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Research Article

Identification and Characterization of Begomoviruses-Whitefly Complex Infecting Soybean in Pakistan

Seema Kanwal[1], Hasan Riaz[1*], Muhammad Ashfaq[1], Shafqat Saeed[1], Faheem Zia[1], Sabeeh Khan[1], Sufyan Raza[1], Muhammad Imran[2], Muhammad Afzal[3], Farah Deeba[4], Mudassar Yasin[5], Farzana Rafeeq[6], Kaneez Fatima[7]

[1] *Institute of Plant Protection, MNS University of Agriculture Multan-Pakistan*
[2] *Department of Soil & Environmental Sciences, MNS University of Agriculture Multan-Pakistan*
[3] *Pest Warning & Quality Control of Pesticides, Agriculture House, Lahore-Pakistan*
[4] *Department of Biochemistry and Biotechnology, The Women University Multan-Pakistan*
[5] *Department of Agriculture Extension, MNS University of Agriculture Multan-Pakistan*
[6] *Department of Horticulture, MNS University of Agriculture Multan-Pakistan*
[7] *Department of Botany, Government College Women University Sialkot-Pakistan.*

ABSTRACT

Begomoviruses are ssDNA viruses exclusively transmitted by whitefly *Bemisia tabaci*. The begomoviruses-whitefly complex is responsible for inflicting severe damage to the crops. Soybean crop, new to Pakistan, remains unexplored regarding the virus-vector complex therefore present study was aimed to identify and determine the genetic variability of begomoviruses infecting soybean crop along with the whitefly mitotype(s) involved in their transmission. Soybean plants displaying symptoms of begomoviruses infection were collected and total genomic DNA was extracted from both symptomatic leaves and viruliferous whiteflies. Diagnostic PCR was employed to detect begomoviruses followed by whole genome amplification of begomoviruses by rolling circle amplification technique. The RCA products were subsequently cloned and sequenced. The partial cytochrome oxidase subunit I (COI) gene of the whiteflies was amplified and directly sequenced in both orientations. The sequences were assembled using Geneious R10 software and BLAST analysis was conducted through online NCBI web portal. Similarity index matrix and phylogenetic trees were constructed using sequence demarcation tool (SDT), MEGA11 and MrBayes software packages. The results proved 98% similarity of begomovirus Multan isolate with *Tomato Leaf Curl New Delhi virus* (ToLCNDV). The COI gene sequence of *Bemisia tabaci* showed 99.9% similarity with mitotype Asia II-I found in Pakistan. The study confirmed the infection of ToLCNDV and Asia-II-I presence on soybean in Multan, Pakistan and would be useful in understanding begomoviruses-whitefly complexes in Soybean crop and Southern Punjab.

Keywords: Begomoviruses, ssDNA viruses, *Bemisia tabaci*, phylogenetic analysis, genetic variability, Soybean.

INTRODUCTION

Soybean crop accounts for the second-largest supply of protein feed as well as vegetable oil globally. The United States, Brazil, Argentina, and China are the four biggest countries where soybean production accounted for 90 percent of global total production in (2005 Workman. 2007). Soybean is among the 16 major crops that primarily grown around the globe, like wheat, sugarcane, barley, groundnut, cassava, potato millet, rice, maize, oil palm, rapeseed, rye, sugar beet, sunflower, sorghum, and soybean (Foley et al., 2011). Soybean crop is typically grown once a year. Few countries, including Pakistan, have the opportunity to cultivate crops in the spring and winter seasons each year. Soybean, also called "king of beans,"

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Correspondence
Hasan Riaz
h.riazz@gmail.com

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is generally stuffed into meals; soy oil is a major component of many edible and non-edible goods, ranging from animal grains, cooking oil, vegetarian food, and other industrial utilization (Thrane et al., 2017). In many countries, like America, begomoviruses pose a significant challenge to the production of common bean, tomato, and pepper production and have been frequently reported in soybean (Morales, 2006).

The genus *Begomovirus* belongs to the Geminiviridae family and is divided into Old and New World begomoviruses. In the Old World (OW Europe, Africa, Asia, and Oceania), begomoviruses were mostly monopartite, and a few bipartite begomoviruses were present, while others were all bipartite and known as New World (NW America) begomoviruses (Fauquet et al., 2008). The composition of begomoviruses was they have typically circular, ssDNA genomes encapsulated in twinned icosahedral virion of 18 nm × 30nm (Zerbini et al., 2017). Begomoviruses can be classified based on their genomic structure as either bipartite, consisting of two genomic components (DNA-A and DNA-B), or monopartite, containing a single genome component (DNA-A).

Begomoviruses are mostly transmitted by vector whitefly through persistent circulative manner. For a successful virus transmission, the insect vector need latent period of 8h between acquisition and inoculation (Ghanim et al., 2001; Czosnek et al., 2017). *B .tabaci* capacity of virus transmission was influenced by its acquisition access period (AAP), sex and age of the vector (Czosnek and Ghanim, 2002). The minimum AAP and maximum period of virus preservation in the whiteflies, which were respectively 3 days for males and 10 days for females, determine the persistence mode. The nymphs of whitefly can acquire with virus from symptomatic leaves but it cannot get in to the eggs. The aptitude of virus transmission in vector cannot be maintained for life time in both male and female whitefly (Karthikeyan et al., 2014). The contact among coat protein of virus and receptors present in whitefly gut and salivary glands depends on particular begomovirus and whiteflies. Whitefly encodes a variety of proteins like molecular chaperone proteins, HSP70 which help in proper virus transmission and circulative (Brown and Czosnek, 2002; Varun and Saxena, 2017). The vector *B. tabaci* was classified into three biotypes (A, B, Q) each of this was likely different enough to be classified a separate species. Because of their incapability to hybridization and deviation in the mitochondrial cytochrome oxidase I (mtCOI) gene, some biotypes, such as (biotype A) NW, Biotype B Middle East-Asian Minor 1(MEAM-1) also Mediterranean (biotype Q) have been planned to be elevated to species status (De Barro et al., 2011). In Pakistan, Ashfaq et al., (2014) were reported the presence and their role in viruses circulated by *B. tabaci* cryptic species like as Asia II-1, MEAM-1, Asia 1, Asia II-5 and Asia II-7. The first two taxa were identified in both Sindh and Punjab, while Asia 1 was only found in Punjab. Additionally, Asia II-5 and Asia II-7 were also detected in Punjab. Indian scientists documented the presence of nine well-known and one newly identified cryptic species of *B. tabaci*. The Asia I as well as Asia II-1 species were more prevalent in northern and central India, while Asia I and Asia II-7 predominated in western India. The most recent research indicates that there were currently 46 cryptic species of *B. tabaci* divided into 11 genetic subgroups (Rehman et al., 2021).

The soybean crop is new to Pakistan and efforts are underway to acclimatize the high yielding genotypes. These genotypes are subjected to biotic stresses mainly whitefly transmitted begomoviruses. It is a novel work in Pakistan, particularly in context of soybean viruses therefore the present study was aimed to identify and determine genetic variability of begomoviruses infecting soybean crop and whitefly mitotype(s) involved in transmission of these viruses in Pakistan.

MATERIALS AND METHODS

PCR Analysis for Begomoviruses and Whiteflies

The Soybean fields of MNS-University of Agriculture, Multan were surveyed (30.1475° N, 71.4436° E). The 10 plants exhibited characteristic viral symptoms including thickening, leaf curling yellowing, mosaic pattern and mottling appearance on leaves were collected randomly and total genomic DNA, from infected leaves, was isolated through CTAB method with few modifications (Doyle & Doyle, 1987). The isolated DNA was quantified by a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Molecular identification of begomoviruses was done by diagnostic PCR, which was performed by using a 5´ GAAGCGACCAGCAGATATAATC ´3 (Forward primer) and 5´ CATCCTGTACATCCTGGGCTT (reverse primer).

