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SHORT REPORTS

Molecular investigations reveal bitter gourd crop is more susceptible to tomato leaf curl New Delhi virus infection in diverse crop cultivation practices

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Abstract
Small- and medium-scale farmer's typically follow polyculture or diverse crop cultivation. However, cultivation of diverse crops in small area can cause cross infection leading to disease spreading across crops. A microplot-based field study was conducted to understand the disease susceptibility and disease mobility across various crops, including tomato, chilli, mungbean, and bitter gourd. The mungbean yellow mosaic virus (MYMV) incidence was noted first in the mungbean crop followed by tomato leaf curl New Delhi virus (ToLCNDV) in tomato and chilli leaf curl virus (ChLCV) in chilli crop. Interestingly, bitter gourd crop was infected lastly with symptoms including yellow and green mottling, severe leaf curling, and stunted growth. However, in bitter gourd crop symptoms, like typical leaf curl virus, could not be conclusively related to a certain type of begomovirus. Molecular diagnosis using begomovirus specific deng primers and coat protein (CP) gene primers specific to begomovirus species revealed the presence of ToLCNDV in bitter gourd samples. The phylogenetic analysis of CP gene sequences revealed 98 per cent nucleotide identity with ToLCNDV. Further cross infectivity assays confirmed the transmission of ToLCNDV from tomato to bitter gourd and vice versa. The cryptic species of whiteflies isolated from the bitter gourd fields were sequence confirmed to belong to Asia-I genetic group that were reported to transmit ToLCNDV previously. Overall, our study suggests the vulnerability of bitter gourd crop for ToLCNDV infection when cultivated by the side of tomato plots.

Keywords Bitter gourd · Cross infectivity · ToLCNDV · Whitefly

Crop diversity is key to agricultural sustainability. This is particularly important in most Asian countries where average land area per holding is minimum, about 1 hectare (Reap 2010; Lowder et al. 2016). In India, small and medium farmers (less than 2 ha per holding) account for nearly 40% of the area. To ensure the household food security and to maximize the agricultural income, small and medium farmers generally adopt intensive cultivation practice (Basantaray and Nancharaiah 2017). This includes either growing diverse crops in different seasons of the year or growing diverse crops in the same season by dividing land into several cultivable areas. Diverse crop cultivation is possible in most South Asian countries due to favorable tropical agroclimatic conditions throughout the year (Hazra 2001). While the cultivation of diverse vegetable crops can provide a means of sustainable income source, there is always a threat of unprecedented insect and pest attack, cross infection, and sudden disease vulnerability (Joshi et al. 2006), more commonly, the threat of virus disease that can spread through the field quickly. Therefore, the studies on identifying the virus disease pattern in diverse crop cultivation would be highly useful toward appropriate crop selection and designing effective management practices. Herein, we have explored the begomovirus disease susceptibility pattern when diverse crops, including tomato, chilli, mungbean, and bitter gourd, were cultivated alongside.

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The genus *Begomovirus* belonging to the family *Geminiviridae* constitutes the largest group of plant-infecting DNA viruses affecting a wide range of host plants, including tomato, chilli, okra, bitter gourd, legumes, and papaya (Varma and Malathi 2003; Nagendran et al. 2014; Leke et al. 2015; Inoue-Nagata et al. 2016; Sharma et al. 2018). Interestingly, the begomoviruses symptom expression varies across different host plants (Vindyashree et al. 2015). This feature of differential host adaptability is more commