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Original Research Article

Insights into the Evolutionary Genetic History of Pak-Turk Bottle Gourds [Lagenaria siceraria (Molina) Standl.]

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Abstract

Bottlegourd [Lagenaria siceraria (Molina) Standl.], a member of Cucurbitaceae family (2*n* = 2*x* = 22) consisting of 90 genera and 750 species, is a monoecious, annual vine vegetable crop with soft pubescence. It is widely grown successfully throughout different geography of the world. Hundreds of accessions are grown showing morphological diversity in plants and fruits, even taste, color, aroma, flesh and juice percent is changed. Plants morpho-botanical traits are always changeable due to various ecological conditions. Various markers have made the genetic diversity clearer among and in between genotypes. SSR and Chloroplast markers are more efficient and reliable than ISSR, SRAP or AFLP markers. Here we have studied SSR analysis of bottle gourd accessions collected from various parts of the world Asia, Africa and USA. One part of this study has already been published, in present, we have added eight landraces collected from Pakistan and compared with the rest US genotypes that showed heterozygosity with other accessions in previous study. A total of 8 SSR primers and 2 Chloroplast markers were used to depict the genetic diversity. Per locus 2-11 alleles were produced. An unweighted pair group method with arithmetic mean dendrogram was constructed. Pakistani landraces clearly showed a close genetic relation with US accessions, which depicts that these might have migrated from Asian origins. Despite geographical locations and morphological dissimilarities all bottle gourd accessions were genetically interrelated with each other with less genetic distance 0.1 among them. One of the landraces from Pakistan showed a heterozygosity character, which can lead to a new breeding approach in Turkey.

Keywords: Bottle gourd, Geographical location, genetic similarities, RNAseq, Chloroplast markers

INTRODUCTION

Bottlegourd [Lagenaria siceraria (Molina) Standl.], a member of Cucurbitaceae family (2*n* = 2*x* = 22) consisting of 90 genera and 750 species, is a monoecious, annual vine vegetable crop with soft pubescence. It is the most popular vegetable in tropics and sub-tropic regions of the world. The leaves are cordate-ovate to reniform–ovate, 15-30 cm across, unlobed. It bears white showy flowers. Mainly it consists of two sub-species; African and Asian (Schlumbaum and Vandorpe (2012), used by various ways in human food in different countries. Bottle gourd is mostly grown for its variable size of fruits. It is used for multi-purposes in the world. Fruits are used as vegetables, as containers for storing liquids, and as medicines. Medicinal values are

far most; lowers the cholesterol, maintains blood pressure, cooling, diuretic and antibilious, dropsy and anthelmintic properties. Carved for traditional craftwith beautiful scenery imprinted designs and dried for making pet house. The gourd can be dried and used as a smoke pipe for tobacco (Bandyopadhyay and Raychaudhuri, 2010).

Currently, an improved rootstock for disease and abiotic stress tolerance for cucurbits is gaining much attention (Davis et al., 2008; King et al., 2008). Biotic and abiotic stress tolerance has been observed in bottle gourd along with controlling fusarium wilt disease (Yetisir et al. 2003; Miguel et al. 2004) and salt tolerance (Yetisir and Uygur, 2010). Further, as rootstock for watermelon has enhanced yield traits (Yetisir and Sari 2003; Karaca et al. 2012). Still, it has become a great interest for breeders to find the best rootstock for melons farming and resistance against diseases.

In a wild form it was first found in tropical Africa (Gautam et al. 2017; Cutler and Whitaker, 1961). South-Africa is considered to be the original places of bottle gourd (Decker-Walters et al., 2004). Stands of *L. siceraria*, as wild plants were reported in Zimbabwe in 2004 (Decker Walters et al., 2004). The mystery of bottle gourd origin remains ambiguous, and how it spreads and acclimatized in different parts of the world. Genetic variationswere found at large among the accessions collected from continents of USA, Africa and Asia. (Levi et al., 2009) which were screened out for their resistance to diseases. Markers assisted selection for particular molecular characterization has still not been studied well, which still needs to be explored (Sarao et al., 2014; Ali et al., 2012).

