GENETIC CHARACTERIZATION OF THE SELECTED PLANTS OF FAMILY *ASTERACEAE* USING SSR MARKERS

Master of Philosophy

in

PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

By

ZULFIQAR ALI

DEPARTMENT OF PLANT SCIENCES,
FACULTY OF BIOLOGICAL SCIENCES,
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN

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A Dissertation Submitted in the Partial Fulfillment of the Requirements for the Degree of Master of Philosophy in PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY

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DEPARTMENT OF PLANT SCIENCES,
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DECLARATION

This is to certify that the dissertation entitled **"GENETIC CHARACTERIZATION OF THE SELECTED PLANTS OF FAMILY *ASTERACEAE* USING SSR MARKERS"** submitted by **ZULFIQAR ALI** is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of M.Phil in PLANT BIOCHEMISTRY AND MOLECULAR BIOLOGY.

Supervisor: _____

Dr. Tariq Mahmood
Chairman Department of Plant Sciences
Quaid-I-Azam University Islamabad

External Examiner: _____

Dr. Ghazala Kaukab
PMAS, Arid Agriculture University
Rawalpindi

Chairman: _____

Dr. Tariq Mahmood
Department of Plant Sciences
Quaid-I-Azam University Islamabad

Date: _____

Dedicated to

my

Beloved and respected Parents, brothers and sisters
particularly my late brother Yahya

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Endless Admirations to **Allah Almighty,** the most Kind and the Gracious and **Prophet Hazrat Muhammad (SAWW).** The most perfect person with the perfect source of enlightenment and knowledge for all humans.

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Zulfiqar Ali

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LIST OF ABBREVIATIONS

EDTA	ethylene diamine tetra acetic acid
Mya	millions year ago
ITS	internal transcribed spacer
Bps	base pairs
RAPD	random amplified polymorphic DNA
AFLP	amplified fragment length polymorphism
SSR	simple sequence repeat
RFLP	restriction fragment length polymorphism
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
DNA	deoxyribonucleic acid
UV	ultraviolet
STRs	short tandem repeats
SSLPs	simple sequence length polymorphism
nuSSR	nuclear SSR
cpSSR	chloroplast SSR
mtSSR	mitochondrial SSR
EMBL	European molecular biology laboratory
QTL	quantitative trait loci
BLAST	basic local alignment search tool
CTAB	cetyl trimethyl ammonium bromide
mM	mili molar
pH	potential of hydrogen
NaCl	sodium chloride

HCl	hydrochloric acid
µL	micro liter
TE	tris ethylene diamine tetra acetic acid
TAE	tris acetate ethylene diamine tetra acetic acid
dNTPs	deoxy nucleoside triphosphate
$MgCl_2$	magnesium chloride
Rpm	revolution per minute
kb	kilo base pair
PAST	paleontological statistics software package
M	molar
A	adenine
T	thymine
C	cytosine
G	guanine

ABSTRACT

Family *Asteraceae* also called "Aster or Daisy" family is the biggest family of plants with extensive distribution and diverse habitats. The family has highest number of species with 25000 divided into 1600 genera comprising 10 % of the flowering plants. *Asteraceae* is classified into 3 subfamilies, *Barnadesioideae*, *Asteroideae* and *Cichorioideae*. *Asteraceae* possess controversial taxonomic position with particular focus on tribes and sister group associations in *Asteroideae* and *Cichorioideae*. Being the largest family on the land is also the biggest family in Pakistan on the bases of number of species having 650 species in 15 genera with some cultivated forms. In the present study 8 species were collected from different regions of Pakistan (Islamabad, Kheora and Peshawar) and their genetic diversity was studied using 10 SSR primers in which 5 primers gave reproducible results. The data was scored using 0, 1 method and edndrogram was constructed using PAST software. The study suggested that the current members of the family went through remarkable diversification through the course of evolution resulting variation in their DNA. Three pairs of species (*Cosmos bipinneta* and *Bidens bitematta*), (*Helianthus annus* and *Lactuca sativa*) and (*Conyzanthus conyzoidus* and *Parthenium hesterophorus*) were the most closely related species, while *Parthenium hesterophorus* and *Cosmos bipinnata* showed the most distant relationship. Further the study suggested that SSR marker can be effectively used to study genetic diversity in these species. In future, large number of species from diverse environment should be collected and resolved through diverse genetic markers including both sequenced and non-sequenced marker to resolve the position of these species in a better way.

INTRODUCTION

We are unaware of the exact diversity of species of flowering plants however, the probable speculations are between 250,000 and 350,000 number of species. On the bases of extant and accepted number of species families of flowering plants are simply categorized into artificial assemblages. There are extra-large angiosperm families like *Asteraceae*, *Orchidaceae* and *Fabaceae* and according to the website of phylogeny of angiosperm (Stevens, 2001). There are 24,000 species of plants in family *Asteraceae* although it is more than 35000, *Orchidaceae* contains 22,000 species while expected no exceeds above 28,000 and *Fabaceae* comprises of 19,500 species. Therefore, it accounts for 19-26 % of known species of flowering plants which are housed in these major families. These families are present in these main clades of angiosperm that are *Asteroids*, monocots and *Rosids* respectively. These major families do not have strict resemblances and their habits are also very diverse in nature. Many problems are faced by these families, of which is the study of different taxa within these families frequently by different researchers applying various approaches like genes and various molecular level investigation coordination and consolidation of these research findings are not difficult but the thorough evolutionary studies among the groups at the level of family becomes difficult.

1.1. *Asteraceae*

Family *Asteraceae* is among the biggest families of angiosperms with extensive distribution and varied habitats (Funk *et al.*, 2009). With the highest number of species of round about 25000 which proportionate 10 % of all flowering plants, *Asteraceae* is the highly diverse and the largest family of Angiosperm (Stevens, 2001; Funk *et al.*, 2005). In the past 40 million years ago the family underwent a quick tribal variations leading to nearly 1600 genera with around 25,000 species, distributed in a wide range of diverse habitats all over the world (Funk *et al.*, 2005; Barker *et al.*, 2008). *Asteraceae* is cosmic with the exception of Antarctica, with over 1600 genera and 23,000 species excluding apomictic micro species (Jeffrey *et al.*, 2007).

Compositae is subdivided into three subfamilies and 17 tribes (Ghafoor, 2002). In Pakistan *Asteraceae* is the biggest family comprising 15 genera with a species of 650 scattered widely across the country and some of the members under farming (Ghafoor,

2002). It is acknowledged that the family originated in south America and its members inhabit various parts of the world from south Africa, southern Brazil, Australia, Mexico, southwest Asia, middle Asia and southwest America (Bremer, 1994). One of the chief centers of origin of the *Asteraceae* is in the dry high-land of central Mexico (Cronquist, 1981). This family is one of the most unique and isolated members of the class *Magnoliopsida* located in a monotypic order *Asterales* (Cronquist, 1981; Bremer, 1994; 1996) classified *Asteraceae* into 3 subfamilies, *Barnadesioideae* (monophyletic and comprises fewer than 1% of the species in *Compositae)*, *Asteroideae* (also monophyletic and includes 65 % of the species in the family) and *Cichorioideae* (35 % of the species in the family) and 17 tribes, on the whole. The phylogeny of *Asteraceae* even now is in a condition of fluctuation.

