Genetic diversity in taro (*Colocasia esculenta*) from Pakistan

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RESEARCH COMPLETION CERTIFICATE

It is certified that research work contained in this thesis entitled: "Genetic diversity in taro (*Colocasia esculenta*) from Pakistan" has been carried out by Abdullah and submitted to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, under the supervision of Dr. Ibrar Ahmed. It is accepted in its present form as satisfying the thesis requirement for the degree of Master of Philosophy in Biochemistry / Molecular Biology.

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DECLARATION

I, Mr. Abdullah, student of M. Phil Biochemistry/ Molecular Biology, session 2014-2016, hereby declare that the matter in the thesis entitled "Genetic diversity in taro (*Colocasia esculenta*) from Pakistan" is my own effort except where others have been acknowledged and that the thesis is my own composition; It has not been publishes, printed or submitted as thesis, research work or publication in any form in any university, research institution etc. in Pakistan or in foreign countries.

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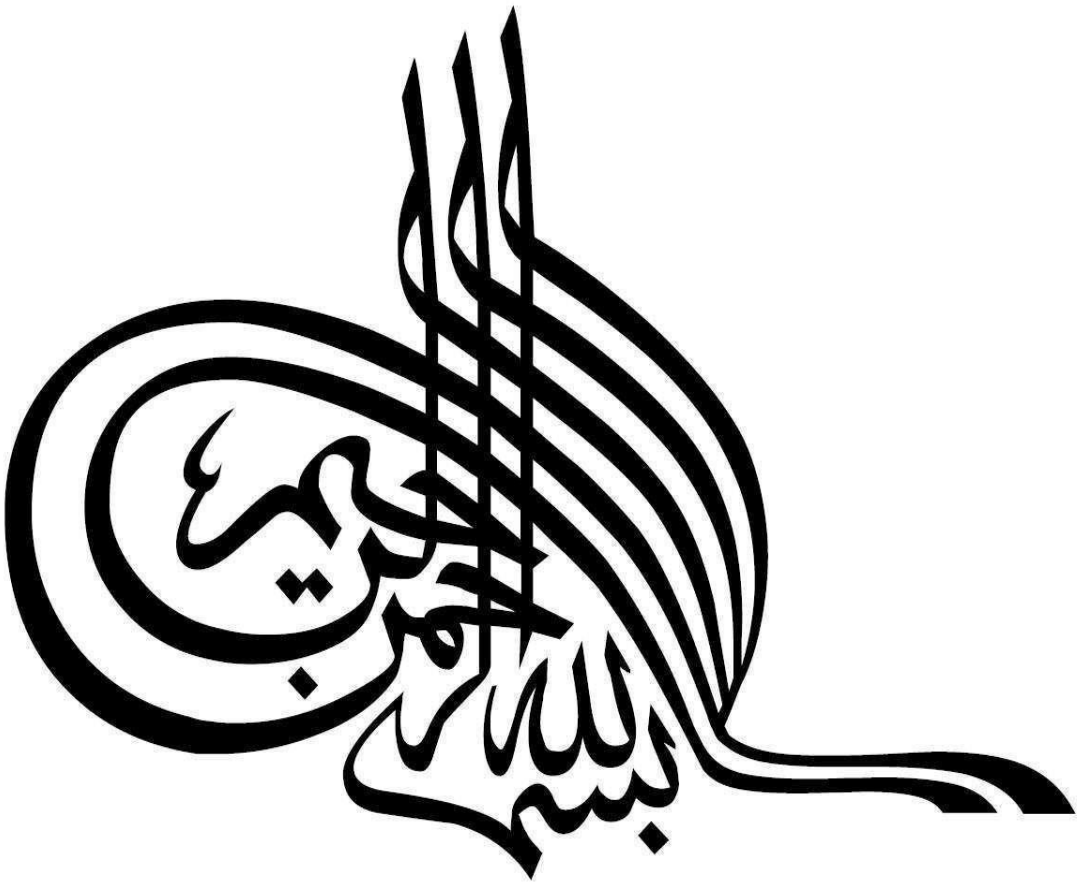

In the name of Allah,
the Most Beneficent,
the Most Merciful

DEDICATED

To

My Loving

Family

Without their Patience, Understanding, Support and most of all Love,

the completion of this work would not have been possible

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List of abbreviation

°C	Degree Centigrade
AFLP	amplified fragment length polymorphism
RFLP	Restriction and fragment length polymorphism
RAPD	Randomly amplified polymorphic DNA
EDTA	ethylene diamine tetra acetate
G	gram
mg	miligram
ug	microgram
ng	nanogram
PCR	polymerase chain reaction
Rpm	revolution per minute
SSR	simple sequence repeat
TBE	tris, boric acid, EDTA
TE	tris, EDTA
uM	micromolar
pM	picomolar
ml	mili liter
L	liter
M	molar
mm	mili meter
bp	base pair
ISSR	inter simple sequence repeat
ITS	Internal transcribed spacer

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Abstract

Colocasia esculenta (L.) Schott, commonly known as taro, is an important tuber crop with historical importance. It belongs to monocot Aroid family (Araceae). This highly polymorphic family consists of 110 genera with over 2500 species. Taro is considered among one of the oldest and widely cultivated crop as starch source. An estimated 400 million people in tropics and subtropics use taro in their diet as a staple food. Taro is mainly grown for its edible corm and leaves although all parts of taro, including corm, cormels, flower, leaves, stalk and rhizome are edible. The aims of current study are assessment of genetic diversity, documentation of morphological / agronomic traits and finding out affiliation of taro in Pakistan with Indo-Pacific superclade. Morphological data revealed significant variation of taro in Pakistan. Character which exhibited variation include plant height, leaf colour, leaf veins colour, leaf length, leaf width, petiole colour, petiole height and petiole sheathed edge area. To find out affiliation of taro in Pakistan with Indo-Pacific superclade, the 592bp fragment of chloroplast from all samples amplified by using ACECP05 primer. The sequencing revealed that all samples belong to Himalayan superclade. Taken together, the results manifested the presence of genetically diverse taro in Pakistan which belongs to Himalayan superclade. Furthermore, Study is needed on taro of Indus basin to further confirm affiliation of Pakistani taro with H-Superclade and IP-superclade.

Keywords: *Colocasia esculenta,* Genetic diversity, Himalayan Superclade, Indo-Pacific superclade, Morphological traits

Introduction and Literature review

Colocasia esculenta (L.) Schott, commonly known as taro, is an important tuber crop with historical importance. It belongs to monocot Aroid family (Araceae). This highly polymorphic family consists of 110 genera with over 2500 species (Beevi, 2013). However, species in few aroid genera have been domesticated as a carbohydrate source: *Colocasia*, *Amorphophallus*, *Xanthosoma Cyrtosperma* and *Alocasia* (Matthews, 1995). Common name taro applies to at least four plant species: *Cocolasia esculenta* (ordinary or true taro), *Alocasia maccrorhiza* (giant taro), *Cyrtosperma chamissonis* (giant swamp taro), and *Xanthosoma sagittiffolium* (tannia) (Ivancic and Lebot, 2000). All four species are grown as ornamentals or as root crops, with the ordinary taro (*Colocasia esculenta*) being the most important in this group (Ivancic and Lebot, 2000). Taro is considered among one of the oldest (Hill, 1939) and widely cultivated crops as a starch source (Matthews, 2004). An estimated 400 million people in tropics and subtropics use taro in their diet as a staple food (Bown, 1988). Taro is mainly grown for its edible corm and leaves (Mace and Godwin, 2002), although all parts of taro, including corm, cormels, flower, leaves, stalk and rhizome are edible (Lakhanpaul *et al*., 2003). In earlier classifications, taro was divided into two morphotypes: eddoe or *C.esculenta* var. *Antiquorum* and dasheen or *C.esculenta* var. *esculenta* (Purseglove, 1972). Owing to marked differences between the two morphotypes, these two types were sometimes considered as two separate species: *C. Antiquorum* and *C. esculenta*.

1.1 Genus *Colocasia:*

The genus *Colocasia* derives its name from qolqas or colcus, which is an ancient name of taro in Egypt (Candolle De, 1885: p74). There is some disagreement among researchers about the name and numbers of species within genus *Colocasia*, and varieties within *Colocasia esculenta* (Hay, 1996), which reveals both the plastic and polymorphic nature of *C. esculenta.* The genus might include up to 20 species (Li and Boyce, 2010).

1.2 Morphology of *Colocasia esculenta*:

Taro is a highly plastic species (Kumazawa *et al*., 1956; Arditti *et al*., 1979; Strauss *et al*., 1979; Hirai *et al*., 1989). Due to prevailing but unstable polymorphism in physio-agronomic characters, only the most important and genetically stable characters need to be described (Ivancic and Lebot, 2000).

Colocasia esculenta is an herb, usually 0.5-1.5 meters tall. Leaves are peltate, somewhat glaucous with hanging leaf blades, and have reticulate secondary venation and collective veins (Hay, 1990). Leaf laminas range from 30-80 cm long and from 20-50 cm wide (Alai and Ivančič, 1995). Leaf petioles are stout, with length from 0.3-1.5 meters. Leaf size is inconstant and is strongly influenced by the environment. Maximum size of taro leaves is associated with beginning of the flowering. As the plant reaches maturity, leaf petioles become short, and leaf blades also become small (Ivancic and Lebot, 2000). Leaf colour is genetically controlled. It varies between different genotypes from light green to dark purple, and is an important descriptor for genotype identification (Ivancic and Lebot, 2000). The type of inflorescence seen in taro is Spadix-spathe inflorescence which is characteristic of nearly all aroid species (Bown, 1988). A section of neutral tissue separates male inflorescence (upper part of spadix) from female inflorescence (lower part of spadix). Numerous ovules in two series arranged on 3-5 parietal placentas are present (Miller, 1975). The plant has stout underground rhizomes with stolons which form colonies. Large, starchy, underground corms are characteristic of taro. Corms vary in size, shape and pigmentation depending on genotype, ecological factors such as soil structure, soil texture, and presence / absence of stones in soil, and type of planting materials. Range of pigmentation of corms varies from white, light yellow, dark yellow, orange, pink, purple and red (Ivancic and Lebot, 2000).

1.3 Taxonomy:

Taro now referred as *Colocasia esculenta* (L.) Schott was originally named *Arum esculentum.* Purseglove (1972) systemize this specie with two botanical varrities: *C. esculenta* (L.) Schott. variety *esculenta*, named as dasheen, and *C. esculenta* (L.) Schott.variety *antiquorum* named as eddoe. According to Plucknett *et al*. (1970) dasheen and eddoe differ in size and shape of corm and cormels: Characteristic of dasheen is large

central edible corm and smaller side cormels or suckers; characteristic of eddoe is small central corms mostly inedible with well-developed side edible cormels. Taro also known by different vernacular names such as eddoe, dasheen, curcas, old cocoyam (Beevi, 2012). Local names ain Pakistan are arvi, arbi and kachalo.