Now a day, bottle gourd farming has been done in different countries with different locations, which depicts variations in fruit size, color, aroma, flesh taste. This variation is might be due to different agroecological conditions present within that particular area or it might be due to the genes inherited within landraces which become dominant or recessive as per environment. The increasing number of varieties and the degree of morphological differences between them have created the need for better systems of identification and characterization of crop varieties. Several attempts have been made on the collection and characterization of bottle gourds (Xu et al., 2011; Levi et al., 2009; Saro et al., 2014), based on geographical places several bottle gourds accessions have been studied on morphological traits. However, their molecular characterization remains undiscovered (Kahraman et al., 2015), various germplasms from different countries were collected and molecular analysis of 60 Turkish bottle gourd accessions along with 31 exotic accessions using SSR markers was conducted (Kahraman et al., 2015), their findings showed a close genetic relationship among all bottle gourd accessions with a very low genetic diversity (0.13).

As we know that most of the perishable vegetable traits are build-up of several biochemical and genetic material complexes which need advanced methods to be detected. Currently, microsatellite analysis along with RFLP, RAPD, AFLP (Saliba-Colombani et al., 2000) and SNP markers (Suliman-Pollatschek et al., 2002) have gained more popularity due to authenticity in the data. Its widely application in different fields is due to; SSR markers reproduce diversity very clear due to high specificity as well as data can be easily incorporated for automatized genetic analyses. Moreover, the application of microsatellite markers has also been successfully applied for QTL pyramiding through which fruit quality can be improved (Mashhid et al., 2016).

Genotyping identification at molecular level have presents more authenticity due to application of SSRs markers, they can generate high polymorphism, extraordinary abundance and fast transferability (Hosseini-Mazinani et al., 2014; Mousavi et al., 2017). Due to application of this technique further insights into gene regulations and molecular sequencees are discovered in cucurbits genomes. Genome sequence is the basis of genome studies for the Cucurbitaceae family. Genetic know how and gene expression enables the breeders to improve more cropping traits for biotic and abiotic stresses (Baloglu, M. C., 2018). AFLP technique looks deeply into genoes for large discrimination besides morphological traits and geographical distribution (Hedre´n et al., 2001 and Kim et al., 2017). Genetic diversity based on geographic areas has also been studied by Ismail et al., 2019 on Typha texa of East Asia through AFLP markers. They found that three AFLP selective primer combinations generated a total of 707 amplification products, of which 704 (99.6%) were polymorphic (Ismail et al., 2019).

Breeders are getting more information due to genetic diversity among or in between crops that benefits crop improvement (Paun and Schönswetter, 2012). A high degree of kinship due to geographic proximity and clonal variations has raised additional identification problems (Nadeem et al., 2018; Ipek et al., 2015). Molecular markers have been used for authentication and identification of plant species by scientists of *Aquilaria* (Thymelaeaceae) (Lee et al. 2011), *Momordica charantia* L. (Paul et al. 2010), Indian mulberry (Thumilan et al., 2016). DNA markers have the capability to measure precisely genetic variations in crops then other markers (Levi et al., 2005).

The present study has also been formulated to explore genetic diversity using the currently available genome resources in Turkey with the landraces grown in Pakistan, this is the first-ever study to be done on landraces of Pakistan, our main idea behind this research was that whether the geographical locations exert an influence on genetic diversity or not regardless of morphological differences. Further, establishing the level and wideness of the genetic variability inside a

Table 1. List of bottle gourd landraces collected from different cities of Pakistan (Sindh and Punjab) for the study.

Bottle gourd Landraces	Cities	Latitude North	Longitude East
TA-1 (Long)	Thatta	24.749731	67.911636
H-1(Long)	Hyderabad	25.416868	68.274307
GK-2 (Round)		25°23'N	68°24'E
KS-1 (Round)	Lahore	31.582045	74.329376
		31°32'N	74°22'E
TJM (Long)	Tando Jan Muhammad	25.067	69.217
		25° 4' 0" North	69° 13' 0" East
JH-1 (Long)	Jhuddo	24.96876870	69.29669330
		24° 58' 7.57"N	69° 17' 48.09"E
TH-1 (Long)	Mithi (Tharparker)	24° 44' 0" North,	69° 48' 0" East.
TH-2 (Long)			



Figure 1. The geographical location of Pakistan with its neighboring countries the latitude and longitude exhibit a diverse range of climatic conditions.

germplasm collection. Here, we have reconstructed the genome of the most recent common ancestor of bottle gourds from Pakistan and USA, which provides insights into the evolutionary genetic history of bottle gourds. Further, this may present a unique platform for further acclimatization and agroecological studies in Turkey to make available this important source of well-defined genotypes to all interested stakeholders and researchers.

MATERIALS AND METHODS

Plant Material

Eight landraces of bottle gourd (Table 1) as plant genomes for this experiment were collected from different locations of Sindh and Punjab provinces of Pakistan, which were compared with the eight US genotypes National Plant Germplasm System (US-1, US-4, US-5, US-6, US-9, US-10, US-11, US-12), DNA of which were taken from Betül-ZiyaEren Genome and Stem Cell Center of Erciyes University, Turkey, their data have already been published (Kahraman et al. 2015). These US genotypes were selected because these were showing the maximum diversity in the data. Seeds of Pakistani landraces were sown in seed trays containing peatmoss at Plant Biotechnology Laboratoryof Betül-ZiyaEren Genome and Stem Cell Center, Erciyes University, Turkey. Leaf samples in duplicate were collected when seedlings developed true leaves, then kept at -80 ^{o}C for further DNA isolation process.

The geographical location of Pakistan and Sindh

Pakistan area consists of the highlands; Indus River (plain) and the plateau. Pakistan lies between 23^{o} 35- 37^{o} 05 North and 60^{o}50-77^{o}50 East longitude (Figure 1). Located in South Asia, Pakistan its borderr with India, China, Iran and Afghanistan runs along its western and northern edge. On Southern with 1,064 km of coastline, Arabian Sea lies. This geographical position makes the Pakistan to grow more and diverse range of crops.

Sindh one of the provinces of Southeastern Pakistan



Figure 2. Map of Sindh showing different cities with their location

(Figure 2) is bordered by the provinces of Baluchistan on the west and north, on the northeast lies Punjab, the Indian states (Gujrat) to the east, and Arabian sea to the south. Sindh is essentially part of the Indus delta and has derived its name from that river, which is known in Pakistan as the Sindhu. It covers 54,407 square miles.With the advent of Islam in the region in the 8th century, groups of Arab, Persian, and Turkish origin settled in Sindh.

The map of Sindh is divided into two parts upper part (Kashmore to Noausharo Feroz) and lower part from where the Bottle gourd landraces were collected (Nawabshah to Thatta and Mithi, Tharparkar)

Protocol for Genomic DNA Isolation

DNA isolation was performed according to procedure designed by He et al. (2003) and Mariotti et al. (2016) with some modifications. The quality and quantity of DNA were detected by NanoDrop (ND 100) spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA) and agarose gel electrophoresis. DNA samples were subsequently diluted to 10 ng/µl for PCR reactions and stored at -20 °C. Sample leaf about 0.5 g were ground in a pestle mortal with liquid nitrogen to get the homogenized material. The ground material was placed in Eppendorf tubes. For DNA extraction buffer 7.00 ml CTAB and 4 ml B- mercaptoethanol per sample was taken and mixed (7X30= 21 ml CTAB+ 4X30= 120 ml B-mercaptoethanol). Buffer about 700 µl was added in each sample tube. Samples were incubated at 65^{o}C for 30 minutes. Mixed with avolume of isoamyl alcohol: chloroform (24: 48, v/v); 2 ml of isoamyl alcohol was added in chloroform containing measuring cylinder. Superannuant was taken out in which 700 µl isoamyl alcohol+chloroform mixture was added into each sample tube. Samples were centrifuged at 13000rpm for 10 minutes. After transference of upper superannuant into fresh tubes 42 µl of autoclaved NACL and 420 µl of isopropynol was added and shaked gently, further samples were centrifuged at 13000 rpm for 20 minutes. liquid was discarding the from each tube. 1 ml of 70% Ethanol added for further clarification of DNA. Centrifuged again at 13000 rmp for 05 minutes. Ethanol was discarded from each sample then tubes were placed inverted on tissue paper to dry out for at least 20 minutes. At the last TE-Buffer 50 µl was added into each tube.

Chloroplast and SSR markers location and source.

The most robust chloroplast markers used by many researchers for the identification of Asian and African genotypes are with 5 bp indels. Whether, the samples are living or fossils, these markers have a strong immunity to many of the post-depositional mutations. chloroplast polymorphism regions reported, as two 5 bp indel, by Erickson et al. (2005) which are the amplifications of primers published by Lee and Wen (2004) and Chung and Stub (2—3). Chloroplast marker LS.Indel1 located in the intergenic regions trnC-trnD, whereas the other chloroplast marker LS.Indel2 located in the trnS-trnG intergenic region. Xu et al. (2011) designated many SSR primers for identification of bottle gourd out of them he applied 14 primers to analyze the genetic diversity of 4 bottle gourd accessions grown in china. In this study, we have used the same SSR primers for bottle gourd molecular analysis. Figure 3

