CHARACTERIZATION AND DIVERSITY ANALYSIS OF WHITE CLOVER *(Trifolium repens)* ACCESSIONS OF DIFFERENT GEOGRAPHICAL ORIGIN

THESIS

By

SARBJIT SINGH

Submitted to

CHAUDHARY SARWAN KUMAR
HIMACHAL PRADESH KRISHI VISHVAVIDYALAYA
PALAMPUR – 176 062 (H.P.) INDIA

IN

Partial fulfilment of the requirements for the degree

OF

MASTER OF SCIENCE IN AGRICULTURE

(PLANT BREEDING)

2003

Dedicated
To
My
Revered
Parents

Dr. T.R. Sharma
Associate Professor

Advanced Centre of Hill
Bioresources & Biotechnology
CSK HP Krishi Vishvavidyalaya,
Palampur 176062 (H.P.) INDIA

CERTIFICATE – I

This is to certify that the thesis entitled **"Characterization and diversity analysis of white clover *(Trifolium repens* ...) accessions of different geographical origin"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science (Agriculture)** in the subject of **Plant Breeding** of Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, is a bonafide research work carried out by **Mr. Sarbjit Singh** son of **S. Taranjit Singh,** under my supervision and that no part of this thesis has been submitted for any other degree or diploma. The assistance and help received during the course of this investigation have been fully acknowledged.

(T.R. Sharma) 11.11.
Chairman
Advisory Committee

Place : Palampur
Dated: the Nov. 11 th, 2003

CERTIFICATE – II

This is to certify that the thesis entitled **"Characterization and diversity analysis of white clover *(Trifolium repens*) accessions of different geographical origin"** submitted by **Mr. Sarbjit Singh** son of **S. Taranjit Singh,** to Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, Palampur, in partial fulfilment of the requirements for the degree of **Master of Science (Agriculture)** in the subject of **Plant Breeding**, has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

9-12-2003

Dr. T.R. Sharma
(Chairman
Advisory Committee)

External Examiner
9/12/2003

Dr. S.K. Sharma
(Member) 9/12/03

9/12/03

Dr. D.K. Banyal
(Member)

9/12/03

Dr. J.C. Bhandari
(Member)

9-12-03

Dr. A.K. Sood
(Dean's nominee)

Head
Department of Plant Breeding & Genetics
CSK HPKV, Palampur

Dean
Post Graduate Studies

ACKNOLEDGEMENT

First of all, I would like to thank soulfully, The Almighty God who bestowed me with his blessing to accomplish this investigation. I feel privileged to express my deep sense of gratitude, appreciation and heartfelt thanks to my major advisor, Dr. T.R. Sharma, Associate Professor, Advanced Centre of Hill Bioresources and Biotechnology for his keen interest, guidance, inspiration and encouragement during the course of this investigation.

I express my heartiest thanks to the members of my advisory committee, Dr. S.K. Sharma, Dr. J.C. Bhandari, Dr. D.K. Baniyal and Dr. R.P. Kaushal for their help in the study. I am also thankful to the Head, Department of Plant Breeding and Genetics for his help during my study.

My sincere thanks are due to all the faculty members of the department for their kind co-operation and continuous encouragement during this investigation.

I am highly thankful to the Programme Director, Advanced Centre of Hill Bioresources and Biotechnology, for providing facilities to undertake RAPD analysis. I am also thankful to Dr. V.K. Gupta, Dean Postgraduate studies, for providing help and facilities for the completion of this investigation. Sincerest thanks are due to Dr. Sindhu Sareen for providing me the required seed material.

I am immensely grateful for the help and encouragement rendered by Dr. Rajeev Rathour, Dr. Rajan Sharma and Dr. Anil Kant during course of my study.

I express my deep gratitude for the help, love and support of Amitoj, Chhabra, Sony, Simran, Purba, Sanjeev, Yadwinder, Ravinder, Navpreet, Gagan, Vikram, Nitish, Bahman, Panditji, Sandy Sir, Nitin Sir, and Salej Sir.

Also sincere thanks are reserved for whole hearted support of Vanita, Madhu, Madam Dorin, Shivali, Anju, Shiwani, Meenakshi throughout my study period.

I am thankful to Harnamji, Trilokji, Amarnathji, Raghuji, Kewalji for their ever-ready help.

I would like to thank my dear parents, my source of strength and inspiration. I can never forget the constant inspiration and support given by my brothers, sisters and Bhabis. My heartfelt thanks and appreciation are for all those to whom I am indebted on various occasions but have not named personally.

Needless to say, all errors and omissions are mine.

Place: Palampur
Date:

Sarbjit Singh 12/11/06
(Sarbjit Singh)

CONTENTS

Chapter	*Title*	*Page*
I	INTRODUCTION	1-3
II	REVIEW OF LITERATURE	4-13
III	MATERIAL AND METHODS	14-27
IV	RESULTS	28-44
V	DISCUSSION	45-56
VI	SUMMARY	57-59
	LITERATURE CITED	60-68
	APPENDIX	69-70

LIST OF TABLES

Table No.	*Title*	*Page*
3.1	Details of the material and seed characteristics used in the present study	15
3.2	List of 10-mer OPERON primers used to generate RAPD bands/ fragments	27
4.1	Analysis of variance	29
4.2	Mean, range and variability parameters of different traits	30
4.3	Non Hierarchial Euclidean Cluster analysis	38
4.3.1	Distance between the cluster centroids	38
4.4.1	Reaction to powdery mildew	40
4.4.2	Reaction of white clover accessions to *Sclerotinia trifoliorum*	41
4.5	Number of scorable and polymorphic RAPD bands obtained in the PCR amplified DNA of white clover accessions	43
5.4	Agro-morphological markers	52-53

LIST OF FIGURES

Table No.	*Title*	*Between pages*
1	Dendrogram of 28 *Trifolium repens* accessions generated by RAPD data using the UPGMA method	43-44

LIST OF PLATES

Plate No.	*Title*	*After page*
1	White clover growing in the field (A) and *Sclerotinia trifoliorum* infected white clover plants (B)	40
2	RAPD profiles of 28 accessions of white clover generated through DNA amplification with primers of OPC20 (A) and OPF15 (B); M denotes the molecular weight marker	42

Introduction

Chapter I

INTRODUCTION

White clover, *Trifolium repens* L. is one of about 250 species belonging to the genus *Trifolium*. It is believed that ancestral forms of *Trifolium* originated in North America, migrated across the Pacific to Eastern Asia, and on to Mediterranean where white clover and other 150 *Trifolium* species evolved (Zohary, 1970).

It is a cross-pollinated tetraploid (2n=4x=32) forage legume due to gametophytic self-incompatibility and is native to Mediterranean (Vavilov, 1951). It is economically important forage legume of temperate and subtropical environments around the world, where soil moisture is adequate for its growth. White clover is distributed from Arctic Circle to cool temperate regions of the world. In India, it is spread over the hilly areas of Himachal Pradesh, Jammu & Kashmir, parts of northeastern states, Uttaranchal and parts of Tamil Nadu. It was spread to other continents by the colonists.

White clover grows in a wide range of habitats including dry meadows, mud flats, banks of river and brooks, plains, semi-desert regions and mountains up to the sub-alpine meadows. It is one of the most nutritious and widely grown forage legumes in the world and contributes a considerable quantity of nitrogen to the sward because of its symbiotic relationship with rhizobia bacteria. Although the annual nitrogen fixation

levels in grazed pastures are extremely variable, ranging from 17Kg N/ha/year in infertile, unimproved hill pastures (Grant and Lambert,1979) to 380Kg N/ha/year in intensively managed pastures (Rumball,1979). Higher clover content in diet increased nutritive value, energy and protein intake (Thomson, 1984; Harris *et al.*1998).

Because of its stoloniferous growth and phenotypic plasticity, it is an ideal companion legume in most grass swards (Woodfield and Caradus, 1994). As feed for the animals, white clover grass mixture is superior to that of grass alone. Clover forage is highly digestible, nutritious and palatable. It is generally high in mineral content. Feed value of white clover is largely attributed to its increased protein content. Though white clover is reported to be most promising forage crop (Doyle, 1989), but wide spread use of this high quality forage is inhibited by problems such as unreliable yield and lack of persistence under intense grazing (Rhodes and Webb, 1993).

Cultivars/ecotypes well adapted to specific environments and management systems can significantly enhance pasture production system. The variability of germplasm substantially influences the choice of breeding material and success of breeding programmes. The low genetic variability necessitates reinforcement of the white clover breeding programmes through introduction of new germplasm and collecting the local ecotypes. The genetic variability in Indian collections of white clover is not very encouraging, due to its localized distribution. So, there is need to collect,

characterize and evaluate local white clover germplasm along with introduced elite material for its use in breeding and conservation programmes. Genetic variation provides the basis for tailoring desirable genotypes. Traditional morphological markers used for characterization and varietal differentiation suffer from environmental influence and are developmental stage specific. In the recent years, several molecular techniques have been used for germplasm characterisation, variety identification, marker development and identification, molecular diagnostics, phylogenetic studies and diversity analysis. Because of its simplicity, rapidity and reliability, the RAPD technique has been used extensively for diversity analysis. This approach utilizes primers of arbitrary sequences, it may be used for different species without previous knowledge of their genomic DNA sequences. Moreover, RAPD markers represent whole of the genome.

Keeping this in view the present study "Characterization and diversity analysis of white clover (*Trifolium repens* L.) accessions of different geographical origin" was under taken with following objectives:

1. Characterization of white clover genotypes of distinct geographical origin.
2. Agronomic evaluation of genotypes for important forage characteristics.
3. Estimation of genetic parameters.
4. Evaluation of genotypes for some forage quality parameters.

Review of Literature

Chapter II

REVIEW OF LITERATURE

Selection is one of the potent tools for the crop improvement programme. However, it is operative and effective only if there is sufficient genetic variability in the crop species. Vavilov (1951) pioneered to recognize the importance of variability in the initial material for developing superior genotypes. In order to achieve the success on long-term basis there is need to characterize and evaluate the material using morphological and agronomic characteristics. More recently, PCR-based molecular markers have found favour among plant scientists for studying patterns and extent of genetic variation in the germplasm accessions.

Various variability parameters that give an idea about the extent and nature of variability in a given crop species include genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability [h^2(bs)], genetic advance (GA) and association (r) among various component characters.

A review of these aspects is given under the following sub-heads:

2.1 Morphological characterization

2.2 Agronomic and quality evaluation

2.3 Component analysis

2.4 Molecular diversity

2.1 Morphological characterization

Characterization consists of recording observations on those characters, which are highly heritable, can be seen by the unaided eye and are uniformly expressed in all environments. Jahufer *et al.* (2002) advocated the exploitation of genetic variation in white clover and also stressed the need to characterize the germplasm collections and to estimate parameters of genetic variability.

Analysis of morphological characters in single spaced plants and their row-sown progenies revealed much greater variation in single spaced plants (Wioncek *et al.,* 1976). Sidhu and Mehndiratta (1976) observed significant differences among the progenies of berseem for all the agro-morphological characters under study, indicating the presence of significant genetic variability.

Harper (1977) and Burdon (1980) observed variation among white clover plants in a single field. Their study showed that in a random sample of 50 plants, nearly every plant was found to differ significantly from every other plant in at least one character as in stolon length, petiole length, peduncle length, leaf area, leaf markings, average number of flower heads etc. Variation in shape, position and intensity of leaf markings in *Trifolium repens* has been reported to be controlled by a complex genetic locus (Corkill, 1971). Further genetic polymorphism and geographic variation in distribution of leaf markings were also observed.

Caradus and Williams (1981) selected *Trifolium repens* lines with small leaves and dense stolon production as these lines performed better than the ones with medium sized leaves on low fertility soils. However, they were inferior on high fertility soils. Caradus (1994) documented genetic variation in white clover for various morpho-physiological and yield related traits, and further stressed to relate this variation to origin in improving white clover performance. Similarly, Jahufer *et al.* (1997) characterized and evaluated 439 collections of white clover for morphological attributes and grouped them accordingly.

Jahufer and Gawler (2000) attributed significant genetic differences for agro-morphological characters in a set of 53 accessions of white clover from distinct geographical origins. Bulked AFLP analysis of leaf samples of *Trifolium repens* showed similar results. Similarly, Sareen (2003) found high agro-morphological variability in 200 populations of white clover having varied geographical origins.

2.2 Agronomic and quality evaluation

It consists of recording observations on potential agronomic characters and quality parameters desired by users of a particular crop. It includes tailoring/branching, time of flowering and maturity, planting data, leaf characters, floral characters, fruiting and seed characters. From quality point of view Wilkin and Humphreys (2003) advocated the breeding of temperate forage species for improving the economic and environmental sustainability and breeding clover with high feeding value, crude protein, *in vitro* dry matter digestibility (IVDMD), water soluble carbohydrates and fibre.

Bhandari *et al.* (1975) studied the performance of 22 diverse white clover accessions and reported sufficient genetic variability for fodder yields. Productive varieties of *T. repens* were reported as having large leaves with fewer stolon whereas persistence types had smaller leaves and many stolons and were less productive (Ahloowalia, 1977). Similarly Annicchiarico (1993) reported sufficient genetic variation for dry matter yield and agronomic traits in white clover land races and natural populations.

Russi and Falcinelli (1999) studied natural populations of 3 different species of *Trifolium* and found that the flowering time, leaf area, stolon length were observed to be the important characteristics for discriminating the populations. Genetic variation for important agronomic characters for eleven Norwegian populations of white clover exhibited considerable genetic variation between populations for spring growth, morphological characteristics, herbage yield, general performance and seed yield (Aasmo *et al.,* 2000).

Lawson (1971) found high heritability values for herbage yield, growth habit in 9 clonal genotypes of *Trifolium pratense.* Seed weight per plant, seeds number/floret and number of heads per plant were the yield components which contributed the most to seed yield. Sidhu and Mehndiratta (1976) found high estimates of heritability for plant height, leaf length, leaf width and moderate for herbage yield. Similarly, Vanbogaert (1977) found that number of florets per head was variable and highly heritable. Similarly, number of seeds per pod was highly heritable in *T. repens.* Salisbury *et al.* (1987) observed that number of days to flowering in *Trifolium* spp. was under polygenic control and this character has shown high heritability estimates.

Phenotypic and genotypic coefficients of variation estimated for seed yield and seed index were lower compared to herbage yields in berseem (Bakheit, 1988). These result in lower estimates of broad sense heritability of seed yield compared to forage yields. The heritability values were high for fresh, dry and protein yield. Similarly Broda (1980) observed highest broad sense heritabilities for seed yield components and leaf area in red clover *(Trifolium pratense).*

Lee *et al.* (1993) evaluated a collection of white clover populations for characters associated with productivity and persistence to assess the extent of genotypic variation. He found that genetic coefficient of variances and broad sense heritability estimates were high for petiole length and leaf area. Similarly, higher heritability values were found for stolon diameter, leaflet width and petiole length and lowest for stolon elongation (Caradus and Chapman, 1996). Avtar *et al.* (1997) found highest GCV for plant height followed by herbage yield in *Trifolium alexandrinum* L.

Hill *et al.* (1988) found that the genetic advance of modern varieties over landraces and old cultivars derived from landraces is relatively low in forage legumes compared with other crops such as grain cereals. The similar results were found by Woodfield and Caradus (1994). Obviously, differences in reproductive system, genetic structure, breeding objective and time span needed for evaluation of materials during the selection process can largely account for such results (Hill *et al.,* 1988; Lorenzetti, 1992). But Annicchiarico

and Piano (1997) suggested that low genetic advance in forage legumes may be due to possible selection, under the severe intraspecific competition occurring naturally during cultivation.

Gill *et al.* (1967) reported that white clover contained, on an average, 25.82 per cent of crude protein as compared to 4.82 to 9.88 per cent of crude protein in herbage from indigenous grasses in sub-tropics. Belliti *et al.* (1972) studied the effect of crude fibre content on digestibility of the diet. They noted that the calves fed on lucerne hay containing 31.34% CF with concentration to give 25.6 or 30.2% CF in the diet, had higher *in vitro* digestibility for low level of CF in diet.

Sharif (1989) reported that acid detergent fibre (ADF) and neutral detergent fibre (NDF) are better indicators of roughage digestibility than CF alone. Frame (1993) observed that white clover is a main pasture legume in many temperate grasslands, and contributes nitrogen through fixation, improves nutritive value of herbage, and complement the growth pattern of the main grass species.

Wilman and Moghaddam (1998) compared the digestibility and neutral detergent fibre content in white clover and other forage legumes species and found that leaflets of *T. repens* had an appreciably lower neutral detergent fibre content than stolons and petioles. Stolons of *T. repens* were much more digestible than stems of *Medicago sativa* and *D. intortum.*

Sareen (2003) evaluated local and exotic white clover collections for various quality parameters. The crude protein varied from 15.36% to 26.68%, ADF (42.98% to 53.26%) and NDF (33.45 to 42.18%) on the dry matter basis.

2.3 Component analysis

Principle component and non-hierarchial euclidean cluster analysis is used for comparing the genotypes. Accessions or genotypes are classified into broad groups. This approach has been used in different crops by various workers (Walia and Garg, 1996; Garg and Gautam, 1997). However there is little information available on its application in fodder crops.

Jatasra and Goyal (1982) grouped 30 strains of alfalfa *(Medicago sativa* L.) in 6 clusters on the basis of D^2-statistics and observed wide genetic diversity for almost all characters investigated. Caradus *et al.* (1989) classified 109 white clover cultivars into 4 groups using Principle Component Analysis (PCA) and cluster analysis. The most important criteria for distinguishing between groups were leaf size, cyanogenesis and combination of these. More than 800 accessions of red clover representing 41 countries were evaluated and large variations were found for most characters over all origins. Clustering produced three distinct groups that corresponded to early, medium and late maturity groups (Kouame and Quesenberry, 1993). Jahufer *et al.* (1997) characterized and evaluated 439 collections of white clover for morphological attributes, viz., internodal length, leaf length, plant height, plant spread and herbage yield. They conducted both cluster analysis and PCA that grouped accessions into 7 groups based on their morphological studies.

Rosso *et al.* (1997) characterized the white clover collections of different countries. Multivariate (Principle Component) analysis of the data showed that there was genetic diversity in the germplasm evaluated. Clustering of accessions based on morphological and physiological traits produced 5 groups of populations with similar phenotypic characteristics, and made it possible to relate these groups to their geographical origin.

Bennett (2000) used Principal Component Analysis (PCA) for analyzing genetic variation in *Trifolium repens* for eleven morphological and flowering characters. He observed that five species could be separated morphologically by principal component analysis and cluster analysis. The most significant source of genetic variation was found to be related to geographical distribution.

Kolliker *et al.* (2001) used bulked AFLP analysis of *Trifolium repens* to assess genetic diversity. A significant amount of genetic variability among 52 cultivars and accessions was detected. However, cluster analysis partially reflected the geographical origin of the accessions and also showed disagreement with cluster analysis based on morpho-physiological characters.

2.4 Molecular diversity

Molecular markers became available in appreciable numbers, a prominent application has been in the assessment of genetic diversity both of wild populations from an ecogeographical point of view, and of crop germplasm collections and varieties from a breeding point of view. Studies

have indicated that RAPD (Random Amplified Polymorphic DNA) is a powerful method for genotype identification, population and pedigree analysis, phylogenetic studies and genetic mapping (Welsh and Mc Celland, 1990; Martin *et al.,* 1991; Mazzarella *et al.,* 1992).

Bullita (1995) advocated the use of RAPD markers for taxonomic studies in *Trifolium* spp. Reproducible amplification products were obtained from different varieties and species belonging to the genus. RAPD markers revealed polymorphism that appeared to be useful for taxonomic studies at population and species level.

Genetic variation was estimated within and between cultivars of red clover *(Trifolium pratens* L.), using morphological, isozymes and Random Amplified Polymorphic DNA (RAPD) markers (Kongkiatngam *et al.,* 1995). Similarly, Bullita and Piluzza (1997) using natural populations of *Trifolium pratense* (red clover) found RAPD variation among the material used. Polymorphism shown by RAPD markers make them useful for germplasm characterization, variety identification and genetic studies.

Gustine and Huff (1999) used RAPD markers to study variation in white clover populations sampled from managed pastures. They found that genetic variability was higher than expected and all populations were significantly different from each other. RAPD technique was used to generate DNA amplified profiles from a set of advanced generation inbred lines of white clover (Joyce *et al.,* 1999). The analysis clearly separated the four major groups with little within group variation.

Crawford (1998) studied genetic variation within and among populations of buffalo clover from throughout its known geographical distribution using RAPD markers. Variation was detected in all populations with levels of diversity in several smaller populations equal to those in the larger one.

Gustine *et al.* (2002) evaluated, quantified and compared genetic variation of white clover populations of 8 different geographical origins. Random amplified polymorphic DNA (RAPD) profiles of these populations revealed similarity of eight populations derived from different climates and geographical regions of the continent that indicated a common European origin for much of the naturalized white clover in North-American pastures.

Bulked AFLP analysis of *Trifolium repens* revealed a significant amount of genetic variability among 52 cultivars and accessions (Kolliker *et al.,* 2001). However, cluster analysis partially reflected the geographical origin of the accessions and also showed disagreement with cluster analysis based on morpho-physiological characters. Gustine and Elwinger (2003) studied the Random Amplified Polymorphic DNA profiles of populations sample of *Trifolium repens* based on a multivariate non parametric approach.

Material and Methods

Chapter III

MATERIAL AND METHODS

3.1 Material

Material for the present investigation comprised of 28 white clover accessions of varied geographical affinities (courtesy: Western Regional Plant Station, WSU, Pullman, USA) including some local germplasm collected from different parts of Himachal Pradesh including a check variety. The material was raised in the randomized complete block design (RBD) using 3 replications, at the Fodder Research Farm of the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur and one set of the material was planted in the pots in the glass house during 2001-2002. Details of the material used are given in the Table 3.1.

3.2 Methods

3.2.1 Field evaluation of experimental material

White clover germplasm comprising of cultivars and accessions of indigenous and exotic origin were maintained at the Fodder Research Farm of the CSK Himachal Pradesh Krishi Vishvavidyalaya Palampur during 2001-02. Row-to-row spacing of 30 cm was maintained in the 2 X 1.5 m^2 plots. Material was characterized and evaluated for important agro-morphological characters using descriptors and descriptor states developed by International Plant Genetic Resources Institute (IPGRI) for white clover. Characterization data were recorded on ten randomly taken plants.

Table 3.1: Details of the material and seed characteristics used in the present study

Sr.No.	Genotypes	Origin	Seed shape	Seed coat Colour
1	TWC-1	Spain	Oval	Brown
2	TWC-2	**	Round	Yellow
3	TWC-3	Bulgaria	Round	Yellow
4	TWC-4	Canada	Oval	Reddish brown
5	TWC-6	Portugal	Oval	Brown
6	TWC-7	Iran	Oval	Yellow
7	TWC-8	**	Oval	Reddish brown
8	TWC-9	Russian Federation	Round	Reddish brown
9	TWC11	Afghanistan	Round	Reddish brown
10	TWC12	Netherland	Oval	Reddish brown
11	TWC13	New Zealand	Oval	Reddish brown
12	TWC14	France	Oval	Brown
13	TWC15	**	Round	Reddish brown
14	TWC16	Turkey	Round	Brown
15	TWC17	Afghanistan	Oval	Brown
16	TWC18	New Zealand	Round	Reddish brown
17	TWC19	Former Soviet Union	Round	Yellow
18	TWC20	Russian Federation	Oval	Yellow
19	TWC21	**	Round	Yellow
20	TWC22	**	Oval	Yellow
21	RRCP-L-65	Joginder Nagar	Oval	Yellow
22	RRCP-L-51	Ghatta	Round	Yellow
23	Palampur composite	Palampur (check)	Oval	Yellow
24	RRCP-L-123	Garsa	Oval	Yellow
25	RRCP-L-17	Baghvda	Oval	Yellow
26	RRCP-L-43	Gadyara	Round	Brown
27	RRCP-L-45	Sukhabagh	Oval	Yellow
28	RRCP-L-42	Sukhabagh	Oval	Yellow

**Not known

3.2.2 Observations recorded

Ten plants were randomly taken to record data on the following traits.

A. Yield and yield related traits

1. **Terminal leaflet length (mm):** This was recorded from base of leaf to the tip with the help of a scale.
2. **Terminal leaflet width (mm):** The width of the terminal leaf was taken by placing the scale breadth-wise.
3. **Leaf shape ratio:** This was calculated as, Terminal leaflet length /Terminal leaflet width (mm)
4. **Leaf area (cm^2):** It was measured by Leaf Area Meter and mean was calculated.
5. **Plant height (cm):** The plant height was recorded with the help of scale, from base of the plant to the tip of main stolon and mean was calculated.
6. **Plant spread (cm^2):** The data were recorded by placing the scale lengthwise and breadthwise.
7. **Petiole length (mm):** The length of the petiole was measured from base to the tip of petiole and mean was calculated.
8. **Peduncle length (mm):** The length of peduncle was measured from base to tip of peduncle and mean was calculated.
9. **Average number of heads per plant:** The number of heads per plant was counted and mean was taken.

10. **Average number of florets per head:** Total numbers of florets per head were counted and mean was calculated.
11. **Pod length (mm):** Pod length was measured and mean was calculated.
12. **Average number of seeds per pod:** The seeds per pod were counted and mean was calculated.
13. **Days to 50% flowering:** The plots were visited daily and days to 50% flowering were noted.
14. **Herbage yield per plant (g):** The whole plant was uprooted and weighed fresh.