1.4 Ploidy:

The species *C. esculenta* exists as two cytotypes: diploids with 2n = 28 chromosomes and triploids with 2n = 42 chromosomes (Ramachandran, 1978; Kuruvilla and Singh, 1981; Coates *et al*., 1988; Sreekumari and Mathew, 1991a; Sreekumari, 1992). Tetraploids are observed sporadically (Isshiki *et al*., 1999) and may be exceptionally sparse in distribution (Matthews, 2004). Diploid varieties are common throughout Oceania and Asia (Matsuda and Nawata, 1999). Triploids have been documented in India, Nepal, Timor,Philippines, Japan, New Caledonia, Australia and New Zealand (Isshiki *et al*., 1999). It is postulated all dasheen types could be diploids, and most eddoe types might be triploids (Irwin *et al*., 1998) although more data are needed for confirmation of this hypothesis (Ivancic and Lebot, 2000). Morphological, biochemical and Cytogenetic studies suggested autopolyploid origin of triploid taro (Isshiki *et al*., 1999), although allopolyploid origin is also possible (Yoshino, 1995; Yoshino *et al*., 1998).

1.5 Basic chromosomes number:

Literature about cytology of taro, especially data from India indicate confusion about basic chromosomes numbers. Darlington and Wylie (1956) reported two basic chromosome numbers: x = 12 and x = 14. Krishnan and Magoon (1977) postulated basic chromosomes numbers x = 7 based on meiosis and karyoa-morphological studies. Parvaain *et al*. (2009) support this postulate by reporting 21 chromosomes in taro variety AshuKachau in Bangladesh. However, subsequent studies on taro from India, Pacific region, New Zealand, Australia, and Papua New Guinea confirmed x=14 as the basic chromosome number, occurring either as diploid with 2n = 2x = 28 or triploids with 2n = 3x = 42 (Vijaya Bai *et al*., 1971; Ramachandran, 1978; Kuruvilla and Singh, 1981; Sreekumari and Mathew, 1991b, a Kokubugata and Konishi 1999). In addition, various

studies report deviations to these chromosome numbers: 2n = 22, 26 and 38 (Sharma and Sarkar 1963); 2n = 32, 44 and 46 (Subramanian 1979), and 2n = 14 (Mookerjea 1955).

1.6 Reproduction:

Principal mode of propagation is vegetative (Ivancic and Lebot, 2000) for which corms, cormels and stolons are involved (Matthews, 1990). Throughout the recorded history, this plant has been regarded a vegetative propagated crop species (Nyman and Arditti, 1985). It has been considered unable to produce flowers and seeds (Plucknett *et al*., 1970) due to it shy flowering habit, especially the cultivated ones (Vijaya Bai *et al*., 1971). Taro also produces flowers and viable seeds (Abraham and Ramachandran, 1960; Vijaya Bai *et al*., 1971; Jackson *et al*., 1977; Strauss *et al*., 1979). Flowering is rare in cultivated plants because most cultivated plants are harvested before flower, fruit and seed maturation (Matthews, 1990). During heavy rainfall or continuous rainy weather, this plant either does not produce flowers or produces infertile flowers (Ivancic and Lebot, 2000). Thus consecutive selection of plants take place without seed setting, mostly in sub-tropical to temperate areas with seasonal cropping systems such as Pakistan (Ahmed, 2014). However, seed can maturate and germinate in tropical regions; seed and fruit production has been reported in tropical parts of Asia and the Pacific, including in Bangladesh (Isshiki *et al*., 1995), New Guinea and Melanesia (Lebot, 1999), Myanmar (Matthews and Naing, 2005), Vanuatu (Caillon *et al*., 2006) and Australia (Hunt *et al*., 2013).

Pardales (1981) observed flowering and seed setting in wild taro in the Philippines, Strauss in Solomon Islands and Kikuta *et al*. (1938) in Hawaii. In India flowering correlates with season starting in mid-June (2-3 months after planting) and lasts till mid-September (Sreekumari *et al*., 2003). Devi (2012) reported that few varrities flowered in February too, four months after planting. In Pakistan flowering starts in August and lasts till September (author's personal observations). Both cross-pollination (Patel and Saelea, 1984) and self-pollination (Carson and Okada, 1982) are documented for taro, although morphology of inflorescences and differential rates of female and male floral developments favour cross pollination in taro (Jackson and Pelomo, 1979).

Meiotic studies sparse in taro due to shy flowering habit, especially the cultivated ones (Vijaya Bai *et al*., 1971; Ramachandran, 1978; Sreekumari and Mathew, 1993). Sreekumari and Mathew (1993) studied meiosis in both diploid and triploid taro and reported normal meiosis in diploid taro with bivalent formation, regular pairing and normal anaphase separation. In triploid taro, meiosis was found to be irregular due to formation of univalent, bivalent and trivalent leading to unbalanced anaphase separation which result in sterility of pollen.

1.7 Origin, domestication and dispersal history:

Taro origin, domestication and dispersal has been studied by numerous researchers including Spier (1951), Yen and Wheeler (1968), Matthews (1990) etc but consensus over centre of origin and dispersal is lacking. Scientist commonly believed that taro originated in Indo-Malayan region (Lakhanpaul *et al*., 2003) probably between Myanmar and Bangladesh (Ivancic and Lebot, 2000). Earlier authors proposed its dispersal from India towards east to Southeast and Eastern Asia and Pacific Islands, and towards west to Madagascar and Africa, from there onward to the Caribbean and the Americas (Yen and Wheeler, 1968). One route suggested for taro dispersal considers that it dispersed in three lines: two forms (28 and 42 chromosome) travelled northwards to Japan via Ryukus, and southwards to New Zealand via Timor and New Caledonia; the third form (28 chromosomes) progressed through Melanesia to Polynesia (Yen and Wheeler, 1968).

The hypothesis of Asian origin of taro and its domestication in the Pacific is associated with prehistoric records of human settlements in the Pacific Islands. Its dispersal has been associated with peoples cultures (Plucknett *et al*., 1970) and human dispersal because of its vegetative propagation and complications in dispersal by seeds (Yen and Wheeler, 1968). Pacific Island people originated in Southeast Asia and then colonized Sahul, the large land area comprising of New Guinea and Australia which was connected by a land bridge to Asia at least 40,000 years ago (Bellwood, 1979).

In disagreement to Asian origin of taro is the presence of wild taro in some Pacific countries, including Indonesia, Solomon Islands, Indonesia, New Caledonia, Papua New Guinea etc. Wild taro genotypes typically have excessive concentrations of

oxalates and extremely small corms due to which can't be used as food. Therefore, it is less expected that wild taro were brought to these countries by the migrating people. Ivancic and Lebot (2000) propose two separate domestications in Asia and New Guinea.

Ahmad (2014) compared 170 cultivated and wild taro accessions from Asian and Oceanic countries using 6 loci of chloroplast and 2 loci of nuclear genome and proposed taro originated in South to Southeast Asia during Miocene to Poliocene period, and reached Australia and New Guinea separately in wild and probably cultivated form. Three main Super-clades in these wider germplasm were identified. These superclades were named (i) a Southeast-Asian Australian superclade, consisting of wild taros only; (ii) a temperate to subtropical Himalayan superclade, consisting of mainly triploid taros, and (iii) a subtropical to tropical Indo-Pacific superclade, consisting of cultivated and wild as well as diploid and triploid taros (Ahmed, 2014).

1.8 Nutritive value of taro:

Taro has high nutritive value and its different parts are use as nutritive source for human and animal. Taro corm supply digestible starch, significant proteins, ascorbic acid, thiamine, niacin, riboflavin and significant value of dietary fibers (Niba, 2003). Taro leaves, consumed as a vegetable, contain β- carotene, proteins, iron, vitamins and folic acid, which prevent anaemia. Corm is source of high energy due to presence of starch. Potassium, calcium, magnesium and phosphorus are abundant mineral in taro. According to Abdulrashi and Nnabuenyi (2009) one meal of taro contain 31.0% dry matter, 7.87% crude protein, 4.75% crude fiber and 3214.91 kcal/kg metabolisable energy on basis of dry matter. The result of proximate analysis of Ogunlakin *et al*., (2012) shows taro contain dry matter 89.53-90.57% which contain carbohydrate 78.7-79.0%, crude protein 4.93-5.17%, crude fibres 2.70-2.97%, ash 2.47-2.87% and ether extract 0.50-0.57%. Taro also contains anti-nutritional nutrients such as saponins, tannins, phytates and oxalates. The anti-nutritional factors such as saponins, tannins, phytates, oxalate and hyrocyanide limit taro nutritive value to animal because of toxic effects. Saponins cause bloat in ruminants, condensed tannins contain flavonoid polymers of flavonol and as astringent bind to protein and reduce protein availability to animals. They also inhibit cellulose digestion and reduce digestion of crude fibres. Phytates decrease utilization of many

elements such as phosphorus, calcium and magnesium –taro's most abundant minerals - because they form insoluble compounds which excrete in faeces (Udeybir *et al*., 2008). Oxalates precipitate calcium as insoluble calcium oxalate in gastrointestinal tract of animals and as a consequence, decrease growth and milk production (Udeybir *et al*., 2008). These toxins are destroyed with different processes such as soaking, cooking, drying and ensiling with which nutritive value of taro can be increased in animal feed (Adejumo *et al*., 2013).

Studies show that differently processed taro is good source of nutrition for animals. It is not only beneficial to animals but also decreases competition between human and animals for use of competitive crops such as maize. Fresh leave and stem fed animals show poor weight gain and feed conversion ratio as compared to taro cooked leaves and stem, while taro leaves and stem show best result when ensiled (Ndabikunze *et al*., 2011).

1.9 Genetic diversity:

The existing of genetic variation within a population is called as genetic diversity. Genetic diversity plays important role in adaptability and survival of species. A species with excessive genetic diversity will incline to produce a wide range of variant offspring, in which some might be better fit variants in a population. In contrast, a species that has little genetic variation will produce offspring that are genetically alike and will be more susceptible to problems or diseases. Hence, lack of genetic variations reduces biological fitness and increases species extinct chances. Genetic diversity within and between populations can be evaluated using morphological, biochemical and molecular characterization and evaluation.

1.9 Morphological Characterization:

Taro has wide variation in morphological characters such as leaves, tubers and flowers as well as chemicals such as aroma and flavour (Lebot and Aradhya, 1991b). Taro genetic variation is evaluated with two approaches: Molecular marker and agro-morphological character, many researchers study genetic diversity using both approaches, they evaluated morphological characters with a list of descriptors. In these descriptors the

colour of leaf, petiole colour, petiole junction colour, petiole height, petiole width, sinus shape, flowering etc are important (Hirai *et al*., 1989; Okpul *et al*., 2004; Singh *et al*., 2007; Trimanto *et al*., 2010; Mulualem Beyene, 2013; Ezeabara *et al*., 2015). Diversity study based on morphology alone has a major disadvantage due to influence of environment. Moreover, due to vegetative propagation of taro for thousands of years, morphotype can be distinct as a result of fixation of somatic mutations (Kuruvilla and Singh, 1981).

1.10 Biochemical and molecular markers:

Molecular tools improve our knowledge about the origin, geographical distribution, phylogenetic relationship, hidden genetic diversity and susceptibility of a crop to any particular disease (Devi, 2012). The knowledge of germplasm characteristics and evolution process is essential to utilize and conserve plant genetic resources (Whitkus *et al*., 1994). Researchers used various molecular markers to access genetic diversity and characterisetaro germplasm in order to broad its cultivar range. A brief review of different molecular markers given below:

1.10.1 Isozyme Markers:

Isozyme markers used earlier than DNA markers. Lebot and Aradhya (1991) studied genetic diversity of taro for the first time using isozyme markers. Their study covered wide geographical region - Oceania and south-east Asia - with 1417 taro (wild and cultivated) sample using enzymes systems isocitrate dehydrogenase, Malate dehydrogenase, phosphoglucoisomerase, malic enzyme, shikimic dehydrogenase, 6-phosphogluconate dehydrogenase and alcohol dehydrogenase. Their study shows Asia having more variation than Pacific, Indonesia is an area of great diversity and majority of Indonesian cultivar shows divergent from Pacific and Philippines cultivar. Prana *et al*. (2000) identified 100 zymotype in 328 taro samples of Indonesia and West Java using 6 enzyme systems, which reflect great variation.

Manzano and Nodals (2001) identify twelve polymorphic loci with twenty-seven alleles for esterase isozyme. Trimanto *et al*. (2010) confirmed morphological characters are independent of altitude with isozyme study using three enzyme systems – esterase,

shikimate and dehydrogenase peroxidase – in taro accessions of Karanganyar district, Central Java.

1.10.2 Amplified fragment length polymorphism (AFLP):

Eck *et al*. (1998) studied genetic diversity of 217 accessions – wild and cultivated accessions – of taro from Oceania and Southeast Asia using AFLP. They observed significant variation with 25% polymorphic markers in diploid and triploid accessions of Indonesia consequently 0.11 and 0.14 and in triploid accessions of Vietnam 0.13. Kreike *et al*. (2004) studied 255 accessions of seven countries – Thailand, Vietnam, Indonesia, Malaysia, Philippines, Papua New Guinea and Vanuatu – with three AFLP primers. They observe high variation 0.19 in wild accessions of Thailand and less variation 0.007 in diploid cultivars of Thailand. Asia and Pacific accessions assemble to separate cluster group which support presence of separate gene pool of Pacific and Asia. They observed AFLP markers couldn't differentiate diploid and triploid accession due to absence of unique bands observed by Irwin *et al* (1998) using RAPD. Caillon *et al*., (2006) distinguish 96 morphotype with insignificant intra-clonal variation.

1.10.3 Microsatellites markers (SSRs):

SSRs are co-dominant and reproducible markers (Devi, 2012). Mace and Godwin (2002) developed forty three primers, from which forty one produce amplify product of expected size. They identify sixteen polymorphic markers – average 3.2 alleles/locus – when compared with restricted germplasm of taro accessions from Oceania and South-east Asia. Godwin *et al*. (2003) used seven polymorphic markers from this set and evaluate genetic polymorphism of 511 taro accessions of Pacific Island countries; seven primers amplify 38 alleles from seven loci with average of 5.4 alleles per locus. Their study suggests SSRs are informative to reveal genetic variation within country and among different countries. Bastide (2000) constructed another enriched microsatellite library; using these markers Noyer *et al*. (2003) studied genetic variation of 105 taro accessions and revealed 100 alleles. Mace *et al*. (2006) amplify 38 alleles from core collection of 515 accession of taro from ten countries of Oceania – Solomon, Vanuatu, Papua New Guinea, Fiji, Palau, Islands, Tonga, New Caledonia, Samoa, Niue and Cook

Islands – using seven SSR markers. They observed 12% variation between population and 88% within population considering accessions of one country as one sub-population. Maximum genetic variations were present in Palau (0.658), Solomon Islands (0.618) and Papua New Guinea (0.589). Moreover, they identify rare alleles from Solomon Islands. Singh *et al*. (2008) established national core collection of taro – first specie whose core collection made by molecular markers – in PNG using SSR markers. They collected 859 taro accessions from 15 provinces of PNG; they selected a core of 20% of these on bases of 30 agro-morphological descriptors and further reduced it to 10% on based on data of seven SSRs markers. Sardos *et al*. (2012) evaluated genetic variation of taro accessions collected from ten villages of South-west pacific situated on different island using nine SSR markers which amplify 89 alleles, six to seventeen alleles per locus with average of 9.89. Moreover, they also detected rare alleles up to 57.3%. The study of Nunes *et al*. (2012) revealed that SSR markers could distinguish between taro cultivars and can be useful for identity of alleles. Molecular data set of SSR revealed that these markers are good tools for monitoring diversity and evolution of taro in future.

1.10.4 RAPD (Randomly amplified polymorphic DNA):

Irwin *et al*. (1998) used RAPD markers to evaluate genetic diversity of forty-four taro accession. They observed high genetic variation in Indonesian taro similar to Lebot and Aradhya (1991) isozyme base study. Moreover, they differentiate between Hawaiian taro and between diploid/triploid taros which were monomorphic with isozyme results. Lakhanpaul *et al*. (2003) evaluate 32 accessions from India belong to 28 morphotype using RAPD. The absence of similarity coefficient equal to one even between two accessions shows high genetic diversity and uniqueness of 32 accessions. They used primers of QWP series which revealed 60-100% polymorphism except QWP-12. Shen *et al*. (2003) observed 88% polymorphism with RAPD in China; Sharma *et al*. (2008) observed 97% polymorphism in India. Moreover, Cultivars of two different parts; North and south formed separate group.

Pillai and Lekha (2008) find high diversity within 45 taro accessions of India using RAPD. Beevi *et al*. (2011) studied genetic diversity of 60 accessions of taro from south India. They point up RAPD also has ability to distinguish between cultivated and

wild accessions. Singh *et al*. (2012) access genetic variation of three commercial verities as reference genotype and 21 taro accessions of Andaman using both ISSR and RAPD, which divided the island and commercial accession into two separate sub clusters, that also revealed correlation with morphological parameters.

Hussain and Tyagi (2006) access genetic stability of an *in vitro* regenerants of taro accession using thirteen RAPD primers and seven ISSR markers that gave 111 and 43 unique band respectively that revealed RAPD primers were useful to find out genetic stability of regenerants.

1.10.5 Chloroplast DNA markers:

Ahmad *et al*. (2012) reported chloroplast genome sequences for two taro morphotypes; Ahmed *et al*. (2013) designed 30 pairs of primers for polymorphic loci of chloroplast to study domestication and phylogeography in taro. Using some of these loci, three main superclades have been documented in wider taro germplasm (Ahmed, 2014).

1.11 Status of research on taro in Pakistan:

In Pakistan, taro has been an ignored crop. Despite its common use as a vegetable, there is not a single report available, documenting genetic diversity in taro. Even standard descriptors to identify different varieties of taro are not available with the Federal Seed Certification and Registration Department, the Government agency with a mandate to record new varieties of crops. However there is one report about its use as a food ingredient in poultry industry (Abdulrashi and Nnabuenyi, 2009).

In a phylogeographic study involving taro germplasm from more than 20 countries, the few taro samples from Pakistan appeared to be part of the Himalayan superclade (Ahmed, 2014). These taros are reported to be triploids, with a vegetative mode of reproduction. The other superclade, Indo-Pacific, which is the most widely distributed superclade in tropics and sub-tropics, and which contains diploid and triploid taros, can also be expected in southern plains of Pakistan (mainly the Indus basin). If diploid taros could be found in Pakistan, these could be used in breeding programs to improve nutritive and cooking value, and to introduce further genetic diversity.

1.12 Aims and Objectives:

The aims and objectives of this study are:

1. Assessment of genetic diversity in taros from Pakistan, by using morphological and molecular techniques.
2. Finding out if the taros with Indo-Pacific superclade affiliation are also found in Pakistani germplasm.
3. Documenting morphological/agronomic traits of importance from taro collected from diverse parts of the country to assess if there exists any variety with genetically stable traits.

Materials and Methods

2.1 Plant sample collection:

Plant samples were collected from three provinces of Pakistan named Khyber Pakhtoon Khwa (KPK), Sindh and Punjab in August, September and October, 2015. Cormels of each sample were planted in Nowshera and Gujar Khan. Morphological and molecular analyses of each sample were evaluated to check genetic diversity whereas chromosomes count was done to study ploidy level of taro in Pakistan. Leaf of each variety was taken for extraction of Deoxy ribonucleic acid (DNA)

Table 2.1: Shows samples numbers, district and province of plant collection, environmental condition, latitude and longitude of each district.

Sample number	District	Province	Latitude (North)	Longitude (East)	Additional note
CESPK01	Swat	KPK	35.48	72.46	Black taro with less hair
CESPK02	Charsada	KPK	34.15	71.74	Thin petiole and small leaf
CESPK03	Lower Dir	KPK	34.85	71.90	Small oval cormel
CESPK04	Haripur	KPK	33.99	72.94	Purple colour at leaf blade margin
CESPK05	Jhang	Punjab	31.26	72.32	Black red skin
CESPK06	Lahore	Punjab	31.55	74.36	Red skin and round cormel
CESPK07	Mardan	KPK	34.20	72.05	
CESPK08	Multan	Punjab	30.20	71.47	Small round cormel
CESPK09	Nawabshah	Sindh	26.24	68.41	
CESPK10	Nowshera	KPK	34.02	71.98	New strain of Nowshera
CESPK11	Nowshera	KPK	34.02	71.98	Old strain of Nowshera
CESPK12	Nowshera	KPK	34.02	71.98	Large cormel
CESPK13	Nowshera	KPK	34.02	71.98	Very large cormel
CESPK14	Punjab	Punjab			
CESPK15	Sukkur	Sindh	27.71	68.85	Large black oval cormel without hair thin in center
CESPK16	Hyderabad	Sindh	25.38	68.37	Small cormels
CESPK17	Swat	KPK	35.48	72.46	Black cormel with no hair
CESPK18	Gujrat	Punjab	32.57	74.1	

2.2 Cultivation of samples:

The samples were cultivated in Nowshera, KPK, Pakistan at the distance of 0.5 meter in rows and five plants were planted in a row. The length of field was 5 X 5 meter. The latitude (N) and longitude of district is 34.02 and 71.98 respectively. The dune cake which was approximately about 3 kg is applied at 2nd month and Urea was applied at fourth month of cultivation to boost up growth. Moreover, humic acid was used in every month from March, 2016 to august, 2016 in order to maintain the softness of field.

2.3 List of descriptors for morphological characterization:

Samples were analyzed by using descriptors of the International Plant Genetic Resources Institute, (IPGRI, 1999), Hirai et al. (1989), and Hawie taro descriptor list. A comprehensive set of descriptors were selected which were useful for general characterization of samples. Table 2 shows the descriptors list which were used for analysis of selected samples. The above ground traits were score at third, fourth and fifth month of planting.

Table 2.2: Morphological character selected from descriptors list

S.No	Trait	Trait code	Description
1	Growth habit	GH	fasciculate , non-fasciculate
2	Plant type	PT	erect, medium, spreading
3	Plant height	PH	< 50cm = Dwarf, 50-100 cm = medium, >100 cm = Tall
4	Plant width	PW	
5	Number of leaf emerge from cormel	LEC	3, 5, 7
6	Leaf colour	LC	light Green, green, dark green
7	Pigment at leaf center	PLC	Absent or present
8	Leaf length	LL	40 cm, 60cm, 80cm
9	Leaf width	LW	20 cm, 30 cm, 40 cm
10	Leaf thickness	LT	.3 mm, .35 mm, .40 mm
11	Depth of leaf base sinus		4 cm, 8 cm, 12 cm
12	Leaf shape	LS	Cordate , cup shape horizontal apex down, oval
13	Tip shape of leaf	TSL	Pointed, intermediate, round
14	Length of leaf from center to margin	LLCM	

15	Length of leaf from center to sinus	LLCS	
16	Length / width ratio	LWR	
17	Length of leaf from center to margin/width	LLCMW	
18	Length of leaf from center to sinus/ width	LLCSW	
19	Length of leaf from sinus to margin	LLSM	
20	Total length/ length of leaf from sinus to leaf margin	LL/LLSM	
21	Pigmentation in leaf veins	PLV	Absent or present
22	Gloss of leaf surface	GLS	Absent, slight, medium, intense
23	Leaf base shape	LBS	Peltate, sagittate, hastate,
24	Leaf blade margin	LBM	Entire, undulate, sinuate
25	Leaf blade colour	LBC	Green, dark green, yellow, purple
26	Leaf blade colour variegation	LBCV	Absent or present
27	Type of variegation	TV	Dark green, greenish
28	Leaf center depth	LCD	
29	Colour of midrib	CM	
30	Petiole length	PL	50 cm, 80 cm, 100 cm
31	Petiole thickness	PTC	6 cm, 12 cm, 18 cm
32	Petiole sheathed edge length	PSL	15 cm, 25 cm, 35 cm
33	Petiole length/ petiole width ratio	PL/PW	
34	Petiole length/ leaf length	PL/LL	
35	Petiole length/ petiole sheathed edge length	PL/PSL	
36	Petiole color-top	PCT	Green, purple, yellow, whitish green
37	Petiole color-bottom	PCB	Green, purple, yellow, whitish green
38	Petiole bending at lamina junction	PBLJ	Absent, partial, medium, deep
39	Pigmentation at basal part	PBP	Absent or present

	of petiole		
40	Pigmentation at sheathed edge of petiole	PSE	Absent or present
41	Number of stolon (side shoot)	NS	5, 9, 13
42	Stolon length	SL	15 cm= small, >15 cm = tall
43	Vein pattern	VP	Y, I, V

2.4 Protocol for ploidy:

Chromosomes number of each sample was counted by following protocol:

i. Collect freshly growing root tip in the morning between 8.00AM- 8:30AM.
ii. Wash the root with distal water to remove dust.
iii. Put the root in 0.05% colchicine solution for 4hrs in a microfuge tube in order to stop the cells in metaphase plate. The microfuge tube was wrapped in aluminum foil in order to maintain dark because colchicine react with light.
iv. Root and root tip was taken from microfuge tube after 4hrs, wash with distilled water, dry and fix in freshly prepared 3:1 (ethanol: acetic acid) fixative for 72 hr.
v. Remove root and root tip from fixative and washed in distilled water.
vi. The root tip was placed in another microfuge tube and hydrolyzed in 1N HCl at 60^{o}C for 10 minutes.
vii. Root tip was washed with distilled water, dried and stained in 1% basic fuchsin for 1hr to stained chromosome.
viii. A small piece of root tip was removed and macerate in 45% acetic acid on slide. Put cover slip on slide and observed under microscope by using 40X lens.
ix. When chromosomes become visible under 40X lens and then observed under 100X lens by using oil immersion capability in order to get clear image of chromosomes for counting.
x. The chromosomes were count and the image was taken by camera.

2.5 DNA extraction:

DNA were extracted by using phenol: chloroform: iso-amyl alcohol methodology of Ahmed *et al*. (2009) and Plant mini Kit protocol. 4µL of each extracted DNA sample was run on 1% Agarose gel stained with Ethidium bromide for 45 minutes at 100V,

whereas 4µL of 1kb mass ladder (Invitrogen) was used for comparison of DNA quantity. Gel was visualized under UV transilluminator (Biometra, Gottingen, Germany); photograph was taken under gel digital camera DC 290 (Kodak, New York, USA).

2.6 DNA Quantification:

UV-spectrophotometer (Biometra, Gottingen, Germany) was used to quantify DNA at optical density of 260 nm. After quantification, stock solution of DNA was diluted for polymerase chain reaction (PCR) up to 20-50 ng/ µl.

2.7 Primer selection:

2.7.1 RAPD primer selection:

High polymorphic RAPD Primer of OPW series reported by Lakhanpaul et al. (2003) and OPA series reported by Beevi et al. (2011) were used to find out genetic diversity of taro in Pakistan. These primers have 100 – 1000 base pairs PCR product. Six primers other than RAPD primer design by Ahmed et al. (2012) were used in this study.

The sequence of RAPD shown in Table No 2, whereas table no 3 shows primer of Ahmed et al. (2012).the primer was purchased from Macrogen, South Korea.

Table 2.3: RAPD primers

Primers	**Sequence (**5′-3′)	**Length**	**Melting Temperature**
OPW-4	CAGAAGCGGA	10	32
OPW-9	GTGACCGACT	10	32
OPW-15	ACACCGGAAC	10	32
OPW-16	CAGCCTACCA	10	32
OPW-17	GTCCTGGGTT	10	32

OPA-03	AATCGGGCTG	10	32
OPA-04	AGGGGTCTTG	10	32
OPA-13	CAGCACCCAC	10	34

Table 2.4: From serial number 1-4 shows chloroplast marker and 5 and 6 shows nuclear marker.

S.No	Primer pair	Sequence	Size
1	ACECP005	F: AAAATGGGGTTCCTAGTGGA R: ACTCGAACTCGAAGAAATGG	631
2	ACECP014	F:AGTACCAAAAGTAGATTCGGG R: GGGGATTACTGACGTTTGTT	977
3	ACECP026	F: ACTACGGTAGAGCGGTTTAT R: AAAGTCATCTCACGTTCACC	478
4	ACECP030	F: AACGCGGTTTTGATCAGTAT R: CAATTCATTGCGCAACTTGT	805
5	ITS(colo)	F: GACACACTCGCGAACGGTTGAC R: GCGTGTGCCACGGCGACGATG	563
6	PhyC	F: AAACATTTCCCTCTTGTG R: TACTTAAGCCTGTAGTAGTA	769

2.8 Amplification of Nuclear genome with RAPD primers:

Polymerase chain reaction (PCR) conditions were optimized by using different dilution of extracted DNA containing 5ng to 30ng DNA, MgCl salt 2-3 mM and Taq DNA polymerase 1-2 U. PCR amplification was carried out in a DNA Thermal cycler (Mygene^{TM} Series Peltier Thermal Cycler, model MG96G) in a final volume of 25ul.
Final 25 µl PCR reaction mixtures were prepared for each sample was consist of 20ng-50ng, 2.5 µL of PCR Buffer (100 M Tris-HCl, 500 mM KCl, 8.3 pH), 2.5mM $MgCl_2$, 200 µM dNTPs, 0.5 µm of primer, 1.5 Unit of Taq DNA Polymerase (Fermentas, UK) and the final volume was adjusted with water.

All PCR tubes were placed in T1 thermocycler (Biometra, Germany) for amplification. The amplified product was run on 2% gel stained with Ethidium bromide to confirm amplification, number and size of bands. The PCR was programmed at the following cycling condition given in table 2.5.

Table 2.5: Optimized conditions for RAPD primers

Steps	Sub-Cycles	Conditions	PCR Cycles
Initial Denaturation		95˚C,10 min	40 cycles
PCR Cycles	Denaturation	95˚C, 1 min	
	Primer annealing	32, 1 min	
	Primer extension	72˚C, 45 sec	
Final Extension		72˚C, 10 min	
Hold		12˚C, ∞	

2.9 Amplification with Chloroplast and Nuclear Marker (ITS and PhyC):

Polymerase chain reaction (PCR) conditions were optimized by using different dilution of extracted DNA containing 10 ng to 50 ng DNA, MgCl salt 2-3 mM and Taq DNA

polymerase 1-2 U. PCR amplification was carried out in a DNA Thermal cycler (Mygene™ Series Peltier Thermal Cycler, model MG96G) in a final volume of 25ul.
Final 25 µL PCR reaction mixtures were prepared for each sample; consist of following components:

- 1.5 µL template DNA sample (20ng-50ng)
- 2.5 µL of PCR Buffer (100 M Tris-HCl, 500 mM KCl, 8.3 pH)
- 1.8 of $MgCl_2$ (25 mM)
- 200 µM dNTPs
- 0.5 µm of each forward and reverse primer (100 µm)
- 0.3µl of Taq DNA Polymerase (5 U/ µL, Fermentas, UK)
- 15.9 µL of PCR water

Once reaction mixture was prepared; mixed it and poured in each PCR tube containing specific DNA template. All PCR tubes were placed in T1 thermocycler (Biometra, Germany) for amplification. The amplified product was run on 2% gel stained with Ethidium bromide to and visualized under transilluminator to confirm amplification and the number and size of bands. The PCR was programmed at the following cycling condition given in table 2.6.