SSRand Cp Marker Analysis

Bottle gourd accessions were initially screened and tested (Kahraman et al. 2015). Samples in this study were analysed with the best performed ten primers (8



Figure 3. Bottle gourd landraces of Pakistan showing morphological variations in mature fruit

Table 2. Description of SSR nuclear markers and two chloroplast loci used for genotypes genetic diversity

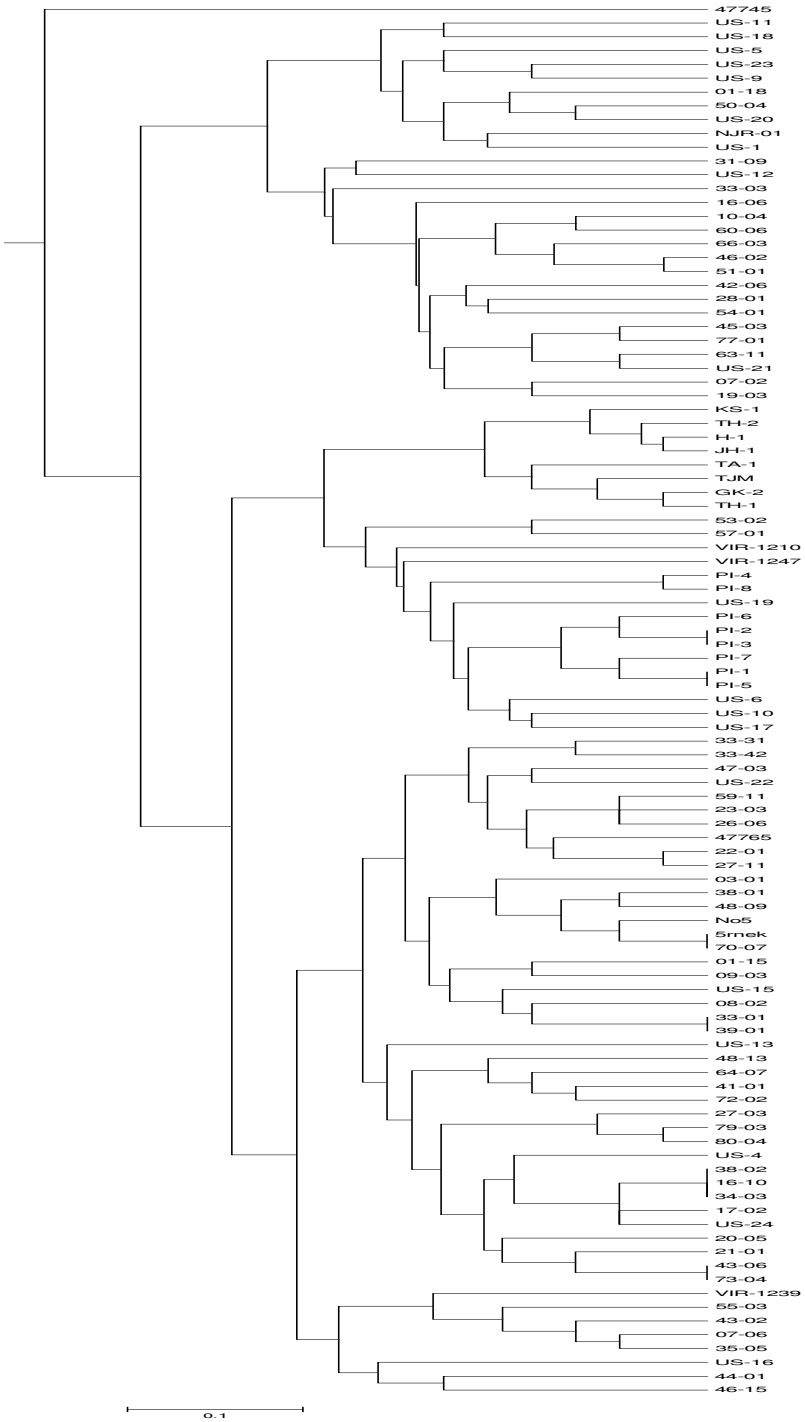
Multiplex group	SSR-Markers with fluorescentlabel	Repeats
1	LSR030-FAM	$(AT)_{10}$
1	LSR015-NED	$(CTT)_{18}$
1	LSR047-VIC	$(TC)_{11}$
1	LSR112-PET	$(TTCT)_5$
2	LSR109-NED	$(GA)_{15}$
2	LSR077-FAM	$(TC)_{11}$
2	LSR108-VIC	$(AG)_{15}$
2	LSR056-PET	$(CTT)_{10}$
1	LS.InDel2-FAM	
2	LS.InDel1-FAM	