B. Morphological Characters

1. **Plant growth habit:** This was recorded on visual observation basis and was classified according to the descriptor states for white clover as:

    a : Erect

    b : Semi-erect

    c : Prostrate

2. **Leaflet shape:** The observation was taken on visual basis according to the descriptor states as following:

    a : Round

    b : Oval

    c : Obovate

    d : Obcordate

    e : Oblong

3. **Leaf colour:** The leaf colour was recorded on visual observation basis and classed according to different descriptor states as:
    1. Light green
    2. Dark green
    3. Variable
4. **Leaf marking uniformity:** This observation was recorded on visual basis and was classed on the basis of different descriptor states as:
    a. Uniform
    b. Variable
5. **Leaf marking:** This was recorded on visual basis and classed on the basis of different descriptor states as:
    1. V-shaped
    2. Both V and other
    3. Other (W- shaped)
6. **Leaf hairiness:** Observation was recorded on visual basis and genotypes were classified as:
    (0). Absent
    (+). Present
7. **Leaf margin:** Shape of the leaf margin was recorded on visual basis and was classified on the basis of different descriptor states as:
    1. Smooth
    2. Faintly toothed
    3. Distinctly toothed
    4. Variable

8. **Petiole hairiness:** Hairiness of petiole was recorded on visual basis and were classed on the basis of different descriptor states as:

    (3). Sparse

    (5). Intermediate

    (7). Dense

9. **Number of stolons:** These were counted manually from primary stem and were classed according to the descriptor states as:

    1. Sparse (<15)
    2. Medium (15-25)
    3. Dense (>25)

10. **Colour of seed coat:** The seed coat colour was recorded on the basis of 100 seeds observed for colour of seed coat.

11. **Seed width (mm):** It was recorded by placing seeds under microscope and were classified as:

    1. Large (>1 mm)
    2. Small (<1 mm)

12. **Seed shape:** The seed shape was recorded by visualizing the seeds under the magnifying lens. The seed shape was recorded as:

    1. Round
    2. Oval
    3. Obovate
    4. Obcordate
    5. Other

C. Quality parameters

a. Protein (AOAC, 1970)

Semi-micro Kjeldahl method was adopted to determine percentage of nitrogen content and a conversion factor of 6.25 was used to calculate crude protein content.

b. Neutral detergent fiber (NDF) (Van soest and Wiens, 1967)

A known quantity of ground sample approximately 0.5g was taken in a spout less beaker and a known quantity of neutral detergent solution (50ml) was added to it. The beaker along with contents was heated to boil for about 1 hour. The contents were filtered through a pre-weighed Gooch crucible under vacuum with 3 washings of hot distilled water and a final washing with acetone. The crucibles were dried to a constant temperature of about 100°C and weighed.

c. Acid detergent fiber (ADF) (Van soest and Wiens, 1967)

Ground sample (0.5g) was heated in spout less beaker with 50ml of acid detergent fiber solution for 1 hour. The contents were filtered through pre-weighed Gooch crucibles under vacuum with 4 washings of hot water and a final washing of petroleum ether. The crucibles were dried to a constant temperature at 100°C and weighed.

3.2.3 Reaction to diseases

3.2.3.I Reaction to powdery mildew (*Erysiphe trifolii*)

The genotypes were screened for reaction to powdery mildew under natural epiphytotic conditions and observations were recorded on the basis of area infected. The genotypes were classified according to the scale 0-5.

3.2.3.II Reaction to clover rot (*Sclerotinia trifoliorum*)

The genotypes were also observed in the field for reaction to the clover rot and mean disease incidence was calculated in percentage.

3.2.4 Statistical Analysis

Mean values were calculated for all the characters studied and used for statistical analysis.

3.2.4.1 Analysis of variance

Data were statistically analyzed as per the procedure given by Panse and Sukhatme (1985). The analysis of variance was based on the following model

$$Y_{ij} = m + g_i + r_j + e_{ij}$$

Where,

Y_{ij} = Phenotypic observation of the ith genotypes in the jth replication;

m = General population mean;

g_i = Effect of ith genotypes;

r_j = Effect of jth replication and

e_{ij} = Error associated with the ith genotypes in the jth replication

Analysis of variance

Source	Degree of Freedom	Mean Squares	F-Value	Expected Mean Squares
Replications	r-1	Mr	Mr/Me	$\sigma^2e+g\sigma^2r$
Genotypes	g-1	Mg	Mg/Me	$\sigma^2e+r\sigma^2g$
Errors	(r-1) (g-1)	Me	-----	σ^2e

Where,

r = Number of replications;

g = Number of genotypes;

$\sigma^2 r$= Variance due to replication;

= (Mr - Me)/g

$\sigma^2 g$= Variance due to genotypes and

$\sigma^2 e$= Error variance

The standard error of mean SE (m), standard error of difference SE(d), and critical difference (CD) for comparing the means of any two lines were computed as follows:

$SE(m) = \pm\sqrt{(Me/r)}$

$SE(d) = \pm\sqrt{(2Me/r)}$

Critical difference (CD) = SE (d) X 't' (5%) value at error degrees of freedom.

Coefficient of variation (CV) was calculated as per the following formula:

$CV(\%) = [(Me)/\bar{x}] \times 100$

Where,

$\bar{x}$ = grand mean

3.2.4.2 Estimation of Parameters of Variability

The genotypic (GCV) and phenotypic (PCV) coefficient of variation were estimated by following Burton and De vane (1953):

$GCV(\%) = \sigma g/\bar{x} \times 100$

$PCV(\%) = \sigma p/\bar{x} \times 100$

Where,

σg = Genotypic standard deviation;

σp = Phenotypic standard deviation and

$\bar{x}$ = Grand mean (Population)

3.2.4.3 Heritability

Heritability in broad sense [h^2 (bs)] was calculated by formula given by Burton and De vane (1953) and Johnson et al.(1955).

Heritability [h^2 (bs)] % = $\sigma^2 g / \sigma^2 g + \sigma^2 e$ X 100

$\sigma^2 g$ = genotypic variance

= [Mg - Me/r]

$\sigma^2 e$ = error variance

$\sigma^2 p$ = phenotypic variance

= $\sigma^2 g + \sigma^2 e$

3.2.4.4 Genetic advance

The expected genetic advance as per cent of mean was calculated as suggested by Burton and De vane (1953) and Johnson et al. (1955).

GA (% of mean) = K X σp X h^2 (bs) / x X 100

Where,

K = Selection differential at 5% selection index i.e.2.06;

σp = Phenotypic standard deviation and

h^2(bs) = Heritability in broad sense

For categorizing the magnitude of different parameters, the following limits were used as:

Genetic Advance, PCV and GCV

>20% = High

10-20% = Moderate

<10% = Low

Heritability

>80% = High

50-80 = Medium

<50 = Low

3.2.5 Cluster analysis

Program for Non-Hierarchical Euclidean Cluster Analysis developed by Beale (1969) for clustering of accessions or genotypes was used through SPAR1 statistical package.

3.2.6 DNA fingerprinting

To generate DNA fingerprints through RAPD markers, accessions of varied geographical origin were used.

3.2.6.1 Isolation of genomic DNA

Ten randomly taken plants were used for DNA extraction. DNA was isolated, using CTAB method of Murray and Thompson (1980). Young leaves were excised, rinsed in de-ionized water and dried on tissue paper. About 300 mg of fresh leaf tissue was ground to fine powder with autoclaved pestle and mortar after freezing the tissue in liquid nitrogen. The powder was transferred to a fresh eppendorf tube containing 800 µl of extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 mM NaCl and 1% PVP at pH 8.0), maintained at 60°C in water bath and mixed vigorously. The tubes were then incubated at

60°C for an hour with an occasional mixing. Equal volume of chloroform: isoamyl alcohol (24: 1) was added to each tube and mixed gently by inverting the tube for 5 minute. After centrifugation at 10,000 rpm for 10 minutes at 4°C, the upper layer (aqueous phase) was transferred to fresh eppendorf tube and 600 µl of pre-chilled isopropanol was added, gently mixed with the aqueous phase and kept at -20°C for 1h. DNA was precipitated by centrifugation at 10,000 rpm for 10 minutes. The supernatant was drained and pellet was retained. The pellet was washed twice with 500 µl of chilled 70 per cent ethanol. The pellet was dried in a laminar air flow cabinet for 3-4 h. Dried DNA pellet was dissolved in 100 µl TE buffer (10 mm Tris-HCl and 0.1 mM EDTA). The dissolved DNA was treated with 2 µl of RNase (10 mg/ml). Electrophoresing in 1 per cent agarose gel checked the yield and quality of DNA.

3.2.6.2 PCR amplification of DNA

DNA amplification was carried out by making final reaction volume of 25 µl containing 2.0 µl dNTP (0.2 mM each of dATP, dGTP, dCTP and dTTP), 0.2 µl *Taq* DNA polymerase (3 U/µl), 2.0 µl (20 ng) DNA template, 1 µl of 5 µM primer, 2.5 µl of 10X PCR buffer, 2.0 µl $MgCl_2$ and 15.3 µl of sterilizing distilled water.

The final reaction mixture was placed in a Thermal Cycler (MJ Research). The PCR programme was set at initial cycle at 94°C for 5 minutes, 37°C for 1 minute, and 72°C for 2 minutes. Further amplification was repeated for 40 times consisting of denaturation at 94°C for 1 minute, annealing at 37°C for 1 min and extension at 72°C for 2 minutes. Final extension of 5 minutes at 72°C was carried out before its rapid cooling to 4°C.

3.2.6.3 Analysis of PCR products

Ten µl of each PCR product mixed with gel loading dye bromophenol blue (0.25% bromophenol blue and 40% sucrose) was electrophoresed using 1.4 per cent agarose gel in 0.5X Tris borate-EDTA (TBE) buffer (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.0). The gel was run at a constant voltage of 100 volts for 2 hours. The gel was stained with ethidium bromide (5µg/ml) for 10 minutes after the completion of electrophoresis.

The gel was destained for 30 minutes by keeping under running tap water. The resolved PCR products were visualized over an ultraviolet transilluminator and a photograph of gel was stored in Gel-Doc (Bio-Rad).

3.2.6.4 RAPD profiling

Forty-eight randomly chosen 10-mer primers were screened but RAPD profiles were generated involving the polymerase chain reaction mediated amplification using 11 randomly chosen10-mer primers only.

3.2.6.5 Data analysis

The band data were scored and used to generate a binary matrix. Numerical Taxonomy System of Multivariate Statistical Program (NTSYS) software package was used for data analysis (Rohlf, 1993). Jaccard's similarity coefficient was used for construction of dendrogram by Unweighted Paired Group Method of Arithmetic Averages (UPGMA).

Table 3.2 List of 10-mer OPERON primers used to generate RAPD bands/ fragments.

Sr.No.	Designation of primers	Base Sequence
1	OPA—13	5'-CAGCACCCAC-3'
2	OPB—07	5'-GGTGACGCAG-3'
3	OPC—13	5'-AAGCCTCGTC-3'
4	OPC—19	5'-GTTGCCAGCC-3'
5	OPC—20	5'-ACTTCGCCAC-3'
6	OPD—08	5'-AAGACCCCTC-3'
7	OPE—11	5'-GAGTCTCAGG-3'
8	OPF—15	5'-CCAGTACTCC-3'
9	OPX—08	5'-CAGGGGTGGA-3'
10	OPX—11	5'-GGAGCCTCAG-3'
11	OPX—14	5'-ACAGGTGCTG-3'

Results

Chapter IV

RESULTS

The results obtained on various aspects of the study are presented hereafter under different heads:

4.1 Analysis of variance

Analysis of variance for the experimental design (Table 4.1) revealed that the mean squares due to genotypes were significant for middle leaflet length (mm), middle leaflet width (mm), petiole length (mm), peduncle length (cm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2), floret length (mm), number of florets, number of seeds per pod, herbage yield (g), NDF % and stolon length (mm), indicating that the material under study had sufficient genetic variability for these characters.