Table 2.6: PCR amplification conditions for chloroplast and nuclear marker

Primer		**Steps**	**Sub-Cycles**	**Conditions**	**PCR Cycles**
ACECP005	50	Initial Denaturation		95˚C,10 min	35
		PCR Cycles	Denaturation	95˚C, 1 min	
			Primer annealing	Mentionedin front of each primer	
ITS(colo)	55	Final Extension		72˚C, 10 min	
PhyC	45	Hold		12˚C, ∞	

The ITS, PhyC and RAPD primer did not give consistent result; therefore it was not used further to evolve genetic diversity.

2.10 Protocol for PCR product cleans up:

The PCR product of Chloroplast markers, ITS and PhyC were cleaned using SAP (shrimp alkaline phosphatase; the enzyme used to de- activate unused dNTPs present in PCR products) and EXO (exonuclease I; the enzyme used to degrade single stranded primers present in PCR products). A standard reaction included 2µL of SAP, 1µL of EXO, and 10 µL PCR products. The PCR products were then placed in thermocycler and "clean-up" or "SAP/EXO" programs were used for cleanup. This consisted of keeping the reaction mixtures at 37°C for 30 minutes, followed by 80°C for 15 minutes.

Results

3.1 Chromosomes counting:

The chromosome counting was done to find out the ploidy level of taros from Pakistan. We succeed in counting of chromosomes of few samples which were triploid and having 42 chromosomes as shown in figure 3.1.

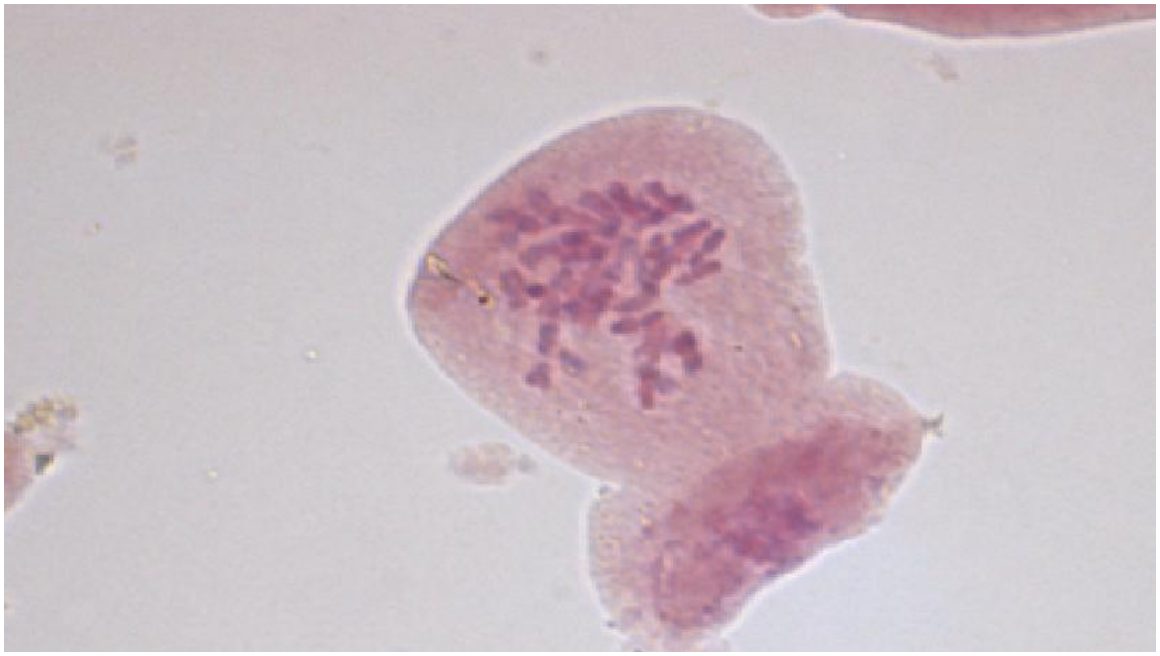

Figure 3.1: Taros from Pakistan were triploid and having 42 chromosomes

3.2 Morphological Data:

To evaluate difference in growth pattern of each sample, morphological data was taken from May 2016-July 2016. Variation in morphological character (according to descriptor list described in table 2.2) was monitored in every month. The leaves of CES01, CES02 and CES17 were thin and had medium glossy appearance. However, all other samples had thick leaves and didn't have glossy appearance. The leaves of all samples were pointing-downward and have reticulate venation. Three strong midribs arose from point of petiole attachment. The primary midrib pointed toward the main lobe whereas the other two midribs pointed toward the two posterior lobes. Four samples

CES01, CES02, CES15 and CES18 exhibited purple pigmentation at the centre of leaf. Moreover, these samples also revealed purple color in petiole sheathed edge area. Primary veins were originated from main midribs. The upper and lower surface of leaf was smooth and leaf margin was entire. The petiole exhibited variation in height and thickness. The color of petiole showed high variation including green, whitish green and dark green color. Five samples CES01, CES02, CES04, CES15, CES18 petiole had purple color dot of 0.5-1 cm exactly on the point of attachment to leaf.

All samples show Y-pattern of vein and the variegation in leaf was absent. In May 2016, samples showed considerable variation in height. CES04 had maximum and CES03 had minimum height of 78cm and 32cm respectively.

The shape of leaf was either ovule or cordate. Among 17 samples, 10 had cordate leaf whereas eight samples had ovule leaf. The length of leaf reflects that 10 samples had leaf length from 23-27.5cm, two samples had 17cm, five samples had 31-35cm and one sample (CES06) had leaf length of 38.5cm. One can observe increase diversity on basis of leaf width in these samples. CES01, CES04 and CES05 which were almost similar on the basis of leaf length showed remarkable difference in leaf width. The lengths of leaf of these samples were 25cm, 27cm and 25.5cm but their leaf width was 14cm, 22cm and 18cm respectively. Additionally, the collected samples also indicated greater variation in length of leaf from center to margin and from center to sinus i.e. the leaf length of CES06 and CES07 was 38.5cm and 25cm respectively. Whereas, the length from center to margin was 17.5cm of each sample and the length of leaf form sinus to margin was 9.2 and 5.5 respectively.

` The ratio of length of leaf and length of leaf from center to margin showed high variation in the samples, the lowest value was 1.4 and the highest value was 2.2. Leaf length to leaf width ratio also revealed greater variation. The CES02 had minimum ratio of 1.3 and CES10 had maximum ratio of 1.85. The ratio of length of leaf from center to margin and width; length of leaf from sinus to margin and width; length of leaf and length of leaf from sinus to margin showed insignificant variation.

Taro showed greater diversity in petiole length, petiole sheeted edge length, petiole upper and bottom colour and at basal part of petiole (IPGRI, 1999). The lowest

petiole length of CES02 was 20cm and the highest petiole length of CES04 was 66 cm. Petiole thicknesses at lower part just above petiole sheeted edge length show insignificant variation among the samples and most samples have same petiole thickness of 3cm. The ratio of petiole length and petiole sheathed edge length indicated that both these characters were not correlate to each other significantly. The ratio for CES02 was 4.75 whereas for CES15 was 1.74. Petiole length and leaf length have correlation in maximum Sample, Some samples i.e CES02 and CES04 showed greater divergence with value of 2.24 and 2.45.

In June 2016, samples exhibited considerable difference in heights. CES09 had maximum and CES05 had minimum height of 78cm and 33cm respectively. Samples CES05, CES06, CES07, CES08 and CES13 height remained almost constant. The width of all samples remained constant and did not reflect any significant increase or decrease. Data also indicates that the length of leaf in maximum varieties was similar to one another but they showed minute increase or decrease. The maximum increase recorded in leaf length of CES09 and CES11 were 5cm and 6cm respectively. Moreover, this increase was distributed almost same in all the part of leaf due to which genetic diversity of samples observed almost same to previous data at level of length of leaf from center to margin and from center to sinus.

Petiole length exhibited high variation because some sample showed rapid increase in petiole length whereas other samples manifested insignificant increase in petiole length. Therefore, on basis of petiole length we observed considerable increase in genetic diversity of samples on this stage. The highest increase recorded in petiole length of CES11 and CES14 which were 18cm and 13cm respectively and became similar to CES06 but reflected greater divergence from at least 6 samples (detailed present in table 3.1). The length of petiole and petiole sheeted edge area was not equally increased i.e the ratio of petiole length and petiole sheeted edge area of CES01, CES02, CES06 was 4.4, 4.75 and 4.25 which decrease to 3.82, 3.37 and 2.15 respectively which manifested that the petiole sheeted edge area showed high increased in length than petiole length.

The last Data were taken in July 2016, almost all character showed increase or decrease with the same rate which was showed in June except characters related to leaf

length and width, other character revealed the presence of genetic diversity similar to previous result. CES09 and CES14 had maximum height 85cm and CES05 had minimum height of 36cm which showed almost similar result. Plant thickness also reflected variation of samples: The lowest value was 5cm and highest value was 11cm.

The plant samples showed complexity in leaf length and leaf width with time. Some samples almost maintain constant leaf length and leaf width, some showed increase, whereas some samples showed decrease in leaf length and width. Samples CES05, CES06 and CES 14 showed decrease in leaf length of 5.5cm, 4.5cm and 7.5cm respectively while the samples CES09, CES11 and CES13 showed increase in leaf length of 4 cm, 6 cm and 4.5 cm respectively, the other samples showed inconsiderable increase or decrease in leaf length.

Leaf width almost remain constant in 8 samples, 5 samples showed increase in leaf width from 2-4.5cm except CES10 which showed decrease in leaf width of 4.5 cm. the highest leaf length 36.5 cm was noted in CES09 and CES12, whereas the lowest leaf length 18cm was noted in CES02. These differences in leaf length and width revealed less genetic diversity on basis of leaf by considering leaf to width ratio. Here we observed genetic diversity of samples almost remains the same to previous data at level of length of leaf from center to margin, center to sinus and on basis of ratio of both with other factor.

The petiole length showed increase or decrease similar to previous data, the highest petiole length 70cm was revealed by CES14 and the lowest 28cm was revealed by CES05. Petiole thickness and petiole sheathed edge length showed inconsiderable difference due to which the other factor related to both also had almost same value to previous data.

The main differences were recorded in plant height, leaf length, leaf width, leaf to width ratio, petiole length, petiole sheathed edge length and petiole length to leaf length ratio showed great variation in these three data, These all data were showed in form of chart below, whereas the table 3.1 revealed information about all characters.