SSR and 2 Chloroplast marker) applied to check the genetic diversity among bottle gourd accessions. PCR was conducted and product was separated on 6% agarose gel. PCR mixture contained 12 pM each of forward and reverse primers, 1 X reaction buffer, 2 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP. 0.25 U TAQ DNA polymerase (Thermo scientific, Finland) with 50 ng templateDNA. PCR annealed to the forward primer using a double step PCR. 3 min an initial denaturation at 92°C,followed by 35 cycles of 30 s denaturation at 94°C, a 40 s annealing step at 55 °C with 1 min extension at 72°C for7 min, the second step (for tail annealing) made up of 20 cycles,with the same conditions of the first step except for annealing temperature (Tm D 55°C), a final elongation at 72_C for 40 min closed the second step PCR. 55 °C annealing temperature produced the bright bands foe all primers.

To confirm the bands of genome before loading to ABI machine PCR products were loaded on 1-mm-thick non-denaturing gels of 6% polyacrylamide (Acr/Bis = 29:1). The electrophoresis buffer contained 1-TBE (100 mM Tris–HCl, 83 mM boric acid, 1 mM Na2EDTA, pH 8.0) (Han et al., 2010); the 20 bp DNA Ladder Dye Plus (Newzealand) was used as a size standard.

Allele Sizing and Diversity Analysis

To amplify the accessions polymorphic primers previously used in the bottle gourd experiments (Kahraman et al. 2015), applied in the present study under the PCR conditions mentioned above. These 10 primer pairs added with forward primer M13 tail (CACGACGTTGTAAAACGAC) to 5` (Schuelke 2000). PCR formulation contained DNA template 40 ng, 2mM$MgCl_2$, 1xPCR buffer, 1pmol reverse primer, 0.2 pmolforwad primer, 1.8 pmol M13 primer fluorescent labelled with 6-FAM, VIC, NED or PET (Applied Biosystems, Foster city, California USA) added with TAQ DNA polymerase (Thermo Scientific, Finland). Distilled water was added to raise the mix up to 15 μL. 6% agarose gel was used to visualize 7μLPCR product. Remaining PCR product was stored for further fragment analysis at -20°C with adding 107μLdistilled water. Each primer was set as a group of two multiplexes. Thus the final volume of PCR was raised up to 85 μLafter completion of each primer PCR. This mixture was further 20 times diluted and 0.75 μL. From this diluted mixture was taken where it was mixed with 9 μL Hi-Di buffer and 0.25 μL LIZ 600 standard dye. To get the final results the



samples were loaded on capillary electrophoresis ABI 3500 (Applied biosystems, Foster city, California USA). GeneMapper 4.1 (USA) was used for DNA fragment size determination. Dendrograms were constructed on the basis qualitative matrices with the method unweight pair group method with arithmetic mean (UPGMA) selected for clustering and to draw genetic trees using softwares MEGA4 (Tamura et al. 2007) and PowerMarker (Liu and Muse 2005).

RESULTS

Results for genetic diversity have already been published (Kahraman et al. 2015). Adding to those results eight genotypes from Pakistan were analyzed and their genetic tree was constructed including the previous landraces (60 Turkish bottle gourds and 31 exotic accessions). Table 2

Various alleles from 1-5bp have been found under 8 SSR primers and 2 chloroplast loci. Chloroplast LS. In

Del2 primer produced two loci 136 for all the Pakistani landraces and 141 for some of the US genotypes showing a 5 bp mutation. Whereas, LS.InDel1 chloroplast generated two alleles 108 and 113 with 5 bp mutation. This represents that landraces from Pakistan have the putative Asian alleles. However, SSR primers LSR047 produced two alleles with 2 bp difference 152-154. Heterozygosity among Pakistani landraces were observed by SSR primers LSR015, that produced two alleles with 3 bp mutation. Further results regarding other Turkish and exotic genotypes showed anAsian and African putative allele (Kahraman et al., 2015).