The whitefly population of 100 whiteflies was collected for cellular analysis. The lysis buffer was used for total genomic DNA isolation of 20 whiteflies. The PCR amplification of mt-COI gene from *Bemisia tabaci* was started by using primers Bt-COI-1F (F-5'-GATCGAAATTTAATTTTATCTTTTTATGATCC-3' and Bt-Cox-1R '5TGTTCTATTGTAAAACTAGCACTATTTTG-3). The PCR conditions, for begomoviruses detection and amplification of mt-CO1 gene, were employed as reported by Afzal et al. (2023).

Whole Genome Amplification of Begomoviruses

The positive PCR samples were subjected to Rolling circle amplification (RCA) as used by (Inoue-Nagata et al. 2004). The reaction was carried out according to the manufacture's protocol (Thermo Fisher Scientific, Waltham, MA, USA). The RCA product was subjected to restriction digestion analysis by *Kpn*I endonuclease and subsequently resolved on 1% agarose gel. The digested products were excised out and purified by silica bead gel extraction kit (Thermo Fisher Scientific, Waltham, MA, USA).

Cloning and Sequencing

The purified products were cloned into non-binary plasmid pUC19 (Addgene) and Dh5-alpha chemically competent *E. coli* cells were transformed with recombinant pUC19 plasmid vector. The transformed Dh5-alpha cells were grown overnight at 37ºC on nutrient agar medium. The white colonies were picked and cultured in liquid nutrient media followed by plasmid isolation by FavorPrep plasmid isolation kit (Favorgen Biotech Corporation, South Korea). The recombinant plasmids and amplified mt-CO1 gene products were sequenced, in both directions, at Macrogen, South Korea.

Genomic Assembly and Phylogenetic Analysis

The sequences of begomoviruses and whitefly were assembled through Geneious R10. The BLAST analysis (available at http://www.ncbi.nlm.nih.gov/BLAST/) was used to evaluate the nucleotide similarity of the target sequences. In case of begomoviruses, the sequence demarcation tool (SDT) was used for pairwise sequence comparison and to determine pairwise similarity scores (Muhire et al. 2014). Phylogenetic analysis was carried out employing maximum likelihood method embedded in MEGA11 with bootstrap value of 1000 replications (Tamura et al. 2011). The selected whitefly CO1 gene sequence was aligned with reference sequences using ClustalW. Phylogenetic analysis was done by MrBayes (Ronquist and Huelsenbeck, 2003).

RESULTS

Molecular Diagnosis and Characterization of Begomoviruses

All the tested samples were found positive exhibiting symptoms of leaf curling, yellowing, and mosaic pattern (Figure 1). The diagnostic PCR analysis yielded 180bp amplifying upstream region of coat protein gene (Figure 2). The RCA digestion resolved on 1% agarose gel and showed approximately 2.8kb band (Figure 3) and the sequencing of recombinant plasmid pUC19, in both directions, resulted in 2734bp. The sequence proved to be an isolate of *Tomato Leaf Curl New Dehli Virus* [U15015] [USA:California:Tomato:1992] also known as *Begomovirus solanumdelhiense* according to new nomenclature.

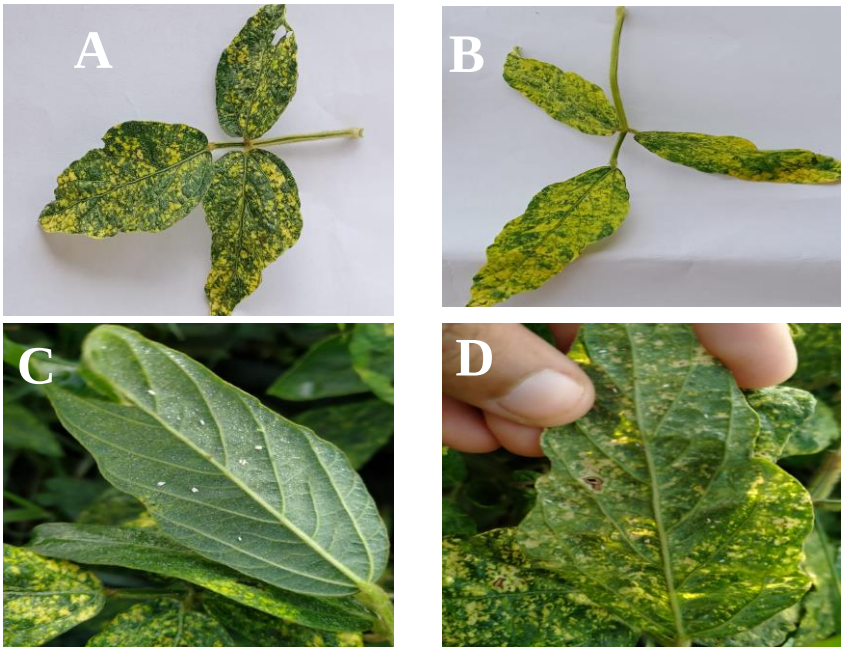

Figure 1. (A) (D) mosaic symptoms; (B) mottling; (C) vein thickening

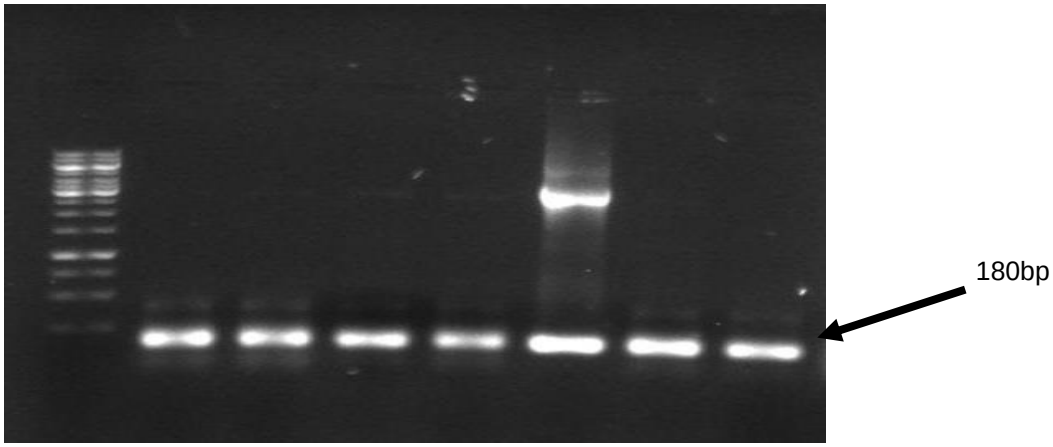

Figure 2: Partial coat protein amplifcation for diagnostic PCR

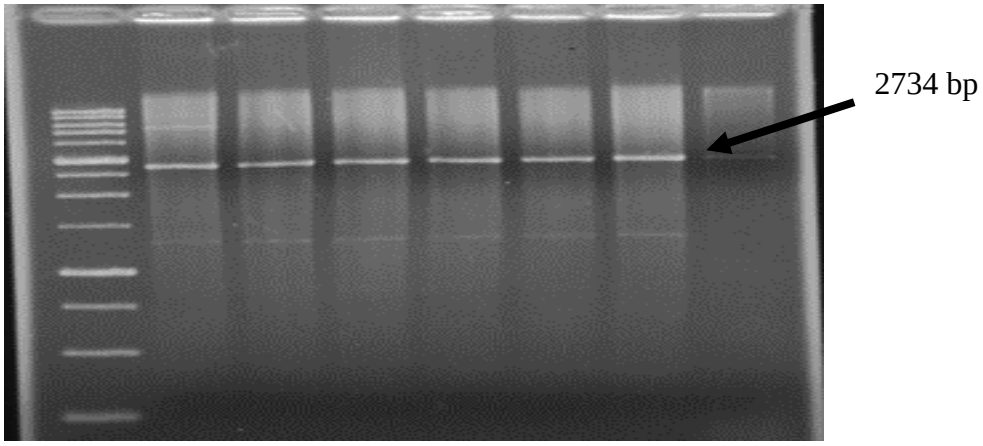

Figure 3: Digested RCA product with *Kpn*1 endonuclease

BLAST analysis revealed homology of 2734bp sequence with DNA-A component of ToLCNDV. The similarity identity matrix was constructed with pairwise sequence analysis, MUSCLE alignment embedded in SDT, showed 98% similarity with sequence *Tomato Leaf Curl New Dehli Virus* (ToLCNDV) isolates with accession numbers HM007120 and KP235542 reported from India in 2008 and 2009 (Table 1) as per demarcation criteria for characterization of begomoviruses.

Table 1. Similarity matrix identity scores with ToLCNDV [Soybean:Multan:2022]

>ToLCNDV_[MH475911][SP1A]	100.001													
>ToLCNDV_[MZ670759][Th8]	95.401	100.001												
>ToLCNDV_[LN887908][14AA116RS57]	95.001	95.201	100.001											
>ToLCNDV_[HM345979]	95.001	95.301	95.901	100.001										
>ToLCNDV_[KF515617][India_Junagadh_2012]_	94.701	94.901	95.801	98.001	100.001									
>ToLCNDV_[MH577013][pBamJ33]_	94.801	95.101	95.801	97.801	98.501	100.001								
>ToLCNDV_[MH577012][pBamM9]_	94.501	94.801	95.601	98.001	98.101	98.301	100.001							
>ToLCNDV_[FN435310]_[Pakistan_Solanum_2009_MS4]_	95.201	95.501	95.701	97.301	96.701	96.701	96.601	100.001						
>ToLCNDV_[AM258977][ToA2]	95.101	95.501	95.701	96.901	96.301	96.201	96.201	98.401	100.001					
>ToLCNDV_[HM159454]	95.101	95.701	95.601	96.901	96.501	96.301	96.201	98.101	97.501	100.001				
>ToLCNDV_[JN129254][Pum_IARI_06]	95.701	95.901	96.101	97.101	96.801	96.601	96.701	97.801	97.201	98.001	100.001			
>ToLCNDV_[DQ169056]	95.701	96.501	96.201	97.301	97.001	97.001	97.001	97.601	97.201	97.401	98.201	100.001		
>ToLCNDV_[DQ116880][PC2No10]	96.101	96.301	96.601	97.101	96.901	96.701	96.801	97.901	97.801	97.601	98.101	98.201	100.001	
>Multan_isolate_[Soybean_2022]	96.401	96.401	96.501	96.701	96.501	96.501	96.601	97.901	97.301	97.701	97.701	97.401	97.001	100.001
>ToLCNDV_[MH577017][pBamSk2]	94.701	95.101	94.901	95.601	95.101	95.401	95.101	95.601	95.101	95.501	95.801	95.501	96.001	97.301
>ToLCNDV_[KP235542]_[VIRO_641]	95.701	96.601	96.201	96.301	95.901	96.101	95.901	96.701	96.201	96.601	96.801	96.601	96.901	98.001
>ToLCNDV_[HM007120_[India_Tumkur_Chilli_2008;pChTumB2]	95.701	96.601	96.201	96.401	95.901	96.101	95.901	96.701	96.301	96.601	96.801	96.701	97.001	98.001
>ToLCNDV_[KP235540]_[VIRO_640]	95.501	96.501	96.101	96.301	95.801	96.001	95.801	96.601	96.201	96.501	96.701	96.601	96.901	97.901
>ToLCNDV_[LT556070]	94.901	94.701	94.901	94.301	94.101	94.001	94.001	94.201	94.501	94.201	94.601	94.801	94.901	96.801

Phylogenetic analysis employing maximum likelihood method, with 1000 bootstrap replications, clustered the ToLCNDV Multan isolate with ToLCNDV isolates from Pakistan reported in 2009 (Figure 4).

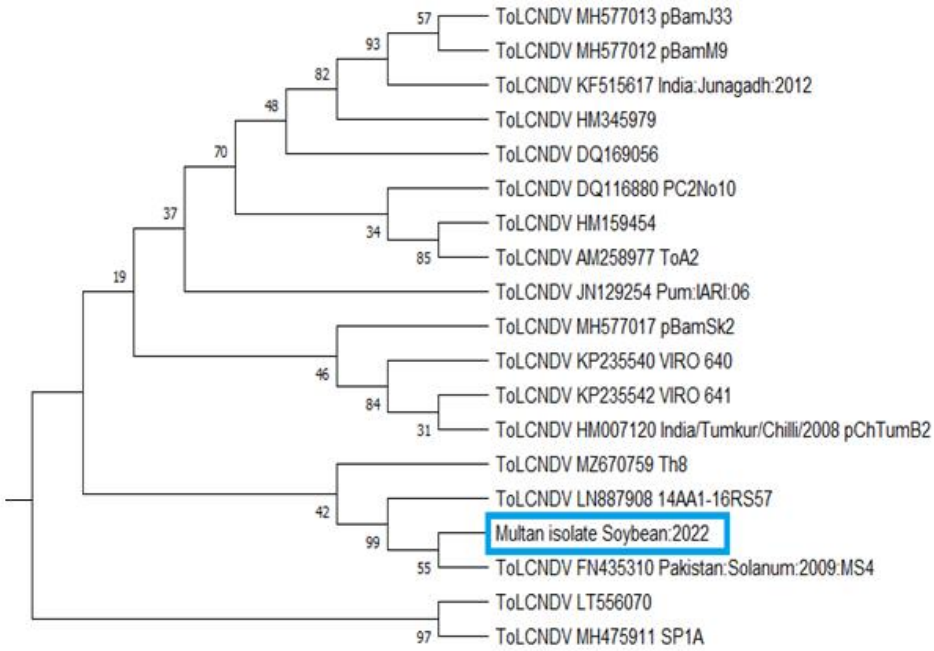

Figure 4. Phylogenetic analysis of Multan isolate of ToLCNDV with reported isolates from across the world

Amplification of Partial COI Subunit Gene and Sequence Analysis

The diagnostic PCR amplified 750bp partial COI gene using Bt-CO1F and Bt-CO2R primer pair (Figure 5). The initial confirmation of whitefly CO1 gene sequence was done by BLAST analysis. The multiple alignment was done through CLUSTALW and identity scores were calculated using MEGA11. The results revealed 99.99% similarity with *B. tabaci* sequences, with accession numbers OL763913 and OL763907 reported from Pakistani cities of Nasirabad and Rahim Yar Khan in 2019 and 2018 respectively (Table 2). Phylogenetic tree was constructed using MrBayes and clustered the whitefly sequence with Asia II-1 mitotypes (Figure 6).

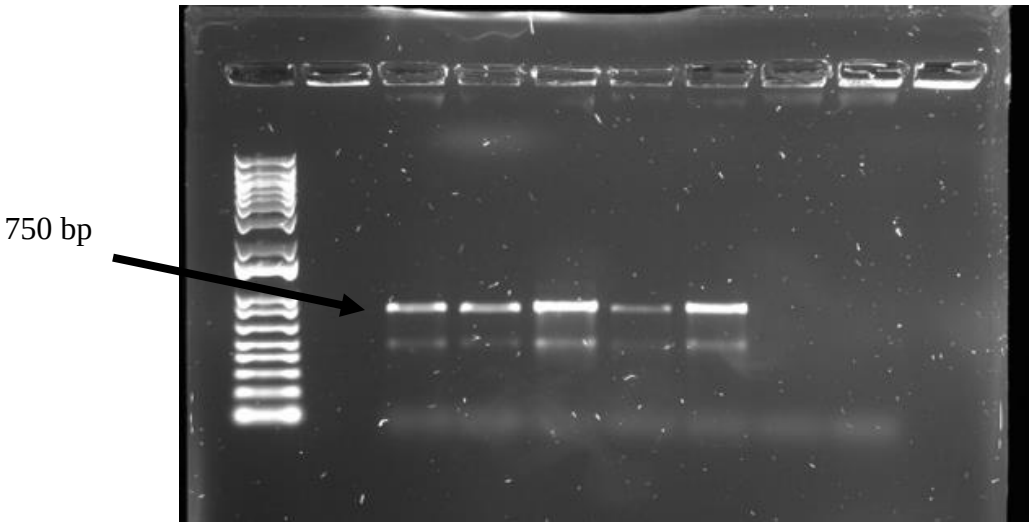

Figure 5. Amplification of partical CO1 gene of whitefly

Table 2. Sequence identity score of Asia II-I mitotype, collected from Soybean, with reported whitefly mitotypes