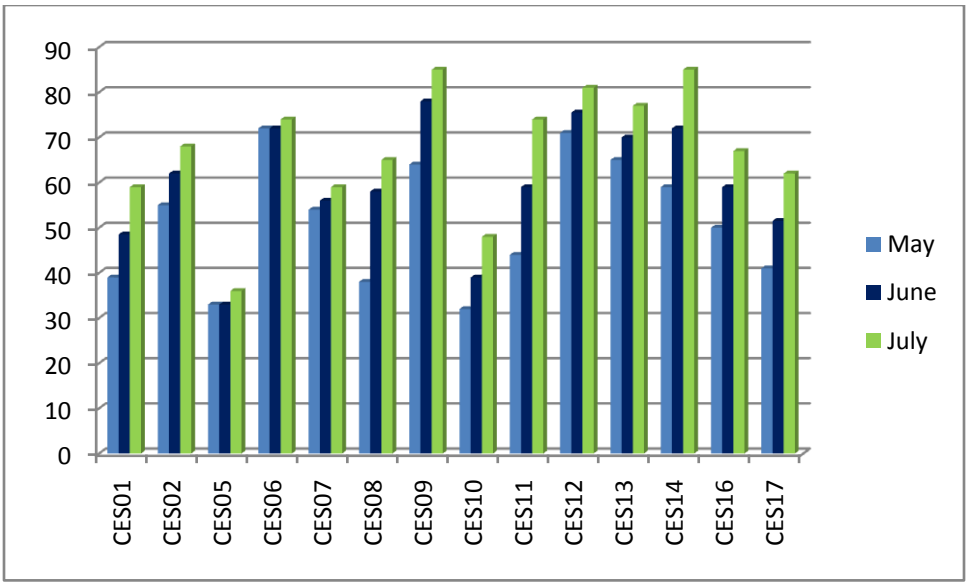

Fig 3.2: Plant height

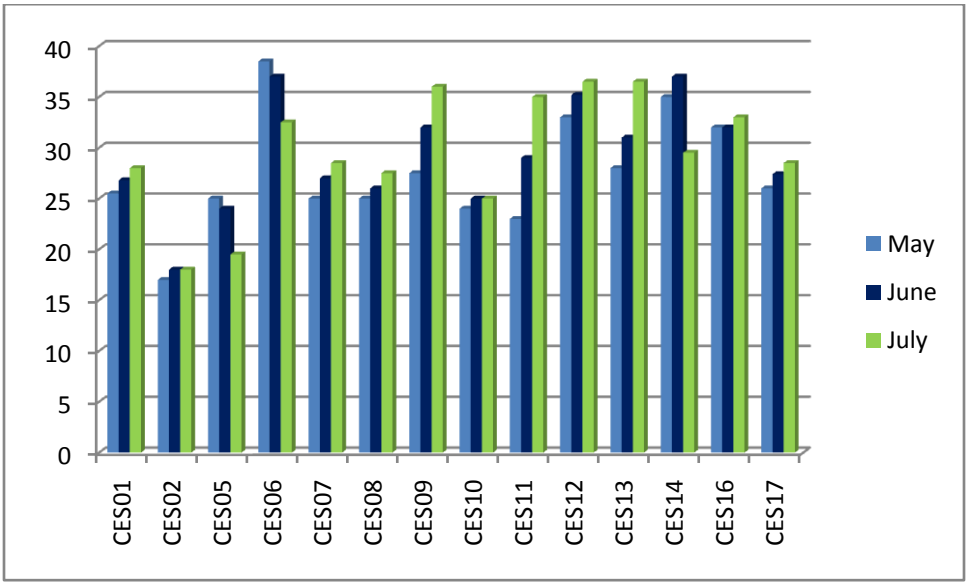

Fig 3.3: leaf length

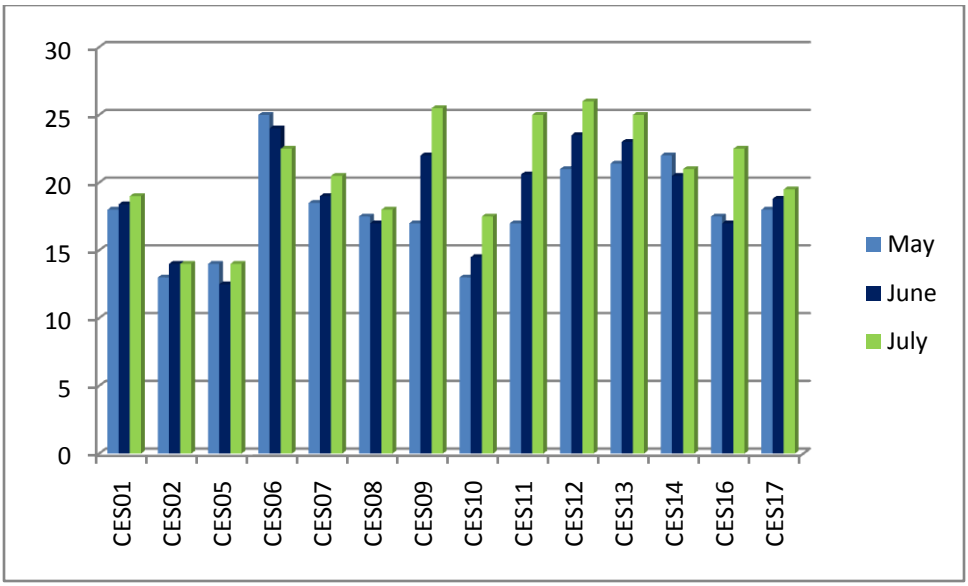

Fig 3.4: leaf width

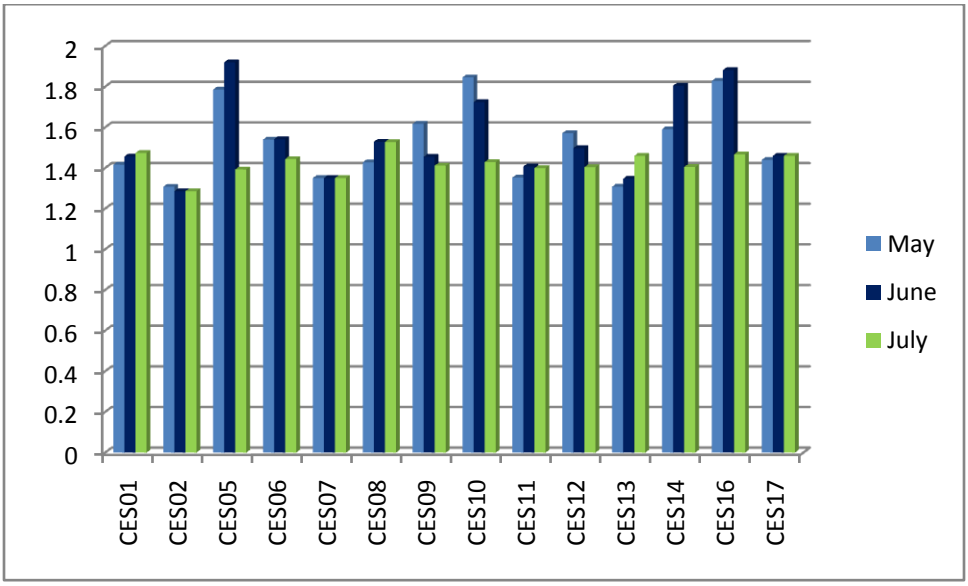

Fig 3.5: Leaf length to width ratio

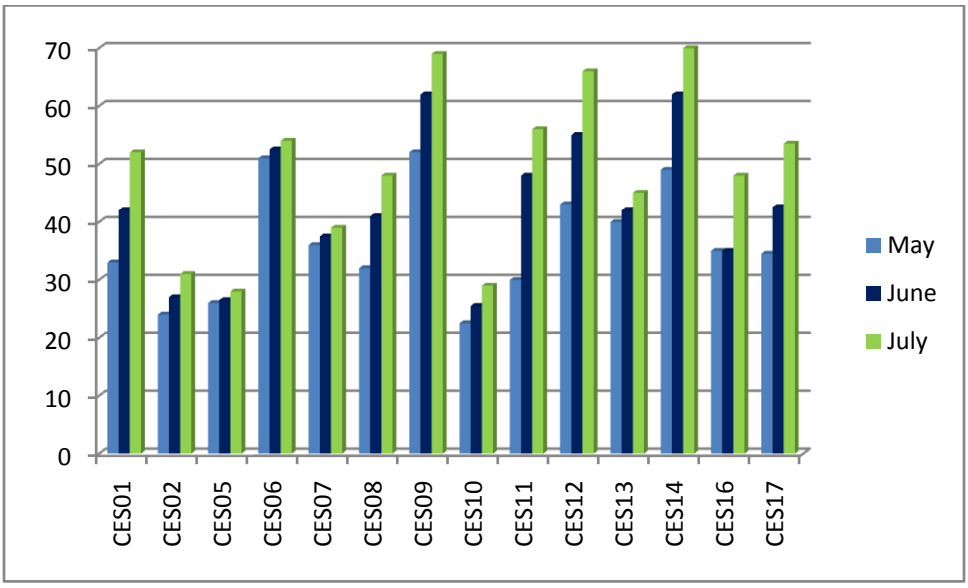

Fig 3.6: Petiole length

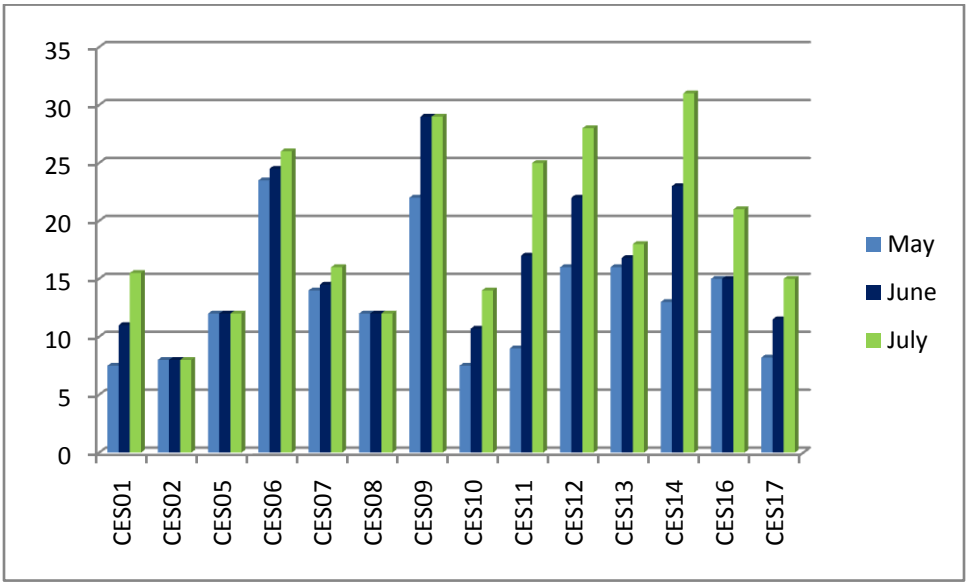

Fig 3.7: Petiole sheathed edge length

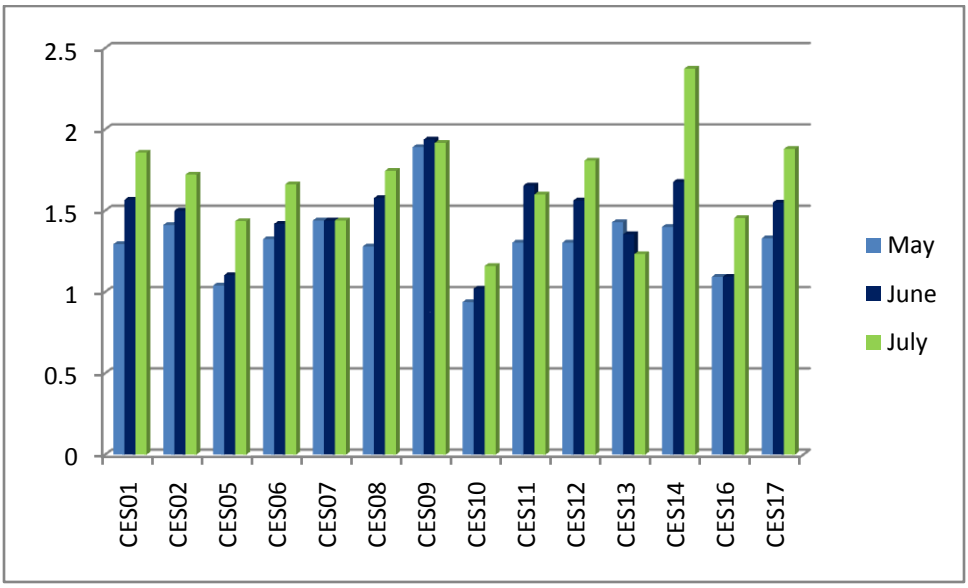

Fig 3.8: Petiole length to leaf length ratio

Table 3.1 shows Morphological data of mature stage taken in July 2016

Character given in vertical and samples names given in horizontal

	CES01	CES02	CES05	CES06	CES07	CES08	CES09	CES10	CES11	CES12	CES13	CES14	CES16
PH (cm)	59	68	36	74	59	65	85	48	74	81	77	85	67
PW(cm)	7.5	5	5	10.5	6	9	11	6.5	9	10	8	7.5	5.5
PLC	P	P	A	A	A	A	A	A	A	A	A	A	A
LL (cm)	28	18	19.5	32.5	28.5	27.5	36	25	35	36.5	36.5	29.5	33
LW (cm)	19	14	14	22.5	20.5	18	25.5	17.5	25	26	25	21	22.5
DLS	7	2	2	4	2	3	4.5	1.5	5.5	2.5	5	2	3.5
LLCM (cm)	18	11	13.5	21	18.5	18.5	24	17.5	22.5	24.5	24	20.5	23
LLCS (cm)	7	5	4	7.5	6.5	6	7.5	6	7	9.5	7.5	7	6.5
L/W	1.48	1.29	1.39	1.45	1.36	1.53	1.42	1.43	1.4	1.41	1.46	1.41	1.47
LLCM/W	0.95	0.79	0.97	0.94	0.95	1.03	0.95	1	0.9	0.95	0.96	0.98	1.03
LLCS/W	0.37	0.36	0.29	0.34	0.29	0.34	0.30	0.35	0.28	0.37	0.3	0.34	0.29
LLSM (cm)	21	16	17.5	28.5	24.5	24.5	31.5	23.5	29.5	34	31.5	27.5	29.5
LL/LLSM	1.56	1.64	1.45	1.55	1.43	1.49	1.5	1.43	1.56	1.49	1.53	1.44	1.44

GLS	M	M	A	A	A	A	A	A	A	A	A	A	SM
LCD	3	3	5	7.5	7	6	8	6	2	11	7.5	7	3.5
PL (cm)	52	31	28	54	39	48	69	29	56	66	45	70	48