4.2 Estimates of mean values and parameters of variability

Estimates of different parameters of variability are presented in Table 4.2. A wide range of variation was observed among different genotypes with regard to different plant traits studied. The mean performance of all the genotypes for various traits and trait-wise description of these parameters is as follows:

Table 4.1 Analysis of variance

Traits	Source	Mean squares		
		Replication	Genotypes	Error
	df	2	27	54
1. Plant height (cm)		9.03	15.62	5.52
2. Middle leaflet length (mm)		1.26	4.84*	1.37
3. Middle leaflet width (mm)		1.97	5.76*	1.29
4. Leaf shape ratio		0.41	0.14	0.44
5. Petiole length (mm)		6.49	721.85*	38.11
6. Peduncle length (mm)		144.61	673.42*	66.28
7. Leaf area (cm^2)		0.18	19.64*	0.12
8. Number of heads per plant		0.40	181.41*	7.38
9. Days to 50% flowering		26.16	599.59*	13.15
10. Mean plant spread (cm)		18523.43	79206.12*	6134.66
11. Floret length (mm)		0.26	0.55*	0.14
12. Stolon length (mm)		362.02	1149.27*	293.80
13. Number of florets		27.85	311.65*	16.54
14. Number of seeds/pod		0.37	1.02*	0.13
15. Herbage yield (g/plant)		3.63	100.51*	18.33
16. ADF (%)		164.17	16.87	6.19
17. NDF (%)		76.47	52.33*	7.84
18. Protein (%)		19.44	4.97	1.50

Table 4.2 Mean, range and variability parameters of different traits

Traits	Mean	Range	PCV (%)	GCV (%)	ECV (%)	H^2bs (%)	Genetic advance (%)
1. Plant height (cm)	18.66	13.97 – 23.50	15.98	9.84	12.59	37.90	12.47
2. Middle leaflet length (mm)	16.47	13.70 – 19.51	9.65	6.53	7.11	45.72	9.09
3. Middle leaflet width (mm)	14.28	11.00 – 17.95	11.67	8.55	7.95	53.67	12.91
4. Leaf shape ratio	1.16	0.99 – 1.32	7.55	4.96	5.69	43.16	6.71
5. Petiole length (mm)	95.1	71.43 – 125.45	17.15	15.87	6.49	85.67	30.27
6. Peduncle length (mm)	85.88	66.00 – 120.42	19.09	16.56	9.48	75.33	29.62
7. Leaf area (cm^2)	8.89	4.21 – 13.28	28.96	28.70	3.85	98.23	58.59
8. Number of heads per plant	25.37	12.30 – 40.14	31.88	30.03	10.70	88.72	58.25
9. Days to 50% flowering	118.75	90.33 – 143.33	12.16	11.77	3.05	93.69	23.48
10. Mean plant spread (cm^2)	448.26	89.41 – 825.25	38.95	34.82	17.49	80.00	64.10
11. Floret length (mm)	7.78	7.01 – 8.68	6.73	4.76	4.76	49.95	6.93
12. Stolon length (mm)	182.04	147.44 – 220.67	13.22	9.28	9.41	49.25	13.41
13. Number of florets	46.65	31.95 – 82.56	22.98	21.26	8.72	85.60	40.52
14. Number of seeds/pod	3.02	1.43 – 4.03	21.57	18.10	11.73	70.43	31.30
15. Herbage yield (g/plant)	26.87	17.07 – 37.04	25.17	21.93	15.93	59.90	31.06
16. NDF (%)	33.29	25.73 – 41.46	14.3	11.56	8.41	65.40	19.27
17. ADF (%)	35.24	30.93 – 39.27	8.86	5.35	7.05	36.55	6.67
18. Protein content (%)	20.59	17.15 – 23.10	7.92	5.22	5.95	43.49	7.09

4.2.1 Plant height (cm)

Plant height ranged from 13.97-23.50 cm with a mean value of 18.66 cm. Check Palampur Composite had the maximum plant height over all the genotypes followed by TWC-17 (22.26 cm), TWC-20 (21.83 cm), RRCP-L-123 (21.09 cm), RRCP-L-42 (20.58), TWC-16 (20.44 cm), RRCP-L-43 (20.32 cm), TWC-8 (20.26 cm), TWC-6 (20.15 cm), TWC-21 (20.02 cm) and TWC-13 (19.94 cm). Values of PCV, ECV and genetic advance in % of mean were found to be moderate as 15.98, 12.59 and 12.47 per cent, respectively. However, GCV and heritability values were low as 9.84 and 37.90 per cent, respectively.

4.2.2 Middle leaflet length (cm)

Middle leaflet length ranged from 13.70-19.51 (mm) with a mean value of 16.47 mm. RRCP-L-43 had the highest middle leaflet length (19.51 mm) followed by TWC-22 (18.10 mm), TWC-18 (18.0 mm), TWC-1 (17.99 mm), TWC-6 (17.8 mm) and RRCP-L-123 (17.69 mm). Low values of PCV (9.65), GCV (6.53), ECV (7.11), heritability (45.72) and genetic advance (9.09) were recorded for this character.

4.2.3 Middle leaflet width (mm)

It varied from 11.0-17.95 mm with mean value of 14.28 mm. TWC-1 had the highest middle leaflet width of 17.95 mm which was followed by TWC-12 (17.16 mm). GCV and ECV were found to be as low as 8.55 and 7.95 per cent, respectively. However, PCV, heritability and genetic advance were moderate with values of 11.67, 53.67 and 12.91 per cent, respectively for this trait.

4.2.4 Leaf shape ratio

Leaf shape ratio ranged from 0.99 to 1.32 with mean value of 1.16. RRCP-L-43 had the highest leaf shape ratio (1.32) followed by RRCP-L-42 (1.25), TWC-18 (1.24), TWC-3 (1.23), TWC-4 (1.22), TWC-6 (1.22), TWC-21 (1.22) and RRCP-L-51 (1.21).

Relatively low values of PCV, GCV, ECV, heritability and genetic advance were recorded for leaf shape ratio.

4.2.5 Petiole length (mm)

It varied from 71.43 to 125.45 mm with a mean value of 95.10 mm. RRCP-L- 123 had the highest petiole length of 125.45 mm followed by RRCP-L-17 (123.12 mm) and TWC-18 (120.37 mm). PCV (17.15%) and GCV (15.87%) were found to be moderate. ECV was found to be low, however, heritability was high (85.67%) coupled with high values of genetic advance (30.27%).

4.2.6 Peduncle length (mm)

This observation ranged from 66.00 to 120.42 mm with a mean value of 85.88 mm. TWC-11 had the highest peduncle length (120.42 mm) followed by TWC-4 (113.55 mm).

PCV (19.09%), GCV (16.56%) and heritability (75.33%) values for peduncle length were found to be moderate. ECV was low with a per cent value of 9.48 and genetic advance was found to be high at 29.62 per cent.

4.2.7 Leaf area (cm^2)

Leaf area varied from 4.21-13.28 cm^2 with an overall mean leaf area of 8.89 cm^2. TWC-9 had the maximum leaf area (13.28 cm^2), followed by TWC-12 (13.00 cm^2). High PCV, GCV, heritability and genetic advance were observed with corresponding values of 28.96, 28.70, 98.23 and 58.59 per cent, respectively. However, ECV was found to be low at 3.85 per cent.

4.2.8 Number of heads per plant

It ranged from 12.30-40.14 with a mean value of 25.37 heads per plant. TWC-11 had the highest number of heads per plant (40.14) followed by the check Palampur Composite (39.34), RRCP-L-123 (37.60) and RRCP-L-43 (36.33).

High PCV (31.88%), GCV (30.02%), heritability (88.72%) and genetic advance (58.25%) were observed for number of heads per plant. ECV was found to be moderate with a value of 10.7 per cent.

4.2.9 Days to 50% flowering

Observation for this character ranged from 90.33 to 143.33 days with a mean value of 118.75 days. RRCP-L-45 took maximum days in attaining days to 50% flowering (143.33 days), which was followed by TWC-11 (141.67 days). TWC-7 and TWC-20, on the other hand, were the earliest in completing days to 50% flowering. The rest of the genotypes were intermediate in days to 50% flowering.

Heritability and genetic advance were found to be high with per cent values of 93.69 and 23.48. PCV and GCV were found to be moderate (12.16 and 11.77 per cent). ECV was low at 3.05 per cent.

4.2.10 Mean plant spread (cm^2)

Mean plant spread ranged from 89.41 to 825.25 cm^2 with a mean value of 448.26 cm^2. RRCP-L-43 a local collection had the highest plant spread (825.25 cm^2), followed by TWC-14 (788.22 cm^2) and TWC-3 (698.04 cm^2).

High values of PCV, GCV, heritability and genetic advance were found with corresponding values of 38.95%, 34.82%, 80% and 64.10%, respectively, whereas ECV was found to be moderate for this character.

4.2.11 Floret length (cm)

Floret length ranged from 7.01 to 8.68 mm with an average value of 7.78 mm. TWC-17 had the maximum floret length of 8.68 mm followed by TWC-12, TWC-11, RRCP-L-65, RRCP-L-43 and check (Palampur Composite) with floret length of 8.61 mm, 8.45 mm, 8.34 mm, 8.13 mm and 8.08 mm, respectively.

Low PCV, GCV, ECV, heritability and genetic advance with corresponding values of 6.73, 4.76, 4.76, 49.95 and 6.93 per cent were recorded for this character.

4.2.12 Stolon length (mm)

Stolon length ranged from 147.44 to 220.67 mm. The mean value was 182.04 mm. Check (Palampur Composite) had the highest stolon length of 220.67 mm followed by TWC-17 (217.25 mm), TWC-8 (200.61 mm), RRCP-L-

42 (200.14 mm), TWC-7 (198.92 mm), TWC-20 (198.66 mm), RRCP-L-123 (194.65 mm), RRCP-L-43 (193.56 mm), TWC-4 (193.25 mm) and TWC-1 (192.9 mm). PCV and genetic advance were found to be moderate with values of 13.22 and 13.41 per cent, whereas GCV, ECV, and heritability were quite low.

4.2.13 Number of florets

Number of florets ranged from 31.95 to 82.56 per inflorescence with a mean value of 46.65. TWC-11 had the highest number of florets and all other genotypes had significantly low number of florets.

Relatively high PCV, GCV, heritability and genetic advance were found with per cent values of 22.98, 21.26, 85.60 and 40.52, respectively.

4.2.14 Number of seeds per pod

Number of seeds per pod ranged from 1.43 to 4.03 with a mean value of 3.02. TWC-4 had the highest number of seeds per pod (4.03) followed by TWC-17 (3.92), TWC-18 (3.78), RRCP-L-123 (3.61), TWC-9 (3.54) and TWC-13 (3.49). Per cent PCV and genetic advance were found to be high with moderate values of GCV, ECV and heritability.

4.2.15 Herbage yield (g)

Herbage yield per plant ranged from 17.07 to 37.04 (g), with a mean value of 26.87 g. RRCP-L-17 was found to be the highest green fodder yielder among all genotypes which was followed by TWC-6 (36.48 g), TWC-1 (34.98 g), TWC-13 (34.04 g), TWC-8 (33.42 g) and the check Palampur Composite

(32.61 g). High per cent values of PCV, GCV and genetic advance were recorded (25.17, 21.93 and 31.06). ECV and heritability were found to be moderate with corresponding values of 15.93 and 59.90 per cent, respectively.

4.2.16 NDF (%)

Per cent neutral detergent fibre ranged from 25.73 to 41.46, with a mean value of 33.29 per cent. RRCP-L-65 had the highest neutral detergent fibre percentage followed by TWC-9 (39.20%), TWC-2 (38.93%), RRCP-L-43 (38.47%), TWC-4 (37.86%) and TWC-16 (37.47%). Moderate values of PCV, GCV, heritability and genetic advance were observed for this trait.

4.2.17 ADF (%)

Acid detergent fibre ranged from 30.93 to 39.27 per cent, with a mean value of 35.24 per cent. TWC-7 and TWC-13 had the highest ADF (39.27%) followed by RRCP-L-43, TWC-9, TWC-21, TWC-4, TWC-22, TWC-17, RRCP-L-42, TWC-16, TWC-18 and Palampur Composite with corresponding values of 38.40, 38.20, 38.20, 37.80, 37.40, 37.26, 37.13, 36.67, 35.46 and 35.40 per cent, respectively. Low values of PCV, GCV, ECV, heritability and genetic advance were observed for this trait.

4.2.18 Protein (%)

Average protein content ranged from 17.15 to 23.10 per cent with a mean of 20.59 per cent. TWC-6 had the highest protein percentage (23.10%) which was followed by TWC-11 (22.38%), RRCP-L-65 (22.28%) and TWC-14 (22.05%). TWC-2, on the other hand, had the lowest protein content (17.15%). Low values of PCV, GCV, ECV, heritability and genetic advance were observed for protein conte

4.3 Cluster analysis

With the help of non-hierarchial euclidean cluster analysis (Table 4.3), 28 accessions were grouped into 7 clusters. Cluster 1 contained three genotypes, 2 of exotic (TWC-4 and TWC-9) and one of indigenous (RRCP-L-65) origin. Cluster 2 had only one genotype TWC-11 of exotic origin, whereas, cluster 3 contained six genotypes, of which five accessions viz., TWC-2, TWC-14, TWC-18, TWC-19 and TWC-22 were of exotic origin while RRCP-L-45 was an indigenous collection. Cluster 4 contained 5 genotypes out of which, four were of exotic (TWC-1, TWC-8, TWC-12 and TWC-15) and one (RRCP-L-17) had indigenous origin. Cluster 5 contained eight accessions, six of exotic (TWC-3, TWC-7, TWC-13, TWC-16, TWC-20 and TWC-21) and two of indigenous (RRCP-L-51 and RRCP-L-42) origin. Sixth cluster had four accessions, two exotic (TWC-6 and TWC-17) and two Indian (Palampur Composite and RRCP-L-123), whereas, Cluster 7 contained only one accession (RRCP-L-43) of indigenous origin.

4.3.1 Cluster distances

Inter cluster distances are given in the Table 4.3.1. Maximum inter cluster centroids distance was found between clusters 7 and 2. Crosses between distant clusters might be more useful if the parents for crosses are carefully selected according to breeding objectives. Minimum inter cluster centroid distance was found between cluster numbers 5 and 3. Attempting crosses within cluster is hardly expected to yield any desirable recombination.

Table 4.3 Non Hierarchial Euclidean Cluster analysis

Cluster number	Number of accessions in each cluster	Genotypes
1.	3	TWC-4, TWC-9, RRCP-L-65
2.	1	TWC-11
3.	6	TWC-2, TWC-14, TWC-18, TWC-19, TWC-22, RRCP-L-45
4.	5	TWC-1, TWC-8, TWC-12, TWC-15, RRCP-L-17
5.	8	TWC-3, TWC-7, TWC-13, TWC-16, TWC-20, TWC-21, RRCP-L-51, RRCP-L-42
6.	4	TWC-6, TWC-17, Palampur Composite, RRCP-L-123
7.	1	RRCP-L-43

Table 4.3.1 Distance between the cluster centroids

Cluster	1.	2.	3.	4.	5.	6.	7.
1.	0.00						
2.	6.11	0.00					
3.	3.94	7.72	0.00				
4.	4.70	6.93	3.68	0.00			
5.	3.57	7.85	3.52	4.26	0.00		
6.	4.80	7.05	5.22	3.95	4.70	0.00	
7.	6.10	9.03	5.65	6.38	5.86	5.71	0.00

Results from present study indicated that cluster analysis is an suitable technique to identify and quantify the genetic distances between strains.

