a shift towards more tolerant *Chenopodium* species.



**Figure 3.13.** Neighbor joining tree of *Chenopodium* populations sampled across the North Central states and selected species based on ITS sequences. Bootstrap values greater than 50% are shown above the branches.



### 3.5 Conclusions

The *Chenopodium* species were investigated using morphological, cytogenetic and molecular data. Some morphological characters such as surface pattern of pericarps and presence or absence of keel on the perianth are informative characters. Many quantitative characters were also used, but due to phenotypic plasticity and similar growth habits, these characters overlapped within and among the species, hence did not prove to be useful in identifying species. The DNA content analysis proved to be an important technique and helped in separating *C. album* (hexaploid) from *C. berlandieri* (tetraploid) and *C. strictum* (diploid). Using the DNA content values is a challenge if used alone because of the presence of polyploidy *C. album*. This means that *C. album* that is a tetraploid will be no different than *C. berlandieri* or *C. quinoa* as both of these species are also tetraploid.

Sequence data from three loci (ITS, *matK* and *trnD-trnT*) all resulted in similar tree topologies. However, the ITS region contained the most polymorphic sites and hence was considered the best marker in the current study. The phylogeny based on ITS region was well resolved and was in accordance with the taxonomic classification of *Chenopodium* genus (Moyaskin and Clements 2004). The only limitation of the ITS molecular markers was that they did not differentiate *C. strictum* from *C. album*. The best explanation for the grouping of *C. album* and *C. strictum* can be deduced from the DNA content values for these two species. Both are in the same subsect. *Chenopodium* and it is possible that diploid *C. strictum* is a recent ancestor of hexaploid *C. album*. Accessions labeled *C. bushianum* and *C. berlandieri* 1 were found to be more closely related to *C. album* than *C. berlandieri*. The molecular data, DNA content values and morphological

characters all indicate that these accessions are actually biotypes of *C. album* and have been misidentified. Another finding was that all the *Chenopodium* populations sampled across the North Central states belong to *C. album*, so decreased sensitivity to glyphosate is due to variation within that species rather than a shift in species.

### **3.6 Sources of Materials**

<sup>1</sup> SAS 9.2 software, SAS Institute Inc., Cary, NC 27513-2414, USA.

<sup>2</sup> Beckman Institute for Advanced Science and Technology 405 North Mathews, Urbana, IL 61801, USA.

<sup>3</sup> Taq polymerase, Promega Corporation 2800 Woods Hollow Road Madison, WI 53711

<sup>4</sup> QUIAquick PCR Purification Kit, 27220 Turnberry Lane Suite 200, Valencia, California 91355, USA.

<sup>5</sup> AB Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., 850 Lincoln Centre Drive, Foster City, CA 94404, USA.

<sup>6</sup> Sequencher™ 4.7 software, Gene Codes Corporation, 775 Technology Drive Suite 100A, Ann Arbor, MI 48108, USA.

<sup>7</sup> W.M. Keck Center for Comparative and Functional Genomics, University of Illinois Biotechnology Center, 340 Edward R. Madigan Laboratory, 1201 W. Gregory Drive, Urbana, IL 61801, USA.

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## CHAPTER 4

### SUMMARY

#### 4.1 Conclusions and Future Directions

Weed management is a significant challenge in agricultural systems. Every year, new weed species are added to the list of resistant weeds (Heap, 2010). There are several ways to manage weeds but the first step is to correctly identify them. In the past, several researchers have systematically investigated complex weed species (Ogg et al. 1981; Schilling 1981), but not much research has been done in understanding the weedy *Chenopodium* complex. The weedy *Chenopodium* complex is not well understood within the North Central states and there are ongoing reports that the complex is becoming more difficult to control with glyphosate.

The overall purpose of this dissertation was to understand the *Chenopodium* complex and to develop markers that can identify and differentiate various species of *Chenopodium*. Prior to this research, several reports were published suggesting increase in tolerance of common lambsquarters populations to glyphosate. Also, we know from the taxonomic studies in *Chenopodium* that several of the weedy *Chenopodium* species are so closely related that it is very difficult to differentiate the species in the field.

Towards our objectives, we first investigated the response of different *Chenopodium* species to glyphosate, followed by screening populations from the North Central to glyphosate. We observed inter- and intra-species variation in response to glyphosate, and within the samples from the North Central states, we observed variability within and among the populations from one state. Although *C. album* is considered as

the main weed present in agricultural fields, we did find other species (*C. ficifolium* and *C. berlandieri*) that were more tolerant than most *C. album* populations.

The variability in response of *Chenopodium* species and populations led to an interesting question. Are the samples that are less sensitive to glyphosate actually *C. album*, or there is shift in species towards more tolerant *Chenopodium* species. To test the hypothesis of shift in weed species, we developed sequence based markers that can be used to differentiate the species. Results from morphological and molecular markers showed that morphological characters are highly variable and of little taxonomic value. Among the three loci examined, ITS sequence data proved to be most useful in segregating *Chenopodium* species. Some species that shared homologous genes were not resolved, however the DNA content analysis helped in discriminating these closely related species that did not differ in ITS sequences.

Implications from this research are that variability in response to glyphosate among populations from different states cannot be attributed to differences among the *Chenopodium* species. Instead, the increased reports of glyphosate resistance in common lambsquarters is due to evolution within *C. album* rather than a species shift. However, some other weedy *Chenopodium* species outperform *C. album* under herbicide treatments, so farmers should remain vigilant as species like *C. ficifolium* and *C. berlandieri* also have the potential of developing high level of tolerance to glyphosate.

Based on the results from this research working with the *Chenopodium* complex, we conclude that the best approach to study this genus includes:

- 1) Studying morphological characters such as seed pericarp structure (honeycomb pitted or smooth), seed orientation on the fruit (vertical or horizontal) and perianth

structure (keeled or not). All these characters were useful in differentiating some species from others. Most of the vegetative characters have high levels of homoplasy and hence cannot be used successfully.

- 2) Using loci that have high number of polymorphic sites. We investigated ITS, *matK* and *trnD-trnT* spacer, but we should look for loci that are more polymorphic to better distinguish closely related species.
- 3) Using DNA content analysis to determine the genome size which proved to be of great help in differentiating those species which have high similarity in the DNA sequences. We were able to differentiate *C. album* from *C. strictum* mainly due to the difference in their genome size.

A take home message from this research is that all the methods described above for species identification can be successfully used to detect one or another species, but a single marker cannot resolve the whole genus. In this research, we took a multi-faceted approach and were successful in understanding the taxonomic makeup of this weedy complex. Future work should incorporate more molecular, morphological and cytogenetic approaches in understanding complex genomes.

Another important aspect of this genus is its reported potential to form hybrids. In the present study, the hybridization potential was not explored, but based on the congruence of results from nuclear and chloroplast markers, we found no evidence for hybrids in our samples. It will also be interesting to include multiple samples of *C. album* that are diploid, tetraploid and hexaploid and then examine whether there are differences associated with ploidy level. It would also be valuable to explore how tetraploid *C. album* differs from *C. berlandieri*, another tetraploid species in the genus.

This research was not aimed at taxonomic revision or providing a detailed phylogenetic relationship among the species, but it again proved that *Chenopodium* is a complex group and taxonomists should work more on this genus to develop easy identification keys and should explore more informative markers to develop a robust phylogeny. In this research, we showed that to investigate complex problems, weed scientists can use molecular, cytogenetic and phylogenetic approaches, and the combined data will yield more conclusive results.

#### **4.2 Literature Cited**

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