PTC	3	3	3	4	3.5	3	4	3	3	4	4	3	3
PSL (cm)	15.5	8	12	26	16	12	29	14	25	28	18	31	21
PL/PW	17.4	10.4	9.34	13.5	10.29	16	17.25	9.67	18.7	16.5	11.25	23.34	16
PL/LL	1.86	1.73	1.44	1.67	1.44	1.75	1.92	1.16	1.6	1.81	1.24	2.38	1.46
PL/PSL	3.36	3.88	2.34	2.08	2.77	4	2.38	2.08	2.24	2.36	2.5	2.26	2.29

3.3 DNA extraction:

Genomic DNA of samples were extracted by using protocol of Ahmed *et al*., (2009) and plant mini kit procedure. The extracted DNA was run on 1% agarose gel which showed intact band of about 10Kb. Extraction result of both samples are shown in Fig. 3.9 and Fig. 3.10.

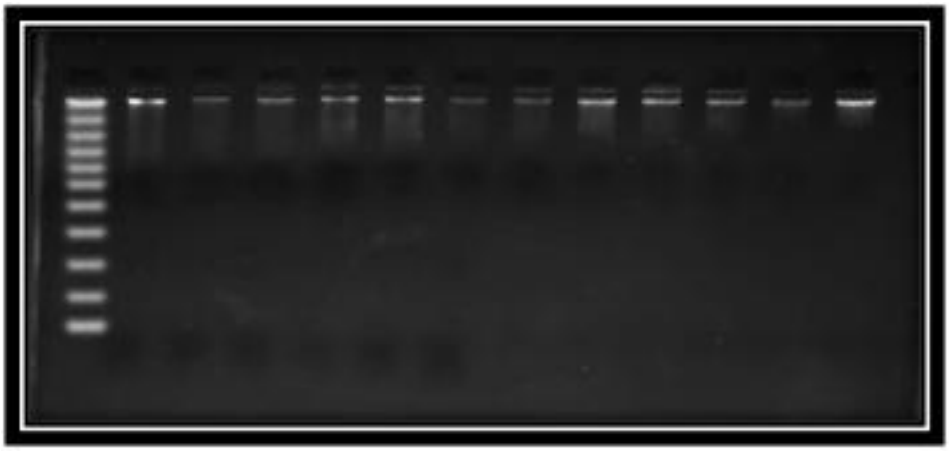

Fig. 3.9: Genomic DNA extraction with Method of Ahmed *et al*. (2009)

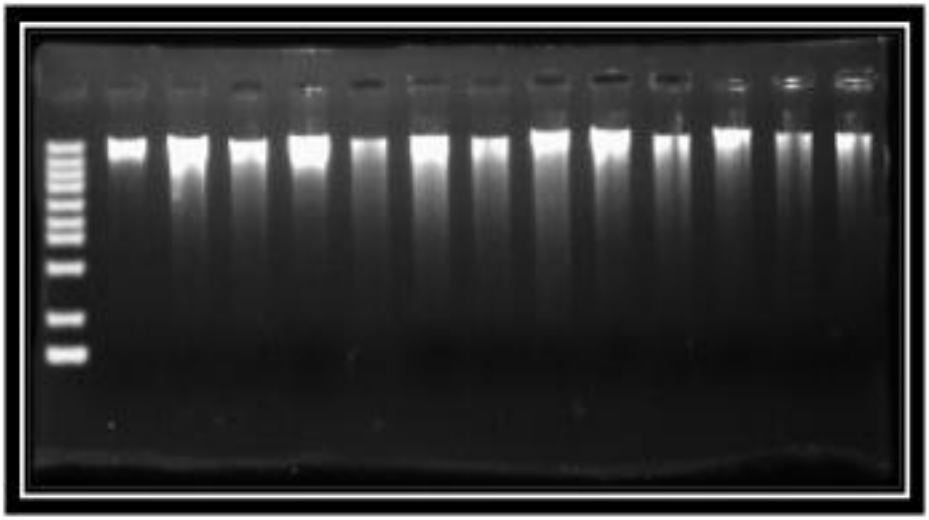

Fig 3.10: Genomic DNA extraction with Plant Mini Kit protocol

3.4 PCR analysis:

3.4.1 ITS and PhyC amplification:

ITS and PhyC primers which amplified fragment of 563 and 769 respectively were used to revealed the genetic variation at nuclear level but both of these primer did not show reproducible results. ITS primer show non-specific amplification and amplify the fragments of 563, 200bp and 80bp. The figure 3.11 shows the presence of 543bp band with non-specific band.

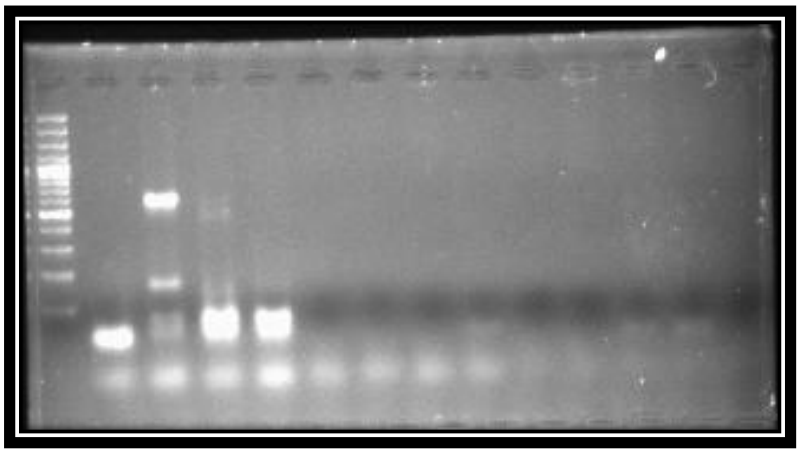

Fig 3.11: Gel image showing the confirmed 563bp fragment in taro sample and 100bp ladder was run for the confirmation of the size of the band.

PhyC primer amplified specific band at annealing temperature of 46ºC and 44ºC, whereas non-specific band of 400bp were also shown at 42ºC along with 759bp.

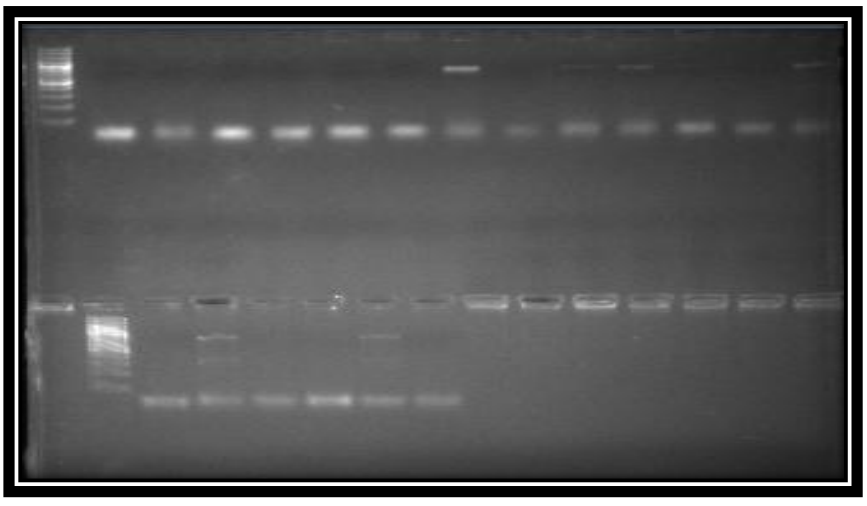

Fig 3.12: Gel image showing the confirmed 769bp fragment in taro sample and 100bp ladder was run for the confirmation of the size of the band.

3.4.2 RAPD primers amplification:

RAPD Primers were also used to find out genetic diversity on basis of nuclear genome but it also did not show reproducible result and amplified 100bp-1500bp fragments of genome.

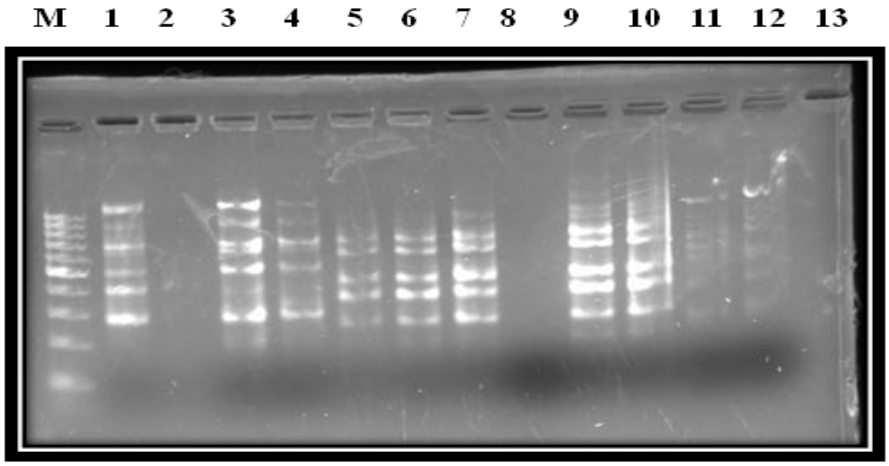

Fig 3.13: Gel image shows amplification with RAPD primers

M = Marker of 100bp; 1,2,3,4 shows amplification with OPW-4; 5,6,7,8,9,10 shows amplification with OPW-9; 11, 12 shows amplification with OPW-15

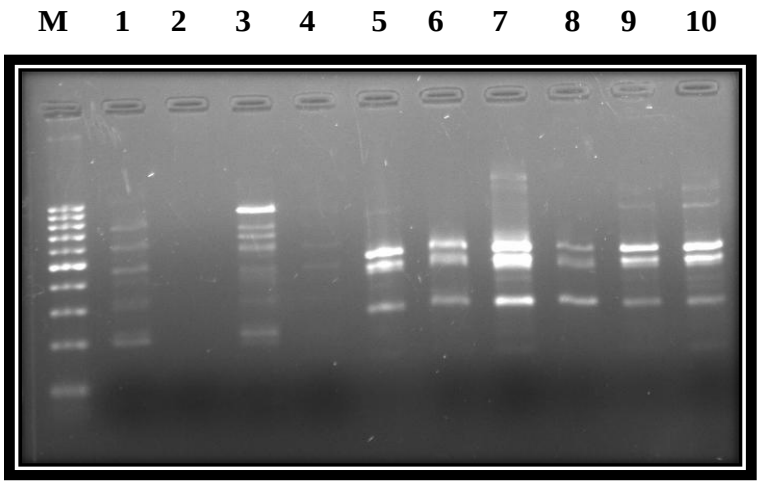

Fig 3.14: Gel image shows amplification with RAPD primers

M show Marker of 100bp; 1,2,3,4 shows amplification with OPW-15; 5,6,7,8,9,10 shows amplification with OPW-16

3.4.3 ACECP05 amplification:

Amplified product of primer ACECP05 was run on 2% gel stained with ethidium bromide which revealed amplification of single band of 592.

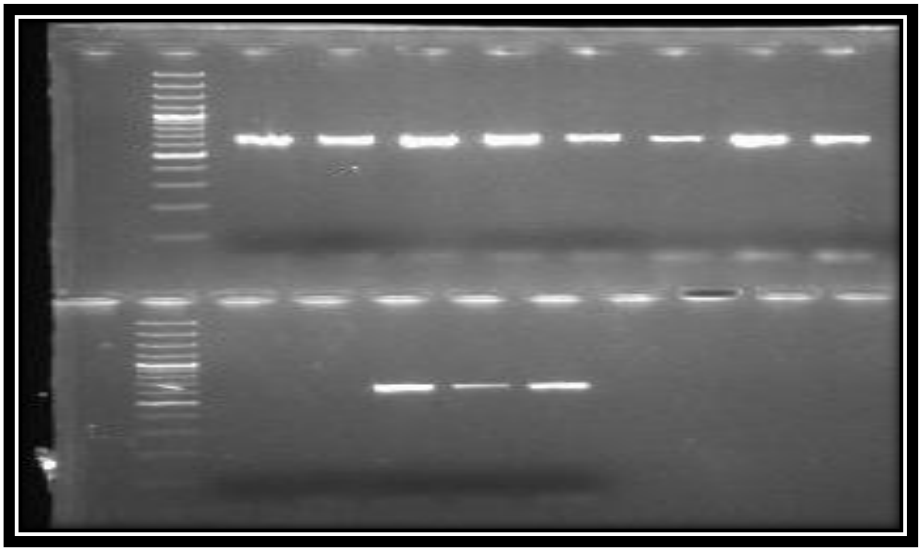

Fig 3.15: Gel image showing the confirmed 592bp fragment in taro sample and 100bp ladder was run for the confirmation of the size of the band.

3.5 Sequencing Result:

The sequence was similar to the sequence of samples of Ahmed (2014). It showed these all samples belong to the H-superclade. The fasta sequence sent by the company shows some SNP between our samples but when we compared chromatogram of all the samples it was shown the sequencing machine read it by mistake and the band shows the result might be similar to other samples, in some places we were sure on our editing while in some cases we guess it due to observing almost two equal graph due to this editing sequence of all the samples become similar to each other.

Discussion

Morphological data of all samples showed different growth habit: 24% of samples were erect, 29% were spreading and 47% were intermediate whereas all 25 accession of Vinutha *et al*. (2015) showed erect growth habit. Leaf of samples were apex down except CES01, CES02 and CES17 which exhibited somewhat horizontal appearance which was according to the result of Vinutha *et al*., (2015) where the 96% leaf was apex down. Upper leaf colour of plant was dark green and green and lower leaf colour was green and whitish green as observed by Vinutha *et al*. (2015). According to their studies, leaf margin of taro in India was undulated and have green, light green, purplish green, purple and dark purple colour whereas in Pakistan entire leaf margin was green and light green color in all sample except CES04 which have purplish green colour at margin. Additionally, the same difference in colour of leaf sinus was observed in taro of both countries. The taro of both countries showed 92% differences on basis of leaf variegation because in Taro of Pakistan variegation in leaf was absent whereas Vinutha *et al*. (2015) observed variegation in 92% samples of their study. The upper colour of vein in Pakistani samples was green and dark green whereas lower colour of vein was light green and whitish green but in India some variety also had purplish green colour (Vinutha *et al*., 2015).

In Pakistan colour of petiole was light green, green and dark green in all parts of it whereas in India the Vinutha *et al*. (2015) observed purplish green, cream and purple shaded cream colour. The plant size, petiole length, petiole sheath edge length and ratio of petiole to leaf give almost same result. Taro grow in Pakistan only once in session and flowering take place in mid-August and last still September (author observation) whereas in India the flowering take place in mid-June and last still September (Sreekumari *et al*., 2003). Devi (2012) reported that few verities flowered in February too, four months after planting. This information clearly indicates that in India taro grow two times annually. It also shows that the flowering time was different in Pakistan and Indian varieties.

These comparison showed that the Indian taro have plastic nature than Pakistani taro. It also reveals that some character between taros of both countries almost different from each other due to which high level variation between the taros of both countries could observed.

When we compare the morphological character of our samples with result of Edison *et al*. (2004) and Vinutha *et al*. (2015) it elaborate that the plant size, leaf colour, leaf length, leaf width, petiole colour and petiole length showed stable character and can be further focused for development of variety in Pakistan.

The sequencing result of samples with primer ACECP05 showed that the Pakistani taro belongs to Himalayan (H) superclade which support the assumption of Ahmed (2014) that the cultivated taro belong to (H) superclade. The result of sequencing was similar to taro of H-superclade which also reflects about the ploidy and we assume that Pakistani taros might be triploid which further confirm assumption of Ahmed *et al*. (2014) that H-superclade contain almost triploid taro. However, the cytology studies can further confirm the sequencing result.

The study of these samples also suggests that the origin of the taro from Pakistan is same and they originate from a common ancestor.

According to Dongxiao (1988) the taro at high altitude was triploid and at lower altitude was diploid. This study also supports our result because our samples were collected from mostly hilly area or from area of high altitude. This study further suggested the chances of taros which affiliate with IP-superclade present in the plain area of Indus basin from where only three samples were included in present study. Moreover, diploids taro present in every parts of India (Edison *et al*., 2004) therefore, it is possible that Indus basin include IP-superclade due to weather condition and climate.

4.1 Conclusion:

The present study concludes that taros from Pakistan shows variation and belong to H-superclade, although some character which are genetically stable showed similarity. All the Pakistani taros samples belong to H-superclade and their affiliation with IP-superclade can be further studied in taro of Indus basin.

4.2 Future Perspective

The morphologically stable characters of taro that does not affected from environment and soil condition can be identified by growing taros in area of different climate and soil. The nuclear genetic diversity can be studies by using nuclear molecular markers to get the information of genetic diversity at genomic level and by combination of morphological and

molecular study variety of taro in Pakistan can be developed. The affiliation with IP-superclade can be further study by collecting high level of samples from area of Indus basin and from the area of Punjab which are nearer to India i.e Bahawalnagar.

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