4.4 Reaction to diseases

4.4.1 Reaction to powdery mildew (*Erysiphe trifolii)*

Twenty-eight accessions were evaluated for their reaction to powdery mildew in pot cultures. Based on leaf area infected and scale used (Table 4.4.1), accessions were classified into different categories. Two accessions viz., TWC-2 and RRCP-L-42 a local collection, were found to be highly resistant. Not a single genotype was classed as resistant, however nine genotypes (TWC-9, TWC-11, TWC-14, TWC-15, TWC-21, TWC-22, RRCP-L-45, RRCP-L-123 and Palampur Composite) were observed moderately resistant. Four genotypes (TWC-7, TWC-16, TWC-18 and RRCP-L-65) were grouped as moderately susceptible and eleven genotypes, viz. (TWC-1, TWC-3, TWC-4, TWC-6, TWC-8, TWC-12, TWC-13, TWC-17, TWC-19 and RRCP-L-17, RRCP-L-51) were found susceptible. The remaining two genotypes (TWC-20 and RRCP-L-43) were found to be highly susceptible to the disease.

4.4.2 Reaction to clover rot *(Sclerotinia trifoliorum)*

Percent disease incidence ranged from 0.00 to 28.70. Maximum incidence of disease was found in TWC-1, TWC-2, TWC-4, TWC-15, TWC-18, RRCP-L-51 and RRCP-L-45 (Plate 1). However, TWC-3, TWC-6, TWC-7, TWC-8, TWC-13, TWC-14, TWC-16, TWC-20 and RRCP-L-42 were found free from disease (Table 4.4.2).

Table 4.4.1 Reaction to powdery mildew

	Categories	Scale	Area infected	Genotypes
1.	Disease free	0	0	TWC-2, RRCP-L-42
2.	Resistant	1	< 1	--
3.	Moderately Resistant	2	1 – 10	TWC-9, TwC-11, TWC-14, TWC-15, TWC-21, TWC-22, RRCP-L-45, RRCP-L-125, Palampur Composite (check)
4.	Moderately susceptible	3	11 – 25	TWC-7, TWC-16, TWC-18, RRCP-L-65
5.	Susceptible	4	26 – 50	TWC-1, TWC-3, TWC-4, RRCP-L-51, TWC-6, TWC-8, TWC-12, TWC-13, TWC-17, TWC-19, RRCP-L-17
6.	Highly Susceptible	5	51 – 100	TWC-20, RRCP-L-43

Plate1. White clover growing in the field (A) and *Sclerotinia trifoliorum* infected white clover plants (B)

Table 4.4.2 Reaction of white clover accessions to *Sclerotinia trifoliorum*

	Genotypes	Disease incidence %	Transformed value (sq. root transformation)
1.	TWC-1	28.70	5.29*
2.	TWC-2	13.00	3.72
3.	TWC-3	0.00	1.00
4.	TWC-4	16.70	4.21
5.	TWC-6	0.00	1.00
6.	TWC-7	0.00	1.00
7.	TWC-8	0.00	1.00
8.	TWC-9	11.10	3.14
9.	TWC-11	11.10	3.14
10.	TWC-12	6.70	2.54
11.	TWC-13	0.00	1.00
12.	TWC-14	0.00	1.00
13.	TWC-15	20.00	4.00
14.	TWC-16	0.00	1.00
15.	TWC-17	2.80	1.70
16.	TWC-18	13.40	3.77
17.	TWC-19	2.77	1.68
18.	TWC-20	0.00	1.00
19.	TWC-21	5.50	2.40
20.	TWC-22	5.50	2.40
21.	RRCP-L-65	5.60	2.07
22.	RRCP-L-51	19.50	4.50
23.	Palampur Composite	2.80	1.68
24.	RRCP-L-123	11.10	2.40
25.	RRCP-L-17	11.10	2.40
26.	RRCP-L-43	2.80	1.68
27.	RRCP-L-45	16.70	4.20
28.	RRCP-L-42	0.00	1.00

CD (5 %) = 1.76

4.5 Molecular characterization

A total of 48 primers (10-mer) were screened for the PCR amplification, out of which 37 failed to give meaningful results.

The RAPD analysis of 28 accessions of white clover was thus, done using 11 primers. The RAPD profiles generated using primers OPC-20 and OPF-15 are presented in Plate 2.

The number of scorable and polymorphic bands obtained with each primer is given in the Table 4.5. RAPD profiles of white clover accessions generated 136 bands, of which 129 were polymorphic. These bands were used to construct a dendrogram (Fig. 1). The cluster analysis of 136 bands showed the formation of 5 groups namely A, B, C, D, and E. Group A comprised of four exotic (TWC-1, TWC-2, TWC-6 and TWC-3) and one indigenous (RRCP-L-123) white clover accessions with 49 per cent similarity. Group B, comprised of two exotic accessions (TWC-18 and TWC-21), showing 57% similarity among themselves. Group C, comprised of four indigenous accessions (RRCP-L-65, RRCP-L-51, RRCP-L-45 and RRCP-L-42), showing 47.5 per cent similarity among themselves. Group D comprised of eight accessions with five exotic (TWC-8, TWC-9, TWC-16, TWC-20 and TWC-22) and three of indigenous (Palampur Composite, RRCP-L-17 and RRCP-L-43) origin representing 46% similarity among themselves. Group E comprised of two accessions (TWC-13 and TWC-14) of exotic origin showing 44% genetic similarity among themselves.

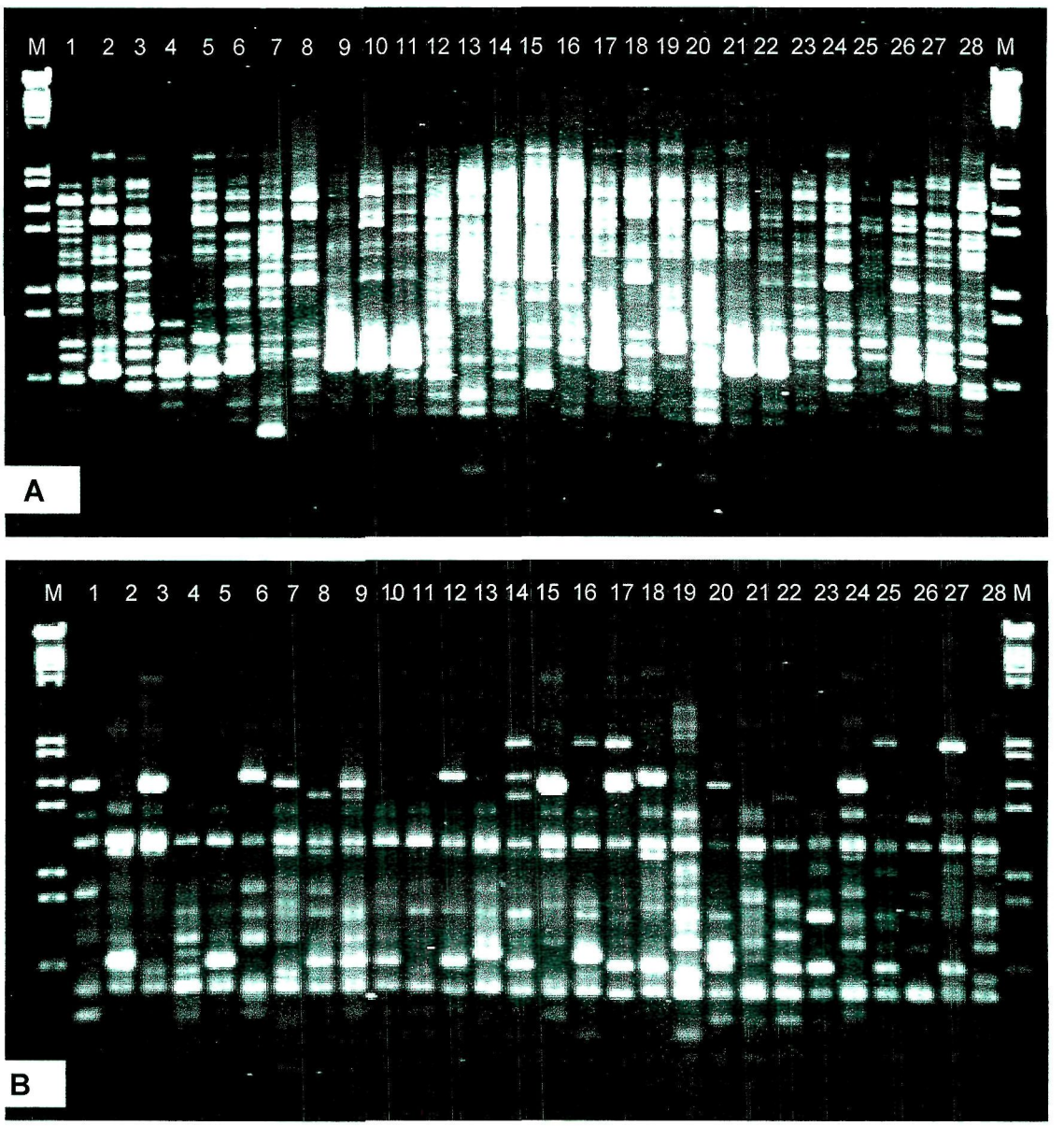

Plate 2. RAPD profiles of 28 accessions of white clover generated through DNA amplification with primers OPC20 (A) and OPF15 (B); M denotes the molecular weight marker

Table 4.5 Number of scorable and polymorphic RAPD bands obtained in the PCR amplified DNA of white clover accessions

Sr. No.	Primers	Numbers of scored bands	Number of polymorphic bands
1.	OPA-13	13	12
2.	OPB-07	16	16
3.	OPC-13	10	10
4.	OPC-19	10	9
5.	OPC-20	10	9
6.	OPD-08	11	10
7.	OPE-11	11	11
8.	OPF-15	18	16
9.	OPX-8	15	14
10.	OPX-11	10	10
11.	OPX-14	12	12
		136	129

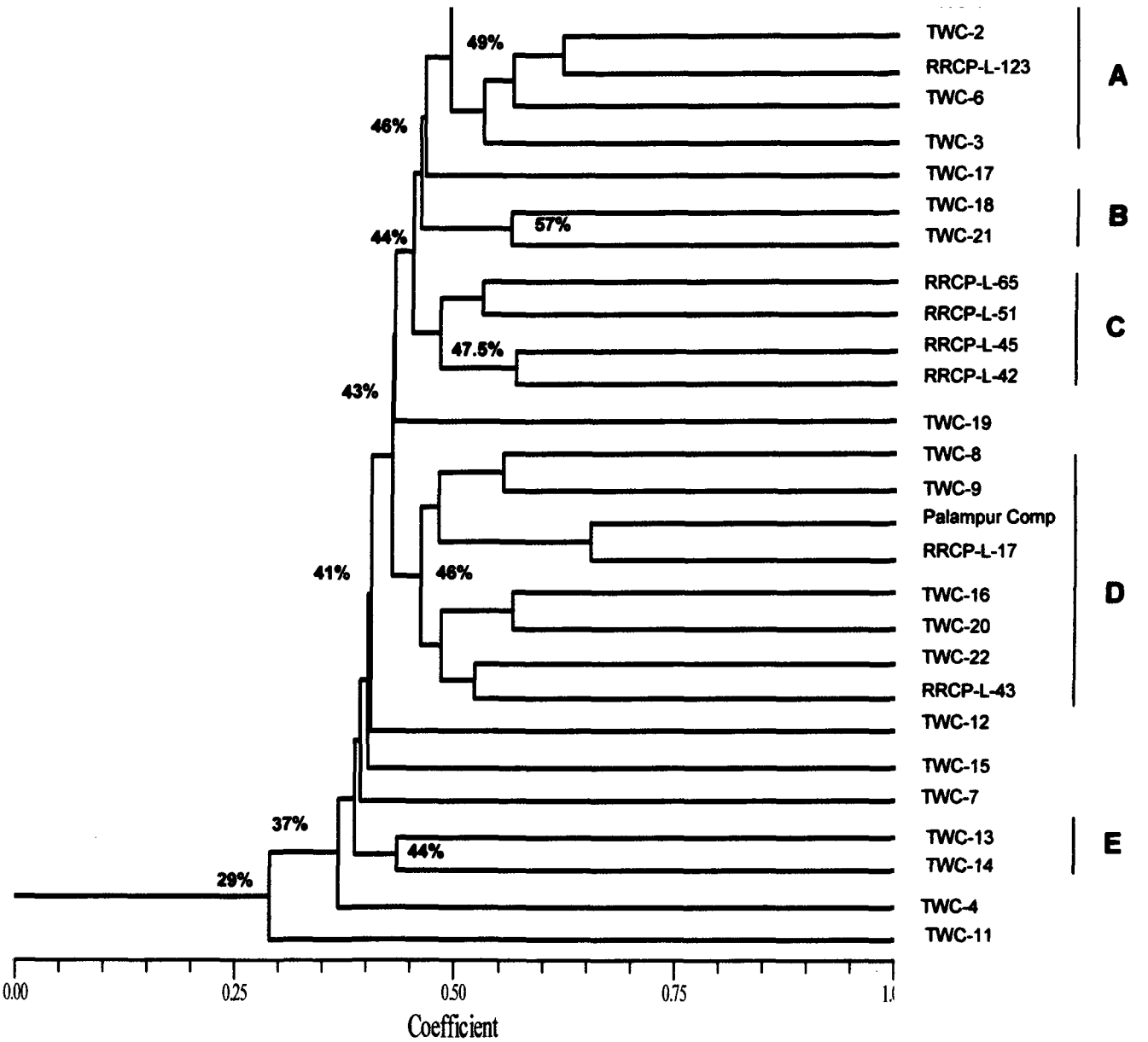

Fig 1. Dendrogram of 28 *Trifolium repens* accessions generated by RAPD data using the UPGMA method

Accessions TWC-17, TWC-19, TWC-12, TWC-15, TWC-7, TWC-4 and TWC-11 did not cluster in any of the above 5 groups. The results showed TWC-4 and TWC-11 to be the most distantly placed accessions. TWC-4 showed 37% similarity with all the 5 groups, whereas, TWC-11 showed only 29% similarity with these groups. The intercluster similarity between group A and B was 46%, between B and C 44%, between C and D 43% and between D and E was 41%.