cytochrome_c_oxidase_subunit_I_partial_sequence_Soybean:2021:Multan																		
B.tabaci_OL763913_Nasirabad:Solanum:melongena:2019	98.2850470208																	
B.tabaci_OL763907_RYKhan:Brassica:oleracea:2018	98.1273074970	0.0038305730																
B.tabaci_KT001155_isolate_Bt4	-553.4986904097	6.6239293858	6.6349220376															
B.tabaci_HG918198	-915.7102277599	10.1950667716	10.1950667716	8.2185144503														
B.tabaci_OM256501_isolate_MM-378	-842.4049839889	10.1565590662	10.1515675671	8.4173909398	4.4223158638													
B.tabaci_KF751573_isolate_SdHd08-2	-652.7497537617	7.0663874979	7.0437153680	9.7370747660	4.7083802166	5.1714419418												
B.tabaci_OL763923_isolate_196_MirpurKhas:Gossypium:hir	97.9590462408	0.0038347953	0.0026210376	6.6348220375	10.2504825310	10.2079224441	7.1288861187											
B.tabaci_OL763921_isolate_199_Ghotki:Gossypium:hirsutur	98.1271117011	0.0013079264	0.0026180021	6.6239290858	10.1950667716	10.1565590662	7.0693381078	0.0026208101										
Bemisia_tabaci_OL763914_TandoAllahyar:Gossypium:hirsut	98.1271117011	0.0013079264	0.0026180021	6.6239290858	10.1950667716	10.1565590662	7.0693381078	0.0026208101	0.0000000000									
B.tabaci_OL763912_RYKhan:Brassica_oleracea:2018	6.5349853941	0.0038305730	0.0026177752	6.6239290858	10.1950667716	10.1565590662	7.0608404358	0.0052507181	0.0026180021	0.0026180021								
Bemisia_tabaci_MT605826_isolate_KA1_1_IQ	-378.9267584614	5.0242649942	5.0242649942	6.6186612395	7.0280355127	7.2161465070	5.1704074542	5.0894788691	5.0187805992	5.0187805992	5.0149294843							
Bemisia_tabaci_MT605822_isolate_KA1_2_IQ	-378.9267584614	5.0242649942	5.0242649942	6.6186612395	7.0280355127	7.2161465070	5.1704074542	5.0894788691	5.0187805992	5.0187805992	5.0149294843	0.0000000000						
Bemisia_tabaci_MT605821_isolate_KA1_3_IQ	-378.9267584614	5.0242649942	5.0242649942	6.6186612395	7.0280355127	7.2161465070	5.1704074542	5.0894788691	5.0187805992	5.0187805992	5.0149294843	0.0000000000	0.0000000000					
Bemisia_tabaci_KX675908	-648.1670680649	6.9197527675	6.9056941468	7.0302457332	10.4969991818	7.1874522662	7.7476300634	6.8321349971	6.9127200099	6.9127200099	6.9056941468	4.7618123952	4.7618123952	4.7618123952				
B.tabaci_KX675907	-338.8387724311	4.5910032349	4.5955410982	7.4680266920	10.0292217137	10.2589036898	6.4680699157	4.5322866079	4.5955410982	4.5955410982	4.6027139134	6.3274580046	6.3274580046	6.3274580046	6.3261158459			
B.tabaci_KX675904	-510.1184150907	6.5110201460	6.5037390282	5.5195689597	6.5645199748	6.5827160409	7.7743580618	6.4114973693	6.5110201460	6.5110201460	6.5139691901	4.2488876870	4.2488876870	4.2488876870	9.8750856999	6.6658635345		
B.tabaci_LN835385	-842.4049839889	10.1498199967	10.1448137707	8.4173909398	4.4185370952	0.0024480922	5.1714419418	10.2012689217	10.1498199967	10.1498199967	10.1498199967	7.2091295324	7.2091295324	7.2091295324	7.1943072212	10.2520820522	6.5827160409	