Most of *Asteraceae* plants are herbaceous, shrubs and trees spread all over the world, growing in sunny places, in temperate and subtropical areas. They occupy almost everywhere on the earth but are abundant in the regions characterized by changing climate such as mountains, deserts, steppes and prairies, however the explosive number is present in temperate and arid areas and tropical and subtropical mountainous parts. *Asteraceae* plants have numerous morphological features such as capitulum inflorescence, extremely modified and small flowers, inferior bicarpellate ovary developing into a cypsela, basal and upright ovule, and connate anthers, supporting its isolated position. In addition, *Asteraceae* has an evolutionary successful status which may perhaps by possessing a different chemical resistance system producing a combination of polyacetylenes and sesquiterpene lactones (Cronquist, 1981). The occurrence of a capitulum inflorescence (head) that looks as a single flower is considered to be the important sign for the successful evolution attained by *Asteraceae* (Gillies *et al.*, 2002). There are ten to hundred number of small florets in the head like inflorescence of *Compositae* frequently adapted in their function and structure (Harris, 1995). *Asteraceae* is a good example in understanding recent adaptive modifications in plants globally.

1.2. Importance

Asteraceae is an economically essential family as some members provide products as well as cooking oils, lettuce, sunflower seed, artichokes, sweetening agent, coffee alternate and tea. The family is also important in herbal medication, with Echinacea,

Grindelia, Yarrow, and various others. Numerous species have come to be thought insistent, most specially in north America and fresh leaves of dandelion is consumed as salad in Europe. *Asteraceae* plants have been reported to contain essential oils, flavonoids (Harris, 1995) among other substances and possess antimicrobial and antitumor actions. Often these constituents are products of secondary metabolism like phenols steroids, alkaloids and tannins that are synthesized and stored in special areas or in the whole plan body (Parekh *et al.*, 2005).

This family has a remarkable phytochemical variety that includes, terpenoids (specially sesquiterpene lactones), flavonoids, alkaloids, polyacetylenes, and different phenolic compounds. The family *Asteraceae* contains chemical compounds having antimicrobial and antioxidant properties. The *Asteraceae* species contain many biologically active compounds and due to their bioactive properties plants from *Asteraceae* are usually used in treatment of a wide range of diseases (Hammad *et al.*, 2007; Maggi *et al.*, 2009; Chengaiah *et al.*, 2010; Darwish *et al.*, 2010; Achika *et al.*, 2014). *Asteraceae* is appropriate for its cosmetic, therapeutic and aromatic properties (Nakajima, 2001; Hattori, 2008).

Asteraceae includes commercially important food crops such as lettuce (*Lactuca sativa),* chicory (*Cichorium intybus),* globe artichoke (*Cynara scolymus*), Jerusalem artichoke (*Helianthus tuberosus*) and Yacon (*Smallanthus sonchifolius*) etc. Besides from consumption, the seeds of *Helianthus annus* (sunflower) and those of *Carthamus tinctorius* (safflower) can be used to manufacture cooking oil. Other commercially significant species of the family *Asteraceae* are members of the *Tanacetum*, *Chrysanthemum* and *Pulicaria* genera either wild or cultivated, their fresh leaves are eaten as salad and on the other hand its roasted roots are used as a substitute of coffee. Aside its medicinal usage, various food stuffs are also obtained from the processed roots, flowers. and leaves. Moreover, its extracts act as flavoring agents in different cooking food items together with frozen desserts, cooked items, candy, cheese, gelatins, puddings and alcoholic drinks etc. (Leung *et al.*, 1996).

Artemisia is commonly uses as food (culinary condiment), ornamental and forage (Pellicer *et al.*, 2011). The leaves have therapeutic properties due to artemisinin, the best natural drug against malaria and fever. Artemisinin extracted from *Artemisia annua* have

anticancer properties against human breast cancer (Singh *et al.*, 2001) and artemisia also produces aromatic oils used as flavorings, vermifuges, hallucinogens and in pharmaceutical industries. Further, oils are used in preparing soaps and coloring materials obtained from *Carthamus tinctorius* and *Serratula tinctoria*. Rubber is present in the tissues of *Parthenium argentatum* and leaves of *Lactuca sativa* and tubers of *Helianthus tuberosus* are edible. *Wedelia calendulacea* is used as hair tonic. Marigold is a multi-use flowering plant of family *Compositae* (Taylor, 2011). The demand for marigold is very high for decorative purposes due to its showy and attractive garden plant (Vasudevan *et al.*, 1997), manufacturing cosmetics and therapeutic industries (Maity *et al.*, 2011), in bioremediation (Sun *et al.*, 2011), as food coloring agent (Barzana *et al.*, 2002) and aroma treatment (Marotti *et al.*, 2004).

Sunflower is a cultivational crop and it is grown not only for ornamental purpose but also its seeds are source of cooking oil and snacks. Since ancient times, safflower has been cultivated for the extraction of oil and dye on a large scale of areas. It is among the earliest domesticated crop plant (Knowles *et al.*, 1989). Today its cultivation has reduced to an area of less than 1 million hectares around the world and has become a minor crop (Yeung *et al.*, 1983; Faostat, 2009). Chrysanthemum has medicinal characteristics as their extracts can be a raw material in tea and dye production. Economically, the varieties and hybrids of chrysanthemums (*Chrysanthemum grandiflorum* and *C. indicum*) are the best among decorative plants around the globe (Dowrick, 1952). Essential oil of *Bidens dracunculifolia* has enormous commercial value in the perfume industry. That contains transnerolidol as its main component, has been regulated through the Food and Drug Administration (FDA) and used normally as a flavoring agent in the food industry (Heywood, 1993; Park *et al.* 2004: Arruda *et al.*, 2005; Gilberti, 2012).

1.3. Taxonomy

Asteraceae is the biggest family of dicotyledonous plants (23,000 species) and has been recognized due to the systematic complexities for very long time. Morphological commonalities among the family members have caused complications to identify conventional features which can be reliable in phylogenetic studies (Carlquist, 1976). Cassini was the first who proposed the natural association in *Asteraceae* and to introduce tribal division in the family dividing it into 19 tribes (Cassini, 1826). Noteworthy efforts

were also made by (Bentham, 1873) who lowered the number of tribes to 13 and Cronquist put tribe *Heliantheae* to the base of his 12 downsized tribes (Cronquist, 1955). In 1980 Hoffmann documented two separated ancestries *Liguliforae* and *Tubuliforae* in *Asteraceae* (Hoffmann, 1890). In *Liguliforae* the only tribe *Lactuceae* was included while in *Tubuliforae* all the rest of the tribes were put together. Succeeding taxonomists have also accepted these two lineages in this family, however circumscription varies considerably. Among these major revisions (Carlquist, 1976) was possibly the first who recognized an extended *Cichorioideae* (*Liguliflorae*) by placing 6 tribes within each of his subfamilies, *Cichorioideae* and *Asteroideae*.

During the beginning of 1980, the discovery and the following investigations of a useful evolutionary chloroplast DNA inversion in *Asteraceae* (Jansen *et al.*, 1987) along with the morphological work by (Bremer, 1987) and others confirmed that the former *Barnadesinae* (in *Mutisieae*) was monophyletic. It was revealed that this subtribe was the basal group in the *Asteraceae* and valuable of being recognized as the archaic subfamily of *Barnadesioideae*. Consequently, from these and other morphological and molecular studies (Bremer, 1987; Kim *et al.*, 1992; Michaels *et al.*, 1993; Gustafsson *et al.*, 1995) it is becoming apparent that *Asteraceae* evolved in South America (Bremer *et al.*, 1992) and is perhaps sister to the South American endemic family *Calyceraceae*. Phylogenetic relationships within the *Asteraceae* have long been an area of dispute starting with (Cassini, 1826) and continuing to the present. The tribal circumscriptions of tribes such as the *Helenieae* and *Eupatorieae* are still very much uncertain. There are 12 accepted subfamilies in the *Asteraceae*, but four of those subfamilies, *Mutisioideae*, *Carduoideae*, *Cichorioideae*, and *Asteroideae*, include 99 % of the species. The family is further classified among 13 tribes, *Arctotideae*, *Calenduleae*, *Astereae, Senecioneae, Mutisieae, Heliantheae, Lactuceae, Cynareae, Liabeae, Anthemideae, Eupactorieae* and *Vernonieae*. Since the introduction of molecular systematic protein-encoding gene sequences have been very helpful for solving higher-order questions of classification (Chase *et al.*, 1993). South America has long been debated to be the place of origin and early evolution of *Asteraceae*. (DeVore *et al.*, 1995; Stuessy, 1996).

Recently, Lundberg (2009) documented an ancestor of *Asteraceae* in Southern South America just north of Patagonia sometime around middle or late Eocene. A quick

radiation occurred globally in *Asteraceae* through the Neogene (Graham, 2003; Barreda *et al.*, 2010). Presently fossil record study from middle Eocene 47.5 Mya (million years ago) northwest Patagonia revealed identification of the first capitulum of *Asteraceae* (Barreda *et al.*, 2010). Furthermore, from the time period of strata containing fossils showed *Asteraceae* diverged from its sister family *Calyceraceae* very long ago contradicting the previous assumptions. According to Bremer (1994), family *Asteraceae* can be separated into three subfamilies *Barnadesioideae, Cichotioideae* and *Asteroideae* in which *Barnadesioideae* is smaller subfamily and other two subfamilies are considerably bigger (Bremer, 1994). Monophyletic nature of *Asteroideae* is substantially proved from molecular (Kim *et al.*, 1995; Bayer *et al.*, 1998) morphological evidences (Bremer, 1987; Karis, 1993; Bremer, 1994;). However, *Cichorioideae* is less supported by morphological data and may be paraphyletic (Karis *et al.*, 1992; Kim *et al.*, 1995).

Taxonomic restrictions of tribes and sister group associations in *Asteroideae* and *Cichorioideae* has been an issue for long time (Cassini, 1826; Bremer, 1994; Heywood *et al.*, 1977) but there are changing views (Bremer, 1994). Monophyletic *Barnadeioideae* appears in the previous studies as parallel group to remaining family (Jansen *et al.*, 1987; Bremer *et al.*, 1992). Earlier systematic works resulted in recognition of main clades (Bremer, 1987; Kim *et al.*, 1992; Kim *et al.*, 1995; Bayer *et al.*, 1998). On the other hand, relationships among some of these clades are not well determined. DNA findings particularly DNA sequences have played significant role to understand classification phylogenetic evolution in *Asteraceae* (Jansen *et al.*, 1994). It is especially correct for the genera that have missing or unclear morphological records (Francisco *et al.*, 1997; Kim *et al.* 1998; Panero *et al.*, 1999; Francisco *et al.*, 2001; Karis *et al.*, 2001; Park *et al.*, 2001). DNA sequence data for taxonomic studies of *Asteraceae* generally gathered from the studies of these regions, trnL-F, ITS and ndhF gene regions (Taberlet *et al.*, 1991). The trnL-F sequence portion although comparatively short (nearly 700 bps) is useful for the study of delimitation in tribes and infrageneric phylogenetic in *Asteraceae* (Bayer *et al.*, 1998; 2000; Fernandez *et al.*, 2001).

Modern angiosperm classification systems (Dahlgren, 1980; Takhtajan, 1980; Cronquist, 1981) stress the phylogenetic seclusion of the family keeping the family in a highest position and monotypic order of class *Dicotyledonae*. Due to largest number of

species, *Asteraceae* is found in extremely diverse locations and has a universal occurrence. Moreover, there is some disagreement relating to the age of the family (Turner, 1977; Boulter *et al.*, 1978), fossil proof (Cronquist, 1977; Muller, 1981) and biogeographical considerations (Raven *et al.*, 1974) which relates the origin of *Asteraceae* about 30 million years ago in Oligocene. During the previous 30 years, six strikingly different schemes of taxonomic interrelationship of *Asteraceae* at tribal and subfamily level have been suggested (Cronquist, 1955; Carlquist, 1976; Wagenitz, 1976; Cronquist, 1977; Bremer, 1987; Jeffrey, 1978; Thorne, 1992). Even though there is common harmony that two distinct subfamilies (*Asteroideae* and *Cichorioideae*) should be recognized but there is no agreement regarding the circumscription of the subfamilies, the number of monophyletic tribes, and the relationships among the 12 to 17 recognized tribes.

1.4. The history of the taxonomic relationships of *Asteraceae*

Despite of replication of whole genome and advances in pappus and capitulum head taken place close to the beginning of the family still earlier ancestors that originated in south America which resembles in background diversification rates with *Calyceraceae* and its sister *Goodeniaceae* in Australia. Rate of diversification instead increased steadily till the time *Asteraceae* has evolved around 69.5 Mya during end of Cretaceous Era. Compared to former studies, remarkable proportion of changes are not tightly interrelated to polyploidization and continental dispersion. The subfamilies *Cichorioideae, Asteroideae, Carduoideae, Gymnarrhenoideae, Pertyoideae* and *Corymbioideae* in Africa has diverged comparatively within short period of 6.5 Mya only through the middle Eocene (Barreda *et al.*, 2010; 2012). Till the Miocene, *Asteraceae* was cosmic nature and its occurrence (Scott *et al.*, 2006) with more than 20000 species at that time and a cosmopolitan distribution (Barreda *et al.*, 2010) so *Asteraceae* is possibly the biggest family (Zavada *et al.*, 2010).

The discovery of a recently well preserved fossil present nearly in biomes from the depositions of Eocene except in Antarctica (Funk *et al.*, 2005). It is evident from its fruits that the family was once part of old *Asteraceae* that are single seeded Cypselae. Numerous other flora that occupied southern Gondwana prior to species could have transformed structure of pappus well suited for dispersal of seeds to greater distances beyond sea borders (Bremer *et al.*, 1997; Katinas *et al.*, 2007). Remarkably just a little number of genera in *Asteraceae* happen to present in southern South America, Australia, New Zealand

and rarely outspreading to Asia (Allan, 1961; Moreira *et al.*, 2007; Ezcurra *et al.* 2008). *Lagenophora cass.* of *Astereae* an example of such genus.

1.5. *Asteraceae* in Pakistan

The *Asteraceae* family includes of 23000 number of plants classified in 1530 genera 17 tribes and 3 subfamilies with its distribution in worldwide and being the largest family on the land is also the biggest family in Pakistan on the bases of number of species and it has 650 species in 15 generas with some cultivated forms (Ghafoor, 2002). In Pakistan the genus *Anaphalis* of tribe *Gnaphalieae* includes 15 species and tribe *Plucheae* consists of 5 genera and 9 special and intraspecific taxa (Qaiser *et al.*, 2003). There is also representation of 49 species in 11 genera of *Inuleae* tribe in Pakistan (Qaiser *et al.*, 2003) and the tribe *Senecioneae* is represented by 39 species in 9 genera (Qaiser *et al.*, 2011). Pakistan is represented by the tribe *Anthemideae* with 91 plant species in 22 genera and 9 subtribes *Artemisinae, Marticarinae, Cancirinae, Achilleinae, Chrysantheminae, Tanacetinae, Leucantheminae, Handelinae* and *Anthemidinae*. Tribe *Anthemideae* of the family *Asteraceae* is recorded in Pakistan with 22 genera and 91 species (Bremer, 1994; Ghafoor, 2002).

1.6. Molecular markers

For the assessment of variation and diversity homologies molecular markers are authentic substitute over phenotypic markers because these are not affected due to environmental factors, developmental stage of the plant or by the type of the tissue (Collard *et al.*, 2005). Molecular markers are helpful method for associating the difference between genotype and phenotype and also evaluating genetic diversity (Varshney *et al.*, 2005). Evaluation of genetic variation using molecular markers is helpful for screening of cultivars and varieties, hybrid selection in breeding and to conserve genetic wealth (Garner *et al.*, 2004). Characteristics for a perfect molecular marker includes codominant, extremely polymorphic, high reproducibility, unbiased and impartial, genomic abundance, simple and quick analyses and easy exchange of data between laboratories (Joshi *et al.*, 1999).

The level of polymorphism and genetic relationship have been studied by different molecular markers i.e. RAPD (Randomly Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length

Polymorphism) and more recently SSR markers (Wünsch *et al.*, 2002). These molecular markers are categorized as (1) PCR-based markers i.e. (AFLPs), (RAPDs), Inter simple sequence repeats (ISSR) and SSR or microsatellites and (2) Markers based upon hybridization i.e. RFLPs, and (3) Sequence based markers i.e. SNP single nucleotide polymorphism (Varshney *et al.*, 2007; Sehgal *et al.*, 2008). Most of the markers are derived or product of either genomic DNA library like (SSR and RFLP) or by random amplifications of genomic DNA through PCR such as RAPDs or both i.e. AFLPs (Varshney *et al.*, 2007).

1.6.1. RFLP (Restriction fragment length polymorphism)

It is technique in which DNA is cut into fragments at particular nucleotide sequences with the use of restriction endonucleases. Different sized fragments are separated through gel electrophoresis followed by southern blotting. Desired fragments are selected and identified through hybridization with radioactive probe. Usually different individual produce varying length of DNA fragments on digestion with endonucleases. It acts as a fingerprint and different genotypes, plant species and sometimes individual plants are identified and differentiated due to this polymorphism (Karp *et al.*, 1998).

1.6.2. RAPD (Random amplified polymorphic DNA)

RAPD is a technique using PCR to amplify random or desired DNA parts by arbitrary primers. The amplification results are products of an area of genome which consists of two short sequences in reverse direction on the complementary strands that match the primer sequence. The amplification products are size fractionated with gel electrophoresis (Jones *et al.*, 1997).

1.6.3. AFLP (Amplified fragment length polymorphism)

Recently the new PCR based method AFLP has been developed. It is actually a transition of RFLP and PCR techniques. Principal of AFLP is to cleave fragments of DNA amplified via PCR. The genomic DNA is cleaved by endonucleases creating restriction sites and adaptors with complementary sequence are ligated to these sites followed by amplification by PCR. Finally, the products are observed by autoradiography or fluorescence method segregated on a non-denaturing polyacrylamide gel (Vos *et al.*, 1995; Jones *et al.*, 1997).

1.6.4. Microsatellites

Microsatellites or also called SSRs are portion of DNA containing recurrent repeats of one, two, three, four and five nucleotides which are present across the whole genomes of almost all eukaryotes (Powell *et al.*, 1996). Due to higher potential of diversity of SSR marker are in particular suitable to discriminate intimately related genotypes also preferred in population studies (Smith *et al.* 1994) and in selection of closely resembling cultivars (Vosman *et al.*, 1992).

1.7. SSR Marker

Microsatellite markers (Litt *et al.*, 1989), are known by different names of simple sequence repeats SSR (Jacob *et al.*, 1991), short tandem repeats STRs (Edwards *et al.*, 1991), simple sequence length polymorphism SSLPs (Tautz, 1989) are short stretch of tandemly repeated sequence ranging from single to six nucleotides with recurrent presence in the genome of prokaryotic and eukaryotic individuals studied so far (Zane *et al.*, 2002). Presence of microsatellites were confirmed by (Hamada *et al.*, 1982) among variety of eukaryotic yeast to vertebrates. Later Delseny *et al.* (1983) and Tautz *et al.* (1984) proved that plants and several other eukaryotes are also rich in SSR. The AC repeats are abundant in animals while plants have majority of AT repeats most (Powell *et al.*, 1996). Microsatellites are found in coding as well as noncoding sites of the whole genome in addition to chloroplast DNA (Provan *et al.*, 2001; Chung *et al.*, 2006) and in genome of mitochondria as well (Soranzo *et al.*, 1999; Rajendrakumar *et al.*, 2007).

Microsatellites have little copy number per locus of 5 to 100 randomly scattered presence of nearly 104 to 105 in every genome (Tautz, 1993) and high level of polymorphic in nature (Zane *et al.*, 2002). Variations in repeat number of SSR motifs cause the greater degree of length polymorphism consequently their detection and reproducibility is easy with PCR. SSR markers are amenable for high throughput genotyping, therefore they are enormously useful for mapping beneficial genes, establishing genetic and evolutionary relations, parentage studies and constructing bulk genomic maps and marker based selection (Parida *et al.*, 2009). For the last two decades, population genetics has been studied employing SSR markers because of codominance and its high polymorphic property (Tautz, 1989). Microsatellites have extensively been used in linkage analysis of genotype at intraspecific level. They have also been extensively used to analyze the genetic

relationships between genotypes at the intraspecific level however are seldom employed in phylogenetic deduction. However, they have seldom employed for deducing taxonomic relations due to cause behind this is the restraint of SSR on the size range of allele (Garza *et al.*, 1996; Goldstein *et al.*, 1997), maximum rates of mutations also scattered in the genomes of chloroplasts and mitochondria and nucleus.

On the bases of position of SSR in the genome, they are grouped into different forms like nuclear SSR (nuSSR), chloroplast SSR (cpSSR) and mitochondrial SSR (mtSSR). Weising *et al.* (1999) reported chloroplast SSR (cpSSR) in *Nicotiana* plant whereas mitochondrial SSR (mtSSR) was confirmed by Soranzo *et al.* (1999) in *Pinus* tree. Amongst the available molecular markers, microsatellites have acquired substantial applications in plant breeding and genomics because of various useful properties such as abundance, codominance, reproducible results, multi allele copy, comparative abundance, wide range genomic existence including presence in organellic genomes, chromosomal specificity, high throughput genotyping and suitability for automation. Maximum level of allelic variations in SSR results in differences in repeats sequences on a locus due to slippage in replication or uneven crossing over in meiotic division.

SSR markers are designed and produced direct from libraries of genomic DNA and can also be derived from libraries rich in particular microsatellites. On the other hand, they can be sourced out via searching public catalogues like EMBL and GenBank and also by transferring across the species. Now among molecular markers, microsatellites are the broadly utilized marker in relation to genetic studies as they have a number of advantages such as technical ease, comparatively low expensive, high genetic resolution power and high polymorphism. Microsatellites are applied to estimate genetic diversity, classification, evolutionary changes, systematic relations and QTL study in various crops (Cho *et al.*, 2010; 2011; Dixit *et al.*, 2010; Moe *et al.*, 2010; 2012; Lu *et al.*, 2012; Yoon *et al.*, 2012; Zhao *et al.*, 2012; Khaing *et al.*, 2013). Furthermore, they are consistent and easy to score (Gupta *et al.*, 2000). Microsatellites being very polymorphic should give information about species barriers. The most essential shortcoming due to increased rate of alteration make them homoplasious marker. Yet studying different loci minimizes the drawback but dominant markers have the advantage of potentially informing hybridization amongst the species (Duminil *et al.*, 2006).

1.8. DNA barcoding

A new means of identification of species developed by Hebert *et al.* (2003; 2004) and his team is known as DNA barcoding. In DNA barcoding technique short sequence of DNA segment is studied from a standard locus for the identification of species. It is a latest technique in the field of biology aimed to identify species fast, precisely and automatically just with the help of a small piece of DNA. This method has been broadly studied particularly for authenticating the Chinese pharmaceutical plants (Hebert *et al.*, 2003; 2004).

Recently the prevalently studied DNA barcodes universally in plants are *ITS*, *matK*, *rpoC1*, *rbcL* and *psbA-trnH* (Yao *et al.*, 2009; Ma *et al.*, 2010). Among different barcodes ITS has confirmed to be the ideal and universal barcode for identifying plants and their close species, since *ITS*2 successfully differentiated 92.7 % cases of above 6600 samples among seven phyla of gymnosperms, algae, mosses, angiosperms, ferns, fungi and liverworts (Chen *et al.*, 2010). Expanding the abilities of barcodes like this taxonomist can significantly increase and ultimately compile a worldwide catalog of diversity of living organisms.

For a strong DNA barcode, it must contain an isolated non-overlapping genetic difference in between inter and intraspecific specimens (Moritz *et al.*, 2004). The taxonomy introduced by Linnaeus will be more manageable and easy to access by implementation of DNA barcoding which will be beneficial to environmentalist, conservators and different agencies involved in controlling alien species, food safety and pest use. In broad sense, the day will come by virtue of DNA barcoding when all inquisitive brains whether biologists or school going students shall definitely have knowledge of nomenclature and biological characteristics of any species in the world. Besides identification of species, the barcoding process will speed up the rate of discovery of new species enabling taxonomists to swiftly arrange species showing diverge taxa so that they can be indicated new species. Although the sequence of the DNA barcode is extremely small compared with the total genomic DNA but these are isolated very rapidly and inexpensively (Kress *et al.*, 2005). The ability of resolution by single at the species level in plants with the help of basic local alignment search tool BLAST (Altschul *et al.*, 1990) was found for matK 99 %, *trnH-psbA* 95 % and *rbcL* 75 %. Using three DNA barcode simultaneously showed above 98 % accurately

identifying 296 tree species, date palm and shrubs (Kress *et al.*, 2009). Presently a team of researchers working on DNA barcode in plants suggested two genes in chloroplast *matK* and *rbcL* collectively is a suitable barcode for plants (Group *et al.*, 2009).

1.9. Objectives

The present work involves the genetic study of plants of *Asteraceae* using SSR marker aimed to find out;

1. The evolutionary and phylogenetic associations among the selected plant species with the help of molecular analysis.
2. To determine effectiveness of SSR as a molecular marker in *Asteraceae*.

MATERIALS AND METHODS

2.1. Collection of plants and preservation

Desired plants were collected from various geographical areas of Pakistan and identified by taxonomist. And the samples were preserved at 04°C with silica gel in zipper bags for long term uses. The plants were identified by experts in taxonomy. Details of plants and their area of collections are given in (Table 2.1). All plant species belong to a single order *Astrale*. *Conyzanthus conyzoidus, Xanthium strumarium, Cosmos bipinnata, Bidens bitemata* and *Parthenium hesterophorus* are members of subfamily *Asteroideae*. *Halianthas annus* belongs to subfamily *Helianthoideae* while *Lactuca sativa* and *Sonchus asper* belong to *Cichorioideae* subfamily.

2.2. Processing of plant specimens

Leaf material was detached from the plants and washed in distilled water and sprayed by 70 % ethanol for sterilization. After this the leaves were left to dry. About 0.4 grams of leaf material was weighed to process the isolation of DNA. The CTAB process with little changes was applied to extract DNA (Richards, 1997).

2.3. Preparation of CTAB

2x CTAB (cetyl trimethyl ammonium bromide) buffer was formed by mixing 20 mM EDTA (ethylene diamine tetra acetic acid) of pH 08, 1.4 M NaCl (sodium chloride) and 100 mM tris HCl (pH 08.) and kept at room temperature for few hours to settle down.

2.4. DNA isolation

Genomic DNA was isolated by CTAB protocol. Leaf tissue was ground with the pestle and mortar mixed with already warmed at 65 °C 2x CTAB buffer as per requisite (1000-1500 µL) and 1 % beta marceptoethanol in the ratio of 10µL per 1000 µL of CTAB buffer. The mixture was crushed and the homogenized mixture was poured into 1.5 ml eppendorf tubes. The tubes were placed on hot plates at 65 °C for about 45 minutes. After this centrifugation of tubes were done for 20 minutes at 10000 rpm. After centrifugation the supernatant was collected and transferred through a micropipette in a new eppendorf tubes.

Equal volume of chloroform isoamylalcohol 24:1 was added into the supernatant and tubes were shacked gradually for two to three times. Tubes were again centrifuged and

supernatant was shifted to new tubes and this step was performed 3 to 4 times until it turns clear white. Later then washing an equal amount of chilled isopropanol was added into the tubes in order to precipitates the DNA. This was followed by gentle shaking and the tubes were kept at -20 for overnight. The next day tubes were again centrifuged for 20 minutes at 12000 rpm. Isopropanol was discarded and white pellet was washed in 70 % ethanol. Pellet was allowed to dry at the room temperature followed by addition of 40 µL 0.1x TE (tris ethylene diamine tetra acetic acid) RNase buffer into the tubes and heated on hot plates for 20 minutes at 37 °C and preserved in -20 to use ahead.

2.4.1. Confirmation of quality of genomic DNA

Absence or presence and quality of DNA was confirmed through gel electrophoresis. 1 % agarose gel was formed in 0.5x TAE (Tris acetate ethylene diamine tetra acetic acid) buffer. Extracted DNA and loading dye (orange blue or bromophenol blue) was mixed together properly and loaded using micropipette in the wells of gel. Gel was run at 80 volts for 30 minutes. When the dye reached about one third of the length of gel electric current was switched off and the gel was stained in ethidium bromide for 20 minutes and finally observed in UV light of gel doc.

2.5. Primer selection

Five SSR primers, MFC1, MFC2, MFC3, MFC4, and MFC 6 were selected to evaluate the genetic diversity among 8 selected plants of *Asteraceae*. Details of these primers are given in (Table 2.2)

Table 2.1: Species name of plants along with taxonomic details and site of collection.

S.N	Spp. Name	Sub-family	Order	Loc. name	Lat. & Long.
1	*Conyzanthus conyzoidus*	*Asteroideae*	*Asterales*	Islamabad	33.7 °N, 73.09 °E
2	*Xanthium strumarium*	*Asteroideae*	*Asterales*	Islamabad	33.7 °N, 73.09 °E
3	*Cosmos bipinnata*	*Asteroideae*	*Asterales*	Islamabad	33.7 °N, 73.09 °E
4	*Bidens bitemata*	*Asteroideae*	*Asterales*	Peshawar	34.01 °N, 71.5 °E
5	*Parthenium hesterophorus*	*Asteroideae*	*Asterales*	Islamabad	33.7 °N, 73.09 °E
6	*Halianthas annus*	*Helianthoideae*	*Asterales*	Kheora	32.6 °N, 73.01 °E
7	*Lactuca sativa*	*Cichorioideae*	*Asterales*	Islamabad	33.7 °N, 73.09 °E
8	*Sonchus asper*	*Cichorioideae*	*Asterales*	Islamabad	33.7 °N, 73.09 °E

Key: S. N; serial number, **Spp;** species, **Loc;** location, **Long;** Longitude, **Lat;** latitude

Table 2.2: Five SSR primers studied in genetic diversity analysis with their sequence, repeat motifs and annealing temperature below.

S. N	Primer	Forward and reverse primers	Rep. mot	Ann. temp
1	MFC1	F: CTAGACTGAAAAAACATTGC	(CT)13	55
		R: TGAGATTGAAAGGAAACGAG		
2	MFC2	F: GCTTCCGATGCTGCTCTTA	(AC)18(AT)7	55
		R: TCGGAGACTTTTGTTCAAT		
3	MFC3	F: GATATTTTCATGTTTAGTTTG	(AC)15TC(AC)8(AT)7	55
		R: GAGGATAGACCAACAACAAC		
4	MFC4	F: CCAAATTTTAGATACAACTT	(AT)4(AC)11	55
		R: TTTCTCAACATATTAACAGG		
5	MFC6	F: AGGCTACTTCAGTGCTACA	(TAA)3(GT)8	50
		R: GCCATAAGTAATAAAAACC		

Key: S.N; Serial number, **Rep. mot;** Repeat motif of each primer, **Ann. temp;** annealing temperature.

2.6. Polymerase chain reaction (PCR)

Five SSR primers were used to quantify the SSR marker region. Details of components of PCR reaction mixture are given in Table 2.3.

Table 2.3: PCR components for amplification of SSR markers.

S. No	Reagents	Concentration	Volume
1	Nano pure water		16.2 µl
2	*Taq* DNA polymerase	1.5 U	0.3 µl
3	Farward primer	25 pmoles	1 µl
4	Reverse primer	25 pmoles	1 µl
5	*Taq* buffer	10 X	2.5 µl
6	$MgCl_2$	25 mM	1.5 µl
7	dNTPs	2 mM	1.5 µl
8	Total volume		25 µl

Key: S. No; Serial No

2.6.1. PCR reaction conditions

Standard PCR conditions for amplification of DNA are

Initial denaturation at 95 °C for 4 minutes

1. subsequently 35 cycles at 94 °C 40 seconds for denaturation
2. primer annealing temperature ranging from 50 °C to 55 °C hold for 45 seconds.
3. Elongation at 72 °C temperature hold for 45 seconds
4. Final elongation for 20 minutes at 72 °C.

Table: 2.4 PCR steps and conditions for the amplification of SSR markers in different species of *Asteraceae*.

S. No	PCR steps	Temperature	Time	
1	Initial denaturation	95 °C	4 minutes	
2	Denaturation	94 °C	40 seconds	⎫
3	Annealing	50 °C	40 seconds	⎬ 35 cycles
4	Extension	72 °C	45 seconds	⎭
5	Final extension	72 °C	20 minutes	

The PCR results were confirmed by gel electrophoresis, running on 2 % agarose gel. Amplified products of PCR were mixed with the loading dye and transferred into wells of gel by micropipette one by one and gel was run along with 1kb DNA ladder to compare the size of the amplified bands. To stain the Gel was immerged in ethidium bromide tank. Finally, stained gel was observed in UV radiations and gel images were saved.

2.7. Data Analysis

The data was scored directly from the gel in the form of "0, 1" method, 0 for absence and 1 for presence and properly arranged in excel sheath (2010). Dendrogram was constructed using Neighbour-joining tree making method by exporting the data to Paleontological Statistics Software Package (PAST) (Hammer *et al.*, 2009).

RESULTS

3.1. Extraction and quantification of genomic DNA

DNA was extracted from fresh leaf tissue of eight plants of family *Asteraceae* by CTAB method with few changes. The isolated genomic DNA was loaded on 1 % gel and observed in UV gel doc to check the presence and quality of DNA for PCR (Figure 3.1). amplified using different SSR primers in PCR machine. The amplified PCR products were run on 2 % agarose gel with 1 kb ladder. different PCR bands of various range size of 980 bp to 1200 bp were produced (Figure 3.2).

3.2. PCR Optimization

Genomic DNA was amplified using a gradient PCR of different range of temperature 50 to 56 °C. Finally, the annealing temperature was optimized and most of the results were amplified at annealing temperature of 55 °C and 50 °C. Primers MFC1, MFC2, MFC3 and MFC4 gave amplification at 55 °C while MFC6 at 50 °C.

3.3. Primers Results

Five SSR primers were applied to determine the genetic diversity among eight plants of *Asteraceae*. The primers were MFC1, MFC2, MFC3, MFC4 and MFC6. The Primer MFC1 showed high polymorphism and genetic diversity. Primer MFC1 produced maximum number of bands (28 amplifications) followed by MFC3 primer which produced (25 amplifications). The MFC4 primer resulted in the lowest number of amplifications while MFC2 and MFC6 gave (11 amplifications) and (9 amplifications) respectively. Storable number of bands were 59. *Lactuca* sativa and *Sonchus asper* gave highest number of bands with MFC1, MFC2, MFC3. *Lactuca sativa* produced the highest number of bands in throughout the four gels. Total amplified alleles were 26 with a mean value 5.2 per primer.

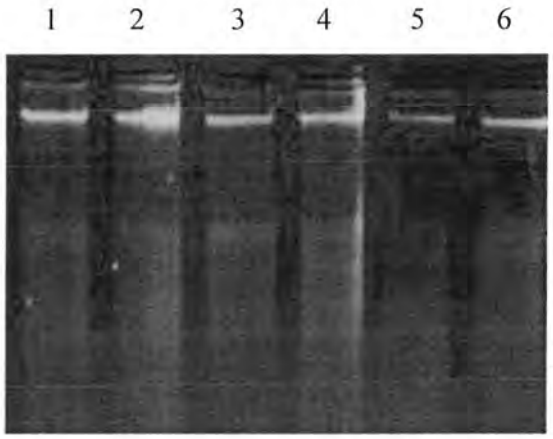

Figure 3.1: Genomic DNA of plant species, 1: *Helianthus annus,*2: *Lactuca sativa,* 3: *Sonchus asper*, 4: *Parthenium hesterophorus,* 5: *Bidens bitemata* 6: *Xanthium strumarium*

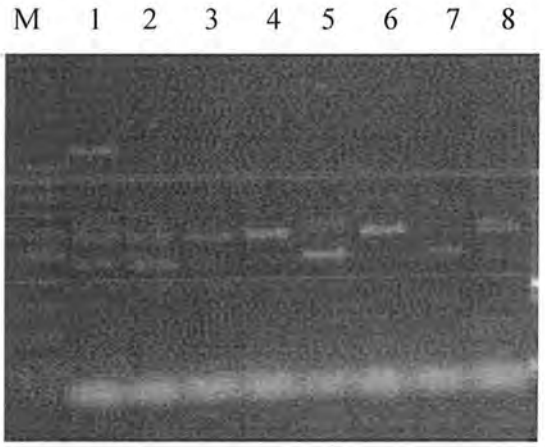

Figure 3.2: PCR results of SSR primer MFC1 of the selected plants of *Asteraceae.* M: marker (1kb ladder), 1: *Conyzanthus conyzoidus*, 2: *Bidens bitemata,* 3: *Cosmos bitemata,* 4: *Lactuca sativa,* 5: *Sonchus asper*, 6: *Parthenium hesterophorus,* 7: *Helianthus annus* 8: *Xanthium strumarium.*

3.4. Phylogram analysis based on SSR primers

A similarity matrix for assessing genetic diversity and affinity was produced with the help of amplified SSR primers data amongst the eight species of *Asteraceae* family. Denrogram of the species was made based upon the genotype results using 0 and 1 methods. It consists of two clades, two clusters and two groups. Two major clades, *clade* 1 and *clade* 2 revealed close resemblance and a genetic diversity of about 5 % among all the specimen was portrayed from the branching length (Figure 3.3).

3.5. Clade 1

Clade 1 comprised of six species which are *Cosmos bipinneta*, *Bidens bitematta*, *Xanthium strumarium*, *Halianthas* annus, *Lactuca sativa* and *Sonchus asper*. The clade 1 was further condensed into cluster 1 and cluster 2. All genotypes displayed 13 % resemblance. Maximum similarity was observed between *C. bipinnata* and *B. bitematta. Sonchus asper* is the most recent species in the clade and overall also. *X. strumarium* is the most primitive species covering little distance on the scale with a genetic diversity of 62.5 %.

3.5.1. Cluster 1

Cluster 1 consists of 3 species i.e. *Cosmos bipinneta*, *Bidens bitematta* and *Xanthium strumarium*. A similarity of 13 % was observed among the species of *cluster* 1. *Cluster* 1 was yet again separated into group 1. *X. strumarium makes sister group with C. bipinneta*, and *B. bitematta.*

3.5.2. Group 1

Group 1 contains two plant specimens *Cosmos bipinneta* and *Bidens bitematta.* These specimens revealed 30 % similarity. Genetic diversity between the two species was about 75 %.

3.5.3. Cluster 2

There are three specimens viz *Halianthas annus, Lactuca sativa* and *Sonchus asper* in the *cluster* 2. Among these 30 % genetic similarity was obtained. *Cluster* 2 is further divided into group 2. Highest genetic diversity was demonstrated in *cluster* 2. Among the species *S. asper* is the most genetically diverse species scoring almost 90 % diversity, hence, perhaps representing more recent species. *S. asper* forms sister group with *H. annus* and *L. sativa* of group 2

3.5.4. Group 2

Group 2 is represented by *Helianthus annus* and *Lactuca sativa*. Overall, the genetic diversity among these members were found to be approximately 80 %. Group 2 indicates 30 % genetic similarity and therefor closely resembles with group 1.

3.6. Clade 2

Clade 2 was represented by only 2 species, *Conyzanthus conyzoidus* and *Parthenium hesterophorus*. Genetic similarity of clade 2 was about 13 % observed between its two species. *C. conyzoidus* shows 80 % genetic diversity and *P. hesterophorus* indicated almost 70 % diversity.

Overall the data from the denrogram showed that all the species carried 75 % diversity using 5 SSR markers. Three pairs of species were the most similar (*Cosmos bipinneta* and *Bidens bitematta*), (*Helianthus annus* and *Lactuca sativa*) and (*Conyzanthus conyzoidus* and *Parthenium hesterophorus*). In addition, *C. bipinneta* and *P. hesterophorus* were the most distant and diverse species forming remote relationship. Furthermore, it can be concluded from the phylogenetic tree that *S. asper* is the most genetically diverse species hence probably more recent species while *X. strumarium* may be the most primitive species with the lowest diversity. *X. strumarium* also forms sister group with group 1.

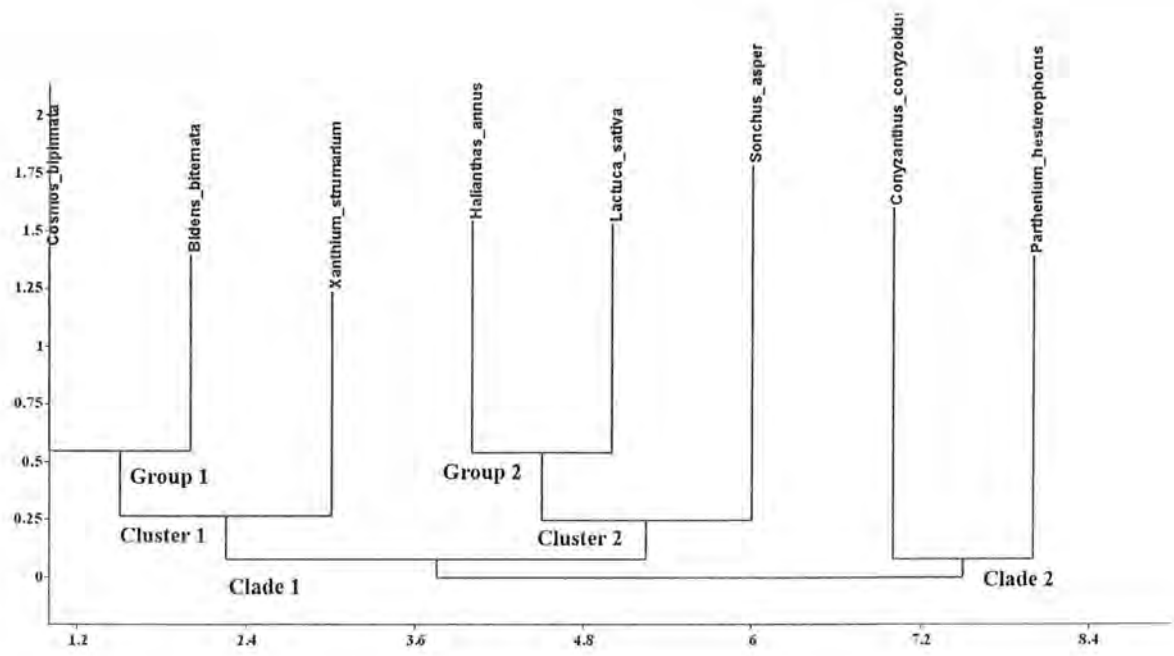

Figure 3.3: Dendrogram showing genetic diversity among various species of family *Asteraceae*.

DISCUSSION

4.1. Molecular diversity based on SSR

Genetic diversity is crucial for conservation and restoration of wild species. Plants with highest genetic diversity can be used to produce breeds that can be incorporated into local cultivars to produce environment friendly and stable species. Up till now, a large number of markers have been studied in assessing genetic variations among wild species of different genera of *Asteraceae* (Lawson *et al.*, 1994; Gong *et al.*, 2010; Wang *et al.*, 2012) for example Gerbera (*Gerbera hybrida*) (Chapman *et al.*, 2009), Safflower (*Carthamus tinctorius* L.), Sunflower cultivar (Solodenko *et al.*, 2005; Hvarleva *et al.*, 2007) and Lettuce (Riar *et al.*, 2011). Family *Asteraceae* is one of the important families that carries considerable importance in stabilizing the natural wild flora with worldwide distribution. Many of them are under serious threat of endangerdness. To our best knowledge no significant study has been conducted using these species with SSR.

As SSR markers have been applied to estimate genetic diversity of plants of *Compositae* (Lawson *et al.*, 1994; Hernández *et al.*, 1999; Beck *et al.*, 2014). The present work also involves the study of genetic diversity of selected plants of family *Asteraceae* using SSR markers. These SSR primers produced 26 alleles with an average of 5.2 each primer. Allele frequency was recorded 11.8. Four of the markers yielded significant variations in allelic diversity.

In the present work first time the MFC series of SSR primers have been used against any plants of *Asteraceae*. Scorable bands of total 59 were amplified. *Helianthus annus* produced highest number of bands of 12. Ten number of bands were scored by *Lactuca sativa* and *Sonchus asper* each. The lowest number of bands amplified were four bands each by *Xanthium strumarium*, *Cosmos bipinnata* and *Parthenium hesterophorus*. Earlier these primers studied in finding genetic diversity in Fig plants (*Ficus carica* L.) (Khadari *et al.*, 2001; Baraket *et al.*, 2011; Abou *et al.*, 2014).

In our study the average alleles per locus was 5.2. This result is in agreement with the results 6.66 alleles per locus in Fig plant (Saddoud *et al.*, 2005) and 2 to 7 alleles per primer in tagetes species (Whankaew *et al.*, 2014). While it is lower than that of 9.5 alleles per primer in Fig plant (Baraket *et al.*, 2011), average 16.3 alleles in *Rheum officinale*

(Wang *et al.*, 2012) and mean 16.7 alleles per locus for chrysanthemums (Khaing *et al.*, 2013) but higher than 1.89 average value of alleles by *Opisthopappus* (*Compositae*) (Wang, 2013). The difference in these results may be due to fewer number of plants and minimum number of primers used.

For five microsatellite loci studied in *Ficus carica* cultivars. Excluding the locus MFC2, experimental heterozygosity was greater as compared to predicted data (Khadari *et al.*, 2001). Alleles scored by six MFC SSR primers for 38 Fig species were 57. For each primer number of alleles ranged from six to fourteen for (MFC7) and (MFC8) primers scored an average 9.5 alleles (Baraket *et al.*, 2011). In a recent study of Fig plants, six SSR primers were applied in identification of seven Fig cultivars which resulted in 51 amplifications of 40 to 740 bp size. Among the 51 bands 36 fragments were polymorphic and rest were monomorphic. MFC1, MFC6 and MFC8 primers yielded the maximum number of bands, while MFC2 and MFC3 produced 2 particular fragments. Moreover, MFC7 failed to generate any bands (Abou *et al.*, 2014). In an another work, MFC2, MFC3, MFC5, MFC6, MFC7 and MFC8 primers were again used in Fig plant. Size range of bands varied from 118 bp for (MFC5) to (MFC2) 228 bp. Different range of alleles were produced by different primers viz, MFC5 and MFC6 four alleles and MFC3 twelve alleles. Average value of alleles per locus was 6.66. It implies occurrence of high level polymorphism of SSR in Fig species. (Saddoud *et al.*, 2005).

In an another study, 153 individuals of two species of *Opisthopappus* were studied using Nine cpSSR primers. Seventeen alleles with 1.89 average per locus was obtained and monomorphic loci scored was 4 on the other hand 5 loci were polymorphic. cpSSR 3, cpSSR 4, cpSSR5, each with 3 alleles were the most variable loci. Further the percent polymorphism of loci extended from 0.00 to 44.44 %, accounting an average of 16.24 % (*Compositae*) (Wang, 2013). SSR markers were employed in sunflower and found useful for the identification of sunflower hybrid genotypes (Lawson *et al.*, 1994). SSR was studied in three *Solidago* subsections, the 14 SSR loci were variable and number of alleles per locus found to be seven to 51, and all loci were polymorphic in all three subsections of *Solidago* (Beck *et al.*, 2014).

In a study of *Rheum officinale* 10 SSR loci found very polymorphic and the number of alleles for respective locus ranged from 8 to 18 hence total 163 alleles were identified

and average number of alleles for every locus was 16.3 which is higher than our results. Average heterozygosity in the seven populations was 0.45 to 0.89 per locus with average 0.66 for all loci (Wang *et al.*, 2012). Eight SSR loci in the lettuce plants were polymorphic among 307 plants of 15 populations. In all members per locus alleles number was in a range of 10 to 44 alleles for Se-194 and Se-136 respectively. Within population number of alleles ranged from 2 to 17 with average of 7.03 (Riar *et al.*, 2011). In the population study of chrysanthemums overall 200 alleles were identified corresponding to a mean value of 16.7 alleles for all loci. Allele size was in a range of 134 to 500 bp (Khaing *et al.*, 2013). In a study of isolating and characterizing SSR primers in *Atractylodes macrocephala,* out of 20 primer pairs, five primers were monomorphic and 15 were polymorphic. The allele sizes varied from 77 to 297 bp and the number of alleles at all loci vary from 2 to 20 with an average 9.9 alleles amongst the four populations (Zheng *et al.*, 2012).

In sunflower Among the 26 loci eight SSR loci were selected due to their polymorphism. These eight loci showed 47 alleles. Number of alleles at every locus was observed in arrange of 2 to 10 alleles. Heterozygosity level was for the eight loci was found 0.217 to 0.755 (Solodenko *et al.*, 2005). In an another study, out of 32 SSRs studied 20 of them generated particular fragments. Among these 20 SSRs eleven SSR primers were polymorphic accounting for 34.38% of the developed SSRs. Number of alleles at every locus was in a range of 2 to 7. Majority of primers yielded 2 alleles for all loci. Majority of the primers amplified two alleles per locus. Every allele showed polymorphism, hence, polymorphism of all loci was 100% in *Tagetes* species (Whankaew *et al.*, 2014)

Microsatellites in plants were first time obtained and duplicated in tropical tree species. Recognized repeats were dinucleotide repeats of Poly (AC) and poly (AG) at a rate of 5×10^3 to 3×10^5 in every genome. The AG repeats were more common relative to AC repeats. On average a repeat > 20 bp exist per 33 kb in the nuclear genome of plants in comparison of after each 6 kb in mammals. TG or AC repeats are maximum in animals while plant genomes are rich in A-T this feature differentiate animal genomes from plant genomes. The capability of using identical primers of SSR in various species of plant is related to degree of the SSR primer flanking sites that are preserved among closely resembling taxa and the consistency of the SSR across evolutionary history (Powell *et al.*, 1996).

Among the DNA markers microsatellites or SSR are the most useful markers due to their high polymorphism, codominant, extremely plentiful, and rationally simple (Gupta *et al.*, 2000; Varshney *et al.*, 2005). Preceding studies have shown that SSR primers generated in a species are applicable for other closely related species (Wang *et al.*, 2006) implying that within a family SSR loci are intact. Other research works also disclosed the sharing SSR loci common among various species and genera (Davierwala *et al.*, 2000; Wang *et al.*, 2006; Zhou *et al.*, 2009), also a great ratio of the SSR which are polymorphic can be cross amplified (Dong *et al.*, 2009; Zhou *et al.*, 2009). Due to enhanced transferability ratio, the SSRs are significant reservoir for polymorphic markers to evaluate the genomics of comparatively unidentified species (Fahima *et al.*, 2002; Wang *et al.*, 2006). It is supposed that SSRs are locus specific (Holton *et al.* 2002) and di- and trinucleotide repeats are the most recurrent types (37.1 and 40.0% respectively) of repeats among SSR, followed by hexa-nucleotide repeats (11.3%). (Gao *et al.*, 2003). Length of the repeats highly correlated with variability of SSR markers (Goldstein *et al.* 1995; Innan *et al.*, 1997).

Dendrogram clearly shows that three pairs of species were the most similar (*Cosmos bipinneta* and *Bidens bitematta*), (*Helianthus annus* and *Lactuca sativa*) and (*Conyzanthus conyzoidus* and *Parthenium hesterophorus*) which may be due to similarity in their environmental conditions through the course of evolution (Bayer *et al.*, 1998) Similarity between *C. Bipinneta* and *B. bitematta* in group 1, is understood and valid because both species belong to same subfamily and order i.e. *Asteroideae* and *Asterales*. Parallel evolution and resemblance between *H. annus* and *L. sativa* in group 2, can be conferred by existing in the same order, *Asterales*, although their subfamilies are different i.e. *Helianthus* is a member of *Helianthoideae* and *Lactuca* belongs to *Cichorioideae* (Grant, 1958). However, *Sonchus asper* and *Xanthium strumerium* showed diverged positions and diversity. This may be because they their subfamilies are different but they exist in the same order *Asterales*. *X. strumerium* belongs to *Asteroideae* and S. *asper* belongs to *Cichorioideae* and also perhaps due to variations in their natural conditions resulting mutation in their genomic sequences. Furthermore, *S. asper* showed primitive position and highest diversity which represented its evolution in a totally separate environment from the rest of the species. Moreover, *Parthenium hesterophorus* and *C.*

bipinneta were the most diverged species separated by a large distance i.e. at et the extreme ends of the tree, despite of presence in the subfamily *Asteroideae* common to both, therefore it may be due to significant variation in their genomic sequence (Anderberg *et al.*, 1992; Stussey *et al.*, 1996; Chen *et al.*, 2010)

4.2. Conclusions

The study suggested that the current member of the family went through remarkable diversification through the course of evolution resulting variation in their genomic DNA. Further, the study revealed significant variation in these species based on SSR marker. three pairs of species (*Cosmos bipinneta* and *Bidens bitematta*) (*Helianthus annus* and *Lactuca sativa*) and (*Conyzanthus conyzoidus* and *Parthenium hesterophorus*) were the most closely related while *Parthenium hesterophorus* and *Cosmos bipinnata* showed the most distant relationship. Further the study suggested that SSR marker can be effectively used to study genetic diversity in these species. In future, large number of species from diverse environment should be collected and resolved through diverse genetic markers including both sequenced and non-sequenced marker to resolve the position of these species in a better way.

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Department of Plant Sciences

Plagiarism certificate

It is certified that **Mr. Zulfiqar Ali S/O Ali Khan** whose registration number 02041413002 has completed his M. Phil. The title of his thesis is **"Genetic characterization of selected plants of family *Asteraceae* using SSR marker"**. His thesis has been checked on "Turnitin" for similarity index and found 6% that lies in the limit provided by HEC (19%).

Dr. Tariq Mahmood

Chairman

Department of Plant Sciences,

Quaid-i-Azam university, Islamabad

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