Discussion

Chapter V

DISCUSSION

The present investigation on "Characterization and diversity analysis of white clover *(Trifolium repens)* accessions of different geographical origin" was undertaken to know the nature and magnitude of variability present in the accessions of white clover of diverse geographic origin using agro-morphological and RAPD markers. The results obtained in the present investigation are discussed under the following heads.

5.1 Studies on variability

Most of the traits which are of interest to plant breeders are quantitative in nature and thus exhibit continuous variation. The continuous variation comprises of heritable and non-heritable components (Fisher, 1918). Therefore, it is necessary to partition the observed variation into heritable and non-heritable components so as to have an idea about the heritable contribution. For the traits with high heritability, selection becomes easy due to close correspondence between phenotype and the genotype. Its estimates are also useful in understanding the amount of genetic progress that can be made during selection process. The estimates of heritability together with the genetic advance are helpful in predicting selection potential in a population.

In the present study, analysis of variance revealed the presence of sufficient variability in the material under study for the traits, viz., middle leaflet length (mm), middle leaflet width (mm), petiole length (mm), peduncle length (mm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2), floret length (mm), number of florets, number of seeds per plant, herbage yield (g/plant), NDF (%) and stolon length (mm). However, plant height (cm), leaf shape ratio, protein content (%) and ADF (%) did not show the presence of sufficient variability. Harper (1977), Burdon (1980), Annicchiarico (1993) and Caradus (1994) also reported wide range of variability for herbage yield and other traits in white clover.

In order to have a comparison of magnitudes at phenotypic and genotypic levels of various traits, the most potential parameters are coefficients of variability. Results from present study indicated that PCV was high (> 20%) for traits viz., leaf area (cm^2), number of heads per plant, mean plant spread (cm^2), number of florets, number of seeds per pod, herbage yield (g/plant) and moderate (10-20%) for plant height (cm), middle leaflet width (mm), petiole length (mm), peduncle length (mm), days to 50% flowering, stolon length (mm) and NDF (%). For remaining characters viz., middle leaflet length (mm), leaf shape ratio, protein content (%), floret length (mm) and ADF (%), the per cent PCV was comparatively low.

High GCV (%) values were observed for leaf area (cm^2), number of heads per plant, number of florets, herbage yield (g/plant), mean plant spread (cm^2) and moderate for petiole length (mm), peduncle length (mm), days to 50% flowering, number of seeds per pod and NDF (%). For remaining characters GCV was found to be comparatively low.

High genotypic coefficient of variation for the characters reflect high magnitude of genetic variation and hence greater scope for improvement through direct selection. GCV values were lower than the corresponding phenotypic coefficients, implying the influence of environment on the genotypes. Environmental coefficient of variation (ECV %) was observed to be low for middle leaflet length (mm), middle leaflet width (mm), leaf shape ratio, petiole length (mm), peduncle length (mm), leaf area (cm^2), protein (%), days to 50% flowering (days), mean plant spread (cm^2), floret length (mm), stolon length (mm), number of florets, NDF (%) and ADF (%), whereas moderate for rest of the traits. Lee *et al.* (1993) also indicated high GCV for leaf area (cm^2) and petiole length (mm). Avtar *et al.* (1997) found high GCV for herbage yield and other component characters in white clover.

Heritability and genetic advance are also two complementary concepts, the former may be used to estimate the expected genetic advance through selection. The effectiveness of a selection programme depends not only on the total available variation, but also on the extent of heritability and

genetic advance. Such parameters have been shown to change from population to population, year to year and environment to environment. The results of the present study revealed that traits like petiole length (mm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2) and number of florets recorded high heritabilities (> 80%) and moderate (50-80%) for middle leaflet width (mm), peduncle length (mm), number of seeds per pod, herbage yield (g/plant) and NDF (%). However, remaining traits exhibited low heritabilities (< 50%). Higher estimates of heritability for the traits implies that the phenotypic selection made for these traits can be relied upon.

For an effective selection, the knowledge of the estimates of the heritability alone is not sufficient and genetic advance, if studied along with heritability, is more useful. In the present study, high genetic advance (>20%) expressed as per cent of mean was observed for petiole length (mm), peduncle length (mm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2), number of florets per head, number of seeds per pod and herbage yield (g/plant); moderate (10-20%) for plant height (cm), middle leaflet width (mm), stolon length (mm), NDF (%) and low (< 10%) for rest of traits viz., middle leaflet length (mm), leaf shape ratio, floret length (mm), ADF (%) and protein (%). Low genetic advance for these traits suggested that the selection based on *per se* performance for the traits may not yield desirable results.

Further, high heritability coupled with high genetic advance for petiole length (mm), peduncle length (mm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2), number of florets indicated the presence of additive gene action for these traits. Hence selection can be quite effective for the improvement of these traits. Contrarily, high heritability with low genetic advance reveals the presence of non-additive gene action.

These results are in conformity with those of previously reported by Lawson (1971), Vanbogaert (1977), Broda (1980), Salisbury *et al.* (1987) and Lee *et al.* (1993) also observed high heritability for herbage yield (g/plant), florets per head, seeds per pod, leaf area, days to flowering and petiole length.

5.2 Cluster analysis

Selection of parents is based on genetic diversity for a successful crop improvement programme. Cluster analysis has often been used for studying the genetic divergence of germplasm. This approach has been used in different crops by various workers (Walia and Garg, 1996; Garg and Gautam, 1997). Therefore, cluster analysis was used for studying the genetic divergence in accessions of white clover *(T. repens)*.

In the present study, 28 accessions of white clover of varied geographical origins (20 exotic and 8 of indigenous origin) were characterized and evaluated using standard descriptors and descriptor states. The cluster

analysis (Non Hierarchical Euclidean Cluster Analysis) grouped accessions into 7 broad clusters (groups), showing high levels of genetic variation. Cluster 1 contained three accessions (2 exotic and 1 indigenous), cluster 2 contained only one accession, exotic in origin. Cluster 3 had six accessions (5 exotic and one indigenous), cluster 4 contained five genotypes (4 exotic and 1 indigenous), cluster 5 contained eight accessions (6 exotic and 2 of indigenous origin), cluster 6 comprised of four accessions (2 exotic and 2 indigenous) and cluster 7 had only one accession of indigenous origin.

Not much association was observed between clustering pattern and geographical origin of the accessions under study. The genotypes of same region were distributed in more than one cluster as the genotypes of heterogeneous region were grouped in the same cluster. The clustering pattern, however, depicted the presence of sufficient genetic diversity, even in the germplasm collections made from similar geographical situations. The similar results were found by Katiyar *et al.* (1998) in forage maize.

5.3 Reaction to diseases

Genotypes were screened for powdery mildew *(Erysiphe trifolii)* resistance under natural epiphytotic conditions and only two accessions of white clover viz., TWC-2 and RRCP-L-42 (a local collection) were found to be highly resistance (Table 4.5.1) which could be used as breeding material for powdery mildew disease resistance germplasm. Field screening conditions for *Sclerotinia trifoliorum* showed the variation in the per cent disease incidence in

the accessions of white clover (Table 4.5.2). Accessions TWC-3, TWC-6, TWC-7, TWC-8, TWC-13, TWC-14, TWC-16, TWC-20 and RRCP-L-42 (a local collection) were found free from disease.

Interestingly a local collection RRCP-L-42 from Sukhabagh (HP) was found highly resistant for powdery mildew and *Sclerotinia*. Therefore, this can be used as one of the parents in breeding for disease resistance in clover.

5.4 Agromorphological characterization

Morphological characterization of genotypes has significant implication in varietal identification, gene bank management, conservation and intellectual property rights issues. Agromorphological characterization was done by using standard descriptors and descriptors states developed by International Plant Genetic Resources Institute (IPGRI) for white clover (Table 5.4). For leaf marking uniformity, most of the accessions were found to be having the uniform type of leaf marking with few accessions showing variable type leaf marking. 'V' shaped leaf marking was predominant in most of the accessions followed by 'W' shaped and both 'V' and 'W' shaped. In most of the genotypes, number of stolons was found to be dense (> 25) followed by medium (15 – 25) and a unique descriptor state sparse stolons (<15) was found in TWC-11.

Dark green leaf colour was found to be prevalent in majority of the accessions followed by variable and light green leaf colour. Majority of the accessions were having large seed size (> 1 mm) than small seed size

Table 5.4 Agro-morphological markers

Genotypes	Leaf marking uniformity		Leaf marking				No. of stolons			Leaf colour			Seed width	
	Uniform	Variable	'V' Shaped	Both v and w	'W' shape	absent	Sparse	medium	Dense	Light green	Dark green	Variable	Small	Large
TWC-1	√				√				√		√			√
TWC-2		√		√					√	√				√
TWC-3	√		√						√		√		√	
TWC-4		√		√				√			√			√
TWC-6	√				√				√		√		√	
TWC-7	√				√				√		√			√
TWC-8		√			√				√		√			√
TWC-9		√	√						√			√		√
TWC-11	√		√				√			√				√
TWC-12	√		√					√			√			√
TWC-13		√		√					√			√		√
TWC-14		√		√					√		√			√
TWC-15		√			√				√			√		√
TWC-16	√		√						√			√	√	
TWC-17	√		√						√		√			√
TWC-18	√		√						√		√			√
TWC-19	√				√				√		√		√	
TWC-20	√				√				√		√			√
TWC-21	√		√						√		√			√
TWC-22		√	√						√	√				√
RRCP-L-65		√			√				√		√			√
RRCP-L-51	√			√					√	√			√	
Palampur composite	√				√				√		√			√
RRCP-L-123	√		√					√				√	√	
RRCP-L-17		√	√						√			√		√
RRCP-I-43	√		√						√		√		√	
RRCP-L-45		√	√					√			√			√
RRCP-L-42	√				√				√		√		√	

Genotypes	Middle leaflet shape					Petiole hairiness			Leaf margin				Plant habit		
	Round	Oval	Obovate	Obco-rdate	Oblong	Sparse	Inter – mediate	Dense	Smoot	Faintly toothed	Distinctly toothed	Variable	Erect	Semi-erect	Prostrate
TWC-1				√		√					√			√	
TWC-2				√		√					√				√
TWC-3				√			√				√				√
TWC-4					√		√			√				√	
TWC-6				√			√				√				√
TWC-7				√		√				√					√
TWC-8				√				√			√				√
TWC-9				√			√				√			√	
TWC-11	√							√		√			√		
TWC-12				√			√			√					√
TWC-13				√			√				√			√	
TWC-14				√			√				√			√	
TWC-15				√		√					√				√
TWC-16				√				√			√				√
TWC-17					√		√				√			√	
TWC-18					√			√		√					√
TWC-19				√		√					√				√
TWC-20				√			√			√				√	
TWC-21				√			√				√			√	
TWC-22				√			√				√				√
RRCP-L-65					√		√				√			√	
RRCP-L-51					√	√					√			√	
Palampur composite					√		√				√		√		
RRCP-L-123					√		√				√		√		
RRCP-L-17				√			√			√					√
RRCP-I-43				√			√				√			√	
RRCP-L-45					√	√					√			√	
RRCP-L-42				4			5				3			2	

(< 1 mm). Middle leaflet shapes in most of the genotypes were found to be obcordate followed by oblong. A unique descriptor state, round shape was found in exotic accession (TWC-11), which differentiated it from others. Leaf hairiness was altogether absent in all the accessions of white clover. Petiole hairiness was found to be intermediate type followed by sparse and dense.

Leaf margin was found to distinctly toothed in most of accessions followed by faintly toothed. Plant habit was predominantly semi-erect followed by prostrate type. Erect plant habit was found in the only accession TWC-11, showing it to be a unique accession.

So, the morphological characterization of the germplasm accessions could be important for cultivar discrimination and gene bank management in white clover.