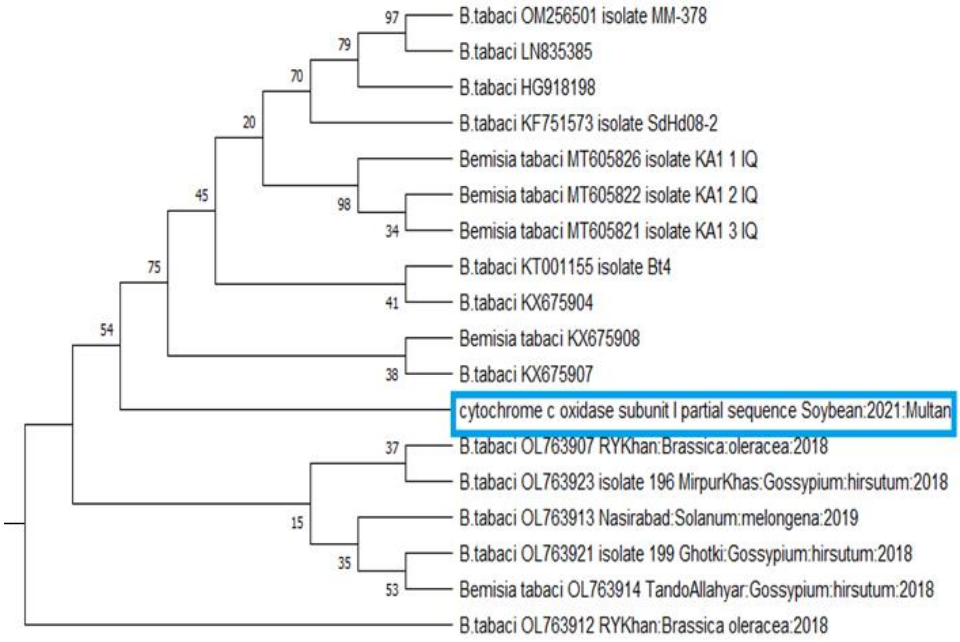

Figure 6. Phylogenetic tree of *Bemisia tabaci*, collected from Soybean, with reported whitefly mitotypes based on cytochrome oxidase subunit gene1

DISCUSSION

Begomoviruses presence in soybean, showing characteristic symptoms, was confirmed through diagnostic PCR followed by whole genome amplification of DNA-A leading to the identification of 2734bp of ToLCNDV isolate which is a first report of ToLCNDV infecting Soybean in Pakistan. The presence of Asia II-1 mitotype is also termed as first report of Asia II-1 presence on Soybean plants in Pakistan. Seven begomoviruses have been reported from soybean crop in the United States including PepGMV (Andrade et al. 2006), CaLCuV reported from Colombia along with documented infections of LeMV, SiMoV and OKMV in Brazil (Fernandes et al. 2019). ToLCNDV is also reported to infect host plants in Pakistan viz. Tomato, wild spinach, Field bindweed, Bitter Gourd, Chilies, Luffa, toothed dock, *Parthenium* spp. and blackberry nightshade (Shih et al. 2006; Brown et al. 2017).

The tomato crop harbors more than 15 begomoviruses species (Shahid et al. 2007; Kumar et al. 2013). While Masood et al., (2017), Islam et al. (2018) and Afzal et al., (2023) documented the spread of Asia II-1 mitotype and

termed it an invasive species in Sindh province over-populating MEAM-1 in areas adjacent to Punjab province. This has a potential impact on the transmission of begomoviruses to new host plants leading to the development of new recombinant species and variants.

CONCLUSION

This study confirms the presence of ToLCNDV and Asia II-1 mitotype on Soybean crop in Pakistan. It is the first report of begomovirus-whitefly complex on Soybean in Pakistan and will be helpful for researchers in the development of potential management strategies to control the spread of begomoviruses-whitefly complex in Pakistan.

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AUTHOR CONTRIBUTIONS

HR and SK conceived the study. SK executed the research work. SK, FZ, SK and SR helped in manuscript write-up. HR, MA, SS, MI, FD, MY and FR reviewed the manuscript.

COMPETING OF INTEREST

The authors declare no competing interest.

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