UPGAMA dendrogram (Figure 4) clustering whole accessions formed four main groups, landraces from Pakistan showed a close relationship with Pakistan and US genotypes, inferring that US genotypes might have been introduced from Asia and Africa. So, our study depicts that bottle gourd accessions collected from different geographical regions of Asia, Africa and USA are genetically close related at 0.1 similarity index. They had avery low genetic distance. In future breeders can use these accessions for improvement or for biotic and abiotic tolerance gene detection purpose

DISCUSSION

Genetic diversity within germplasm has been widely determined by the molecular markers SSR. Exploring genetic diversity among germplasm is paramount in crop breeding and maintaining germplasm (Benor et al., 2008). Bottle gourd is widely consumed throughout the world especially in Asia and Africa continents. It represents the earliest emergent member clade of Cucurbitaceae. Genome insights explore the most insights about molecular and biological traits of bottle gourd. In this study, we have added landraces of bottle gourds from Pakistan with the US-bottle gourds. As in previous studies about genetic diversity in bottle gourds (Kahraman et al., 2015; Yetisir et al., 2008 and 2012) collected from USA, India, Turkey and Russia found very low genetic diversity (0.13). Our study is the addition to that one conducted by Kahraman et al. (2015). Genetic diversity regarding Pakistani bottle gourds has not been studied previously. However, several studies about genetic diversity in bottle gourds have been studied by different scientists (Cui, et al., 2020; Xu, et al., 2011; Yetisir, et al., 2012). Morphological data showed less heterozygosity among bottle gourd germplasm (Yetisir, et al., 2008). 166 *M. charantia* geographically differentiated germplasms was found and identified 710, 412, and 290 candidate domestication genes in South Asia, Southeast Asia, and China populations, their study is in controversial with ours study (Cui, et al., 2020). 400 SSR loci were used in the Chinese bottle gourds study (Xu, et al., 2011), their results provided an average of 3.64 allel per locus. Same loci were also used by Sarao et al., (2014) for 29 bottle gourd accessions and reported 2.6 allels per loci. In our study, we also used the same pairs of SSR loci to depict genetic diversity among Pakistani landraces and USA genotypes. Our study showed a very low genetic distance (0.1) among bottle gourd genotypes collected from different geographical regions of Asia and USA. Saengprajak and Saensouk (2012) used RAPD technique with twenty 10-mer primers produced 212 RAPD fragments, ranging from approximately 120 to 2531 bp in Cucumerinae (Cucurbitaceae) belonging to the four northeastern provinces of Thailand. Based on the sequence of Cm (OPJ11700), SCAR primer pair; Cm (SCJ11516)-17F and Cm (SCJ11516)-17R was designed. Genomic DNA from all the *C. melo* specimens was found to be fragmented. Therefore, Cm (SCJ11516)-17F and Cm (SCJ11516)-17R were designed to amplify a small region (516 bp) of Cm (OPJ11700) to widen their application. Cm (SCJ11516)-17F and Cm (SCJ11516)-17R generated 516 bp bands in all *C. melo* specimens, while no amplification was observed in other Cucumerinae species. In our results, SSR amplifications were very strong as seen through gel documentations and scores. The reasons behind low genetic diversity are might be due to strong ribosomal and mitochondrial DNA makeup that did not change due to domestication process of bottle gourds throughout geographical regions. These findings support the hypothesis that bottle gourds might have been domesticated in South Asia, wild and cultivated accessions might have been dispersed together which might be the reason for showing low genetic diversity. Two of the genotypes from Pakistan showed heterozygosity which might lead to new genetic improvement and resistance gene development in Cucurbitaceae members. level of polymorphism shown by Cucurbitaceae: 86.98% in cucumber (Ping et al., 2002), 23.2% in sweet gourd (Gwanama et al., 2000), 41.34% in bitter gourd (Behera et al., 2012), 60.29% in bottle gourd (Srivastava *et al*., 2014). However, the development of species-specific markers has become an objective of high priority in the breeding program of bottle gourds. Further, gene expression should be followed up to know the exact genes within collected genotypes, this will explore the fundamental paths towards resistant recombinant gene manipulation within varieties.

CONCLUSION

Despite morphological and physico-chemical differences among bottle gourd accessions that exists due to geographical variations, all the accessions collected from different geographical regions of world showed 0.1% genetic diversity with a close kinship. Most of them had a genetic closeness from Asia and Africa.

Author Contributions

Tanveer Fatima Miano and HalitYetisir organized the research, collected the sample and wrote the experimental subject. Özhan Şimşek participated in the research, guidance and paper modification, Ilyas Kılınçer participated in the research and Tahseen Fatima Miano participated in growing plant materials.

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Conflict of Interest

The authors declare no conflicts of interest.

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