5.5 RAPD analysis

The selection of plant varieties based on morphological markers only is not very reliable because many characters of interest have low heritabilities and are genetically complex. However, genome characterization based on DNA markers is more reliable. Williams *et al.* (1990) established a DNA polymorphism assay based on PCR amplification of random DNA segments with single primers of arbitrary nucleotide sequence (RAPD). Different studies have indicated that RAPD is a powerful method of genotype identification, diversity analysis, population and pedigree analysis, phylogenetic studies and genetic mapping (Welsh and McCelland, 1990; Yu and Nguyen, 1994; Raghunathachari *et al.,* 2000).

In the present study, the dendrogram obtained by analyzing the RAPD fingerprints generated through eleven 10-mer primers on 28 genotypes, clearly indicated the presence of sufficient genetic variation. The variation, thus indicated, could be very useful for white clover improvement programme.

Molecular analysis clustered the genotypes in five groups. Group A contained 4 accessions of exotic origin and one accession of indigenous origin. Group B contained both accessions of exotic origin. Group C contained 4 accessions, all of indigenous origin. Group D contained 5 accessions of exotic and 3 accessions of indigenous origin. Group E had 2 accessions of exotic origin. Seven accessions of exotic origin did not cluster in any group. This represented a high genetic diversity in exotic accessions. TWC-11 and TWC-4 (exotic origin) were placed distantly in the dendrogram and showed least similarity between other five groups. Largest intracluster similarity was found in the group B (57%) followed by group A (49%), group C (47.5%), group D (46%) and group E (44%). Largest intercluster per cent similarity was found between groups A and B (46%) followed by groups B and C (44%), groups C and D (43%) and groups D and E (41%). The molecular analysis showed the lack of variability in the indiegenous collection and it therefore warrants the need to introduce exotic germplasm in our environment to increase the genetic diversity in the indigenous white clover gene pool. Like agromorphological descriptors, the white clover germplasm did not show any definite correspondence between clustering pattern and geographically affinity with

molecular markers. However, group C had all accessions of local origin. The lack of variability in the white clover germplasm could be because of limited flow of material to India. The similar results have been reported by Kolliker *et al.* (2001). Gustine and Huff (1999) and Gustine *et al.* (2002) found very high genetic variability in white clover using Random Amplified Polymorphic DNA (RAPD) markers.

Summary

Chapter VI

SUMMARY

Twenty-eight accessions of white clover *(Trifolium repens* L.) were evaluated at the Fodder Research Farm of the Department of Plant Breeding & Genetics, CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur during 2001-2002. The genotypes were grown in randomized block design with three replications. Analysis of variance revealed sufficient differences among the genotypes for different traits indicating the existence of high magnitude of genetic variability. In general, phenotypic coefficients of variability were higher than the corresponding genotypic coefficients of variability indicating the presence of environmental effects on the performance of genotypes. High genotypic coefficients of variation (GCV %) were observed for leaf area (cm^2), number of heads per plant, number of florets, herbage yield (g/plant) and mean plant spread (cm^2), which reflected high magnitude of genetic variation and hence greater scope for improvement through direct selection.

The effectiveness of a selection programme depends not only on the total variation available but also on the extent of heritability and genetic advance. The results of the present study revealed that traits viz., petiole length (mm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2) and number of florets recorded high heritability, thereby indicating lesser effect of environment. Thus, the selection for these traits on the basis of phenotype could be relied upon.

Further, high heritability coupled with high genetic advance for petiole length (mm), peduncle length (cm), leaf area (cm^2), number of heads per plant, days to 50% flowering, mean plant spread (cm^2), number of florets indicated the presence of high additive gene action for these traits. Hence, selection could be quite effective for improvement of these traits.

Cluster analysis was done for studying the genetic divergence in accessions of white clover *(T. repens)*, using various agro-morphological attributes based on standard IPGRI descriptors. The cluster analysis (Non Hierarchial Euclidean Cluster analysis) grouped accessions into 7 broad clusters, showing high level of genetic divergence. Clusters 7 and 2 were found to be distantly placed. The accessions clustered arbitrarily with genotypes of the same region were found distributed in more than one cluster, while the genotypes of heterogeneous region were grouped in the same cluster. The clustering pattern depicted the presence of sufficient genetic diversity and showed the lack of correspondence with geographical affinities of accessions. The morphological characterization, done by using standard descriptors and descriptor states developed by IPGRI, proved useful for cultivar discrimination and diversity analysis and gene bank management for white clover. Under natural epiphytotic conditions only two accessions viz., TWC-2 (exotic), RRCP-L-42 (a local collection) were found to be highly resistant to powdery mildew *(Erysiphe trifolii)*. Field screening of clover rot *(Sclerotinia trifoliorum)* showed that genotypes TWC-3, TWC-6, TWC-7, TWC-8, TWC-13, TWC-14, TWC-16, TWC-20 and RRCP-L-42 were found free from this disease.

A local collection (RRCP-L-42) from Sukhabagh (HP) was found highly resistant for powdery mildew and clover rot, therefore, this can be used as one of the parents in breeding for disease resistance in clover.

Dendrogram constructed by using molecular data, illustrated that genetic diversity and geographical origin of accessions did not show much correlation. The genotypes of the same regions were distributed in more than one cluster and also the genotypes of heterogeneous regions were grouped in the same clusters. However, group C had all the accessions of local origin. Molecular variation of higher order was resolved in the exotic accession compared to indigenous germplasm. This suggests the need to introduce the exotic germplasm in our environment to increase the genetic diversity in the indigenous white clover gene pool.

Literature Cited

LITERATURE CITED

A.O.A.C. 1970. Official methods of Analysis of the association of Official Analytical Chemists. 11th Edn. Washington, D.C.

Aasmo Finne, M., Roguli, O.A. and Schjelderup, I. 2000. Genetic variation in a Norwegian germplasm collection of white clover *(Trifolium repens* L.). Euphytica 112 : 33-44.

Ahloowalia, B.S. 1977. Strategy of forage crop improvement. Farm and Food Research 8 : 120-122.

Annicchiarico, P. 1993. Variation for dry matter yield, seed yield and other agronomic traits in white clover land races and natural populations. Euphytica 71 : 131-141.

Annicchiarico, P. and Piano, E. 1997. Effect of selection under cultivation on morphological traits and yield of Ladino white clover landraces. Genetic Resources and Crop Evolution 44 : 405-410.

Avtar, R., Khatri, R.S., Yadav, R., Arora, R.N. and Jatasra, D.S. 1997. Germplasm evaluation in Egyptian clover for fodder yield and components. Forage Research 22 : 279-284.

Bakheit, B.R. 1988. Selection for seed yield in Egyptian clover *(Trifolium alexandrinum* L.). Forage Research 14 : 39-47.

Beale, E.M.L. 1969. Euclidean cluster analysis. 37th session of the International Statistical Institute, U.K.

Bellitti, E., Bufano, G., Celi, R. and Ciruzzi, B. 1972. The effect of crude protein content on the digestibility of diet. Herbage Abstract 44 : 3533.

Bennett, S.J. 2000. Genetic variation of five species of *Trifolium* L. from South-West Turkey. Genetic Resources and Crop Evolution 47 : 81-91.

Bhandari, J.C., Singh, L.N. and Katoch, D.C. 1975. Performance of white clover in substropics of Himachal Pradesh. Forage Research 1 : 123-125.

Broda, Z. 1980. Heritability of characters in multifloriate mutants of diploid red clover *(Trifolium pratense* L.) Genetica – Polonica 21 : 275-281.

Bullitta, S. 1995. Development of RAPD markers of potential taxonomic use in the genus *Trifolium.* Journal of Genetics and Breeding 49 : 313-318.

Bullitta, S. and Piluzza, G. 1997. The use of RAPD molecular markers for evaluation of genetic variability between and within natural populations of *Trifolium pratense* L. Rivista-di-Agronomia (Italy) 31 : 137-140.

Burdon, J.J. 1980. Intraspecific diversity in a natural population of *Trifolium repens.* Journal of Ecology 68 : 717-735.

Burton, G.W. and De vane, E.W. 1953. Estimating heritability in tall fescue *(Festuca arundinaceae)* from replicated clonal material. Agronomy Journal 45 : 478-481.

Caradus, J.R. 1994. Genetic diversity within white clover *(Trifolium repens* L.). Proceedings-Annual-Conference-Agronomy-Society-of-New Zealand 24 : 1-7.

Caradus, J.R. and Chapman, D.F. 1996. Selection and heritability of stolon characteristics in two cultivars of white clover. Crop Science 36 : 900-904.

Caradus, J.R. and W.M. Williams. 1981. Breeding for improved white clover production in New Zealand hill country. In : Plant Physiology and Herbage Production. Ed. (E. Pbnght). PP. 163-168. Occas. Symp. British Grasslands Society 13.

Caradus, J.R., Mackay, A.C., Woodfield, D.R., Bosch, J.V. and Wewala, S. 1989. Classification of a world collection of white clover cultivars. Euphytica 42 : 183-196.

Corkill, L. 1971. Leaf markings in white clover. Journal of Heredity 62 : 307-310.

Crawford, D.J., Esselman, E.J., Windus, J.L. and Pabin, C.S. 1998. Genetic variation in running buffalo clover *(T. stoloniferum)* using random amplified polymorphic DNA markers (RAPDs). Proceedings of first Midwestern Rare Plant Conference 85 : 81-89.

Doyle, C.J. 1989. Economics of forage legumes. In : Legumes in farming system. eds. Plancquert and Hagger 148-155.

Fisher, R.A. 1918. The correlation between relatives on the supposition of mendelian inheritance. Transaction from Royal Society, Edinburgh 52 : 399-433.

Frame, J. (ed.). 1993. White clover in Europe – State of the Art. FAO REUR Technical Series 29 : 153.

Garg, D.K. and Gautam, P.L. 1997. Genetic divergence studies in wheat germplasm using non-hiearchicai euclidean cluster analysis. Indian Journal of Plant Genetic Resources 10 : 11-15.

Gill, R.S., Sharma, D.D., Chander, S. and Negi, S.S. 1967. Chemical composition of a few perennial forages grown in Kangra district. P.A.U. Journal Research 4 : 240-243.

Grant, D.A. and Lambert, M.G. 1979. Nitrogen fixation in pasture Unimproved North Island Hill Country, 'Ballantrae'. New Zealand Journal of Experimental Agriculture 7 : 19-22.

Gustine, D.L. and Elwinger, F.G. 2003. Spatio-temporal genetic structure within white clover populations in grazed swards. Crop Science 43 : 337-344.

Gustine, D.L. and Huff, D.R. 1999. Genetic variation within and among white clover populations from managed permanent pastures of North Western USA. Crop Science 39 : 524-530.

Gustine, D.L., Voigt, P.W., Brummer, E.C. and Papadopoulous, Y.A. 2002. Genetic variation of RAPD markers for North American white clover collections and cultivars. Crop Science 42 (2) : 343-347.

Harper, J.L. 1977. Populations biology of plants. London, U.K., Academic press. 892.

Harris, S.L., Auldist, M.J., Clark, D.A. and Jansen, E.B.L. 1998. Effect of white clover content in the diet on herbage intake, milk production and milk composition of New Zealand dairy cows housed indoors. Journal of Dairy Research 65 (3) : 389-400.

Hill, R.R., Shenk, J.S. and Branes, R.F. 1988. Breeding for yield and quality. Alfalfa and Alfalfa improvement 809-825, ASA, CSSA & SSSA, Madison.

Jahufer, M.Z.Z. and Gawler, F.I. 2000. Genotypic variation for seed yield components in white clover *(Trifolium repens* L.). Australian Journal of Agricultural Research 51 : 657-663.

Jahufer, M.Z.Z., Cooper, M. and Harch, B.D. 1997. Pattern analysis of the diversity of morphological plant attributes and herbage yield in a world collection of white clover *(Trifolium repens* L.) germplasm characterized in a summer moisture stress environment of Australia. Genetic Resources and Crop Evolution 44 : 289-300.

Jahufer, M.Z.Z., Cooper, M., Ayres, J.F. and Bray, R.A. 2002. Identification of research to improve the efficiency of breeding strategies for white clover in Australia. Australian Journal of Agricultural Research 53 : 239-257.

Jatasra, D.S. and Goyal, K.C. 1982. Genetic divergence in Alfalfa. Forage Research 8 : 117-120.

Johnson, H.W., Robinson, H.F. and Comstock, R.E. 1955. Estimates of genetic and environment variability in soybeans. Agronomy Journal 47 : 314-318.

Joyce, T.A., Abberton, M.T., Michaelson Yates, T.P.T. and Forster, J.W. 1999. Relationships between genetic distance measured by RAPD-PCR and heterosis in inbred lines of white clover *(Trifolium repens* L.). Euphytica 107 : 159-163.

Katiyar, P.K., Choubey, R.N. and Chaudhary, B.S. 1998. Eco-geographical diversity vis-à-vis agro-morphological divergence in germplasm of forage maize *(Zea mays* L.). Crop improvement 25 : 191-196.

Kolliker, R., Jones, E.S., Jahufer, M.Z.Z. and Forester, J.W. 2001. Bulked AFLP analysis for assessment of genetic diversity in white clover *(Trifolium repens* L.). Euphytica 121 : 305-315.

Kongkiatngam, P., Waterway, M.J., Fortin, M.G. and Coulman, B.E. 1995. Genetic variation within and between two cultivars of red clover *(Trifolium pratense* L.) : Comparison of morphological, isozymes, and RAPD markers. Euphytica 84 : 237-246.

Kouame, C.N. and Quesenberry, K.H. 1993. Cluster analysis of a world collection of red clover germplasm. Genetic Resources and Crop Evolution 40 : 9-47.

Lawson, N.C. 1971. Improvement of red clover and other species. Recherches-Agronomiques 16 : 69.

Lee, C.K., Eagles, H.A., Mc Farlane, N.M. and Kelly, K.B. 1993. Genetic variation within white clover *(Trifolium repens* L.) populations from North-Central Victoria. Australian Journal of Experimental Agriculture 33 (3) : 333-336.

Lorenzetti, F. 1992. Methods and procedures in the variety constitution. In : P. Rotili, P. and L. Zannone (Eds.), Proc. 10^{th} Int. Conf. EUCARPIA, Medicago spp. Group, pp : 494-498.

Martin, G.B., Williams, J.G.K. and Tanksley, S.D. 1991. Rapid identification of markers linked to a pseudomonas resistance gene in tomato by using random primers and near isogenic lines. Proceedings of National Academy of Sciences, USA 88 : 2336-2340.

Mazzarella, R., Montanaro, V., Keve, J., Reinbold, R., Coccidicola, A., D'Urso, M. and Schlessinger, D. 1992. Conserved sequence tagged sites : a phylogenetic approach to genome mapping. Proceedings of National Academy of Sciences, USA 89 : 3682-3685.

Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Research 8 : 4321-4325.

Panse, V.G. and Sukhatme, P.V. 1985. Statistical Methods for Agricultural Workers. Indian Council of Agricultural Research, New delhi, 359.

Raghunathachari, P., Khanna, V.K., Singh, V.S. and Singh, N.K. 2000. RAPD analysis of genetic variability in Indian scented rice germplasm *(Oryza sativa* L.). Current Science 79 : 994-998.

Rhodes, I. and Webb, K.B. 1993. Improvement of white clover. Outlook on Agriculture 22 : 189-194.

Rohlf, F.J. 1993. NTSYS-pc : Numerical Taxonomy and Multivariate Analysis System, Version 1.80. Exeter Software : Setauket, New York.

Rosso, B.S., Pagano, E.M. and Frutos, E. 1997. Evaluation of a collection of white clover *(T. repens* L.). Informe-Technico-Estacion-Experimental. Agropecuaria-Pergamin 17 : 316.

Rumball, P.J. 1979. Nitrogen fixation in pasture II. Northland warm temperate, Kaikohe. New Zealand Journal of Experimental Agriculture 7 : 7-9.

Russi, L. and Falcinelli, M. 1999. Characterization and potential role of annual clover landraces in the farming systems of Mediterranean areas. Journal of Agricultural Sciences 132 : 387-397.

Salisbury, P.A., Aitken, Y. and Halloran, G.M. 1987. Genetic control of flowering time and its component process in subterranean clover *(Trifolium subterranum* L.). Euphytica 36 : 887-902.

Sharif, B.S. 1989. Values of acid detergent fibre (ADF) and neutral detergent fibre (NDF) are better indicators of roughage digestibilities than crude fibre (C.F.) values alone. Journal Sckolah Technology – Makmal-Veteriner 5 (3) : 29-32.

Sidhu, B.S. and Mehndiratta, P.D. 1976. Genetic analysis of factors influencing forage yield in berseem. Indian Journal of Agricultural Sciences 46 : 168-170.

Sareen, S. 2003. Variability in white clover from the Indian Himalaya. http://www.fao.org/ag/AGP/AGPC/doc/bulletin/white clover.htm.

Thomson, D.J. 1984. The nutritive value of white clover. British Grassland Society Occasional Symposium 16 : 78-92.

Van Soest, P.J. and Wiens, R.H. 1967. Use of detergents in the analysis of fibrous feeds. Determination of plant cell wall constituents. Journal of Agricultural Chemistry 50 : 50-55.

Vavilov, N.I. 1951. The origin, variation, immunity and breeding of cultivated plants. Chronica Botanica, Waltham, Mass, USA. Journal of Hereditary 46 : 109-114.

Vavilov, N.I. 1951. The origin, variation, immunity and breeding of cultivated plants (selected writings). Chronica Botanica 13, (1/6) Waltham, Mass. U.S.A.

Vonbogaert, G. 1977. Factors affecting seed yield in white clover. Euphytica 26 : 233-239.

Walia, D.P. and D.K. Garg. 1996. Evaluation and genetic divergence in wheat *(Triticum aestivum* L.) germplasm. Indian Journal of Genetics & Plant Breeding 56 (4) : 452-457.

Welsh, J. and Mc Celland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18 : 7213-7218.

Wilkins, P.W. and Humphreys, M.O. 2003. Progress in breeding perennial forage grasses for temperate agriculture. Journal of Agricultural Science 140 : 129-150.

Williams, J.G.K., Kubelik, A.R., Livek, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research 18 : 6531-6535.

Wilman, D. and Moghaddam, P.R. 1998. *In vitro* digestibility and neutral detergent fibre and lignin contents of plant parts of nine forage species. The Journal of Agricultural Sciences 131 : 151-158.

Wioncek, J., Krzaczek, M., Marcinek, K. and Hulewicz, T. 1976. Phenotypic correlations with some characters of mother plants and their offspring in red clover *(Trifolium repens* L.). Genetica Polonica 17 (2) : 171-179.

Woodfield, D.R. and Caradus, J.R. 1994. Genetic improvement in white clover representing six decades of plant breeding. Crop Science 34 : 1205-1213.

Yu, L.X. and Nguyen, H.T. 1994. Genetic variation detected with RAPD markers among upland and low land cultivars *(Oryza sativa* L.). Theoretical and Applied Genetics 87 : 668-672.

Zohary, M. 1970. Trifolium. In Davis, P.H. (Ed.) Flora of Turkey, Vol. 3. Edinburgh, UK; Edinburgh University Press, 384-448.

Appendix

APPENDIX – I

Mean performance of genotypes

	Traits / Genotypes	Plant height (cm)	Middle leaflet length (mm)	Middle leaflet width (mm)	Leaf shape ratio	Petiole length (mm)	Peduncle length (mm)	Leaf area (cm^2)	Number of heads per plant	Days to 50% flowering
1.	TWC-1	18.67	17.99	17.94	1.10	85.97	101.28	9.96	22.79	122.00
2.	TWC-2	15.87	15.96	14.62	1.09	74.89	69.83	7.07	21.83	128.33
3.	TWC-3	17.11	16.66	13.67	1.23	77.83	67.83	7.69	20.26	100.33
4.	TWC-4	19.60	16.53	13.59	1.22	80.04	113.55	4.86	28.95	112.67
5.	TWC-6	20.15	17.80	14.64	1.22	99.83	67.33	11.17	32.82	127.67
6.	TWC-7	18.21	16.48	14.14	1.17	105.13	70.53	4.21	18.75	90.33
7.	TWC-8	20.26	17.13	15.28	1.12	99.48	85.86	10.08	23.56	115.67
8.	TWC-9	16.19	15.79	14.48	1.09	80.16	104.78	13.28	33.52	122.33
9.	TWC-11	17.58	15.51	14.00	1.11	80.23	120.42	10.21	40.15	141.67
10.	TWC-12	16.40	17.06	17.16	0.99	82.14	95.73	13.00	21.51	97.67
11.	TWC-13	19.94	16.22	14.59	1.11	74.76	78.43	11.09	18.10	102.00
12.	TWC-14	16.66	16.84	14.22	1.18	90.98	86.55	8.18	22.10	137.00
13.	TWC-15	17.65	16.00	15.31	1.04	92.45	88.50	8.22	32.96	124.67
14.	TWC-16	20.44	14.51	13.09	1.10	89.82	71.00	9.39	22.29	122.00
15.	TWC-17	22.26	16.31	13.65	1.19	109.28	99.40	11.15	30.89	126.00
16.	TWC-18	17.75	18.00	14.46	1.24	120.37	75.23	10.35	12.29	99.67
17.	TWC-19	14.96	16.97	14.80	1.15	105.68	66.00	9.07	16.32	112.33
18.	TWC-20	21.83	15.38	13.28	1.17	83.43	76.36	5.64	21.92	95.00
19.	TWC-21	20.02	14.96	12.25	1.22	89.83	74.50	6.51	29.88	112.33
20.	TWC-22	13.97	18.10	15.19	1.19	96.42	78.00	4.31	27.04	136.33
21.	RRCP-L-65	17.81	15.27	13.07	1.17	90.47	92.99	5.61	15.67	127.33
22.	RRCP-L-51	18.10	15.10	12.52	1.21	105.11	101.12	11.24	15.88	112.33
23.	Palampur Composite	23.49	15.37	13.40	1.14	115.22	103.26	9.16	39.34	125.00
24.	RRCP-L-123	21.09	17.69	15.00	1.18	125.45	92.84	10.95	37.59	132.33
25.	RRCP-L-17	18.84	17.40	15.57	1.12	123.13	82.36	12.56	25.64	116.67
26.	RRCP-L-43	20.33	19.52	14.87	1.32	113.08	91.62	7.19	36.33	126.00
27.	RRCP-L-45	16.62	16.88	14.02	1.20	100.12	69.22	8.56	25.15	143.33
28.	RRCP-L-42	20.58	13.70	11.00	1.25	71.43	80.07	8.08	16.73	116.00
	CD 5%	3.85	1.92	1.86	0.11	10.11	13.34	0.56	4.45	5.94

	Traits / Genotypes	Mean plant spread (cm^2)	Floret length (mm)	Stolon length (mm)	Number of florets per head	Number of seeds per pod	Herbage yield (g/plant)	NDF (%)	ADF (%)	Protein (%)
1.	TWC-1	543.32	7.26	192.90	47.27	3.27	34.98	28.4	30.93	20.65
2.	TWC-2	410.66	7.11	173.35	38.57	3.29	20.51	38.93	35.13	17.15
3.	TWC-3	698.04	7.01	186.13	41.94	1.66	26.12	29.40	32.87	21.23
4.	TWC-4	231.83	7.52	193.24	57.90	4.02	18.44	37.87	37.80	20.42
5.	TWC-6	609.57	7.84	180.73	37.33	3.14	36.47	27.00	32.80	23.10
6.	TWC-7	679.18	7.28	198.92	31.95	3.04	25.03	36.80	39.27	21.00
7.	TWC-8	509.26	7.59	200.61	39.45	3.03	33.42	35.67	33.73	19.25
8.	TWC-9	430.51	7.60	178.33	50.25	3.54	25.11	39.20	38.20	21.23
9.	TWC-11	89.40	8.44	176.33	82.56	1.43	20.61	33.13	34.13	22.38
10.	TWC-12	369.06	8.61	162.37	60.86	3.12	28.56	30.60	34.87	20.53
11.	TWC-13	464.53	7.56	189.06	42.25	3.48	34.03	36.40	39.27	19.37
12.	TWC-14	788.22	7.35	155.57	47.07	2.93	24.47	29.73	34.46	22.05
13.	TWC-15	536.16	7.84	155.62	61.09	2.67	26.18	27.67	33.46	21.58
14.	TWC-16	365.74	7.44	189.96	41.17	3.43	22.65	37.47	36.67	21.35
15.	TWC-17	418.81	8.68	217.25	55.86	3.92	32.59	32.46	37.27	21.82
16.	TWC-18	513.41	8.07	162.96	41.89	3.78	19.54	34.40	35.47	20.42
17.	TWC-19	369.58	[illegible]06	147.56	41.04	3.02	28.08	30.06	31.73	19.37
18.	TWC-20	308.49	[illegible]63	198.65	37.79	2.83	20.94	34.87	34.00	19.60
19.	TWC-21	449.29	[illegible]00	187.24	44.37	3.06	29.47	32.40	38.20	20.77
20.	TWC-22	389.07	[illegible]85	154.01	45.55	3.18	20.18	34.27	37.40	20.77
21.	RRCP-L-65	339.21	[illegible]34	191.97	47.53	2.90	17.07	41.46	31.87	22.28
22.	RRCP-L-51	478.64	[illegible]81	171.46	48.62	2.42	24.30	30.60	35.07	20.18
23.	Palampur Composite	325.98	[illegible]8	220.67	53.30	3.23	32.61	28.46	35.40	21.00
24.	RRCP-L-123	380.59	[illegible]	194.65	50.37	3.61	32.01	31.60	32.67	21.70
25.	RRCP-L-17	375.83	[illegible]	176.31	40.32	2.51	37.04	25.73	34.27	18.63
26.	RRCP-L-43	825.25	8.13	193.56	42.27	2.58	24.27	38.47	38.40	19.48
27.	RRCP-L-45	313.15	7.40	147.44	40.29	2.75	26.15	33.20	34.46	19.48
28.	RRCP-L-42	338.45	7.68	200.14	37.36	2.64	31.38	36.00	37.13	19.83
	CD 5%	128.38	0.61	28.09	6.67	0.58	7.01	4.59	4.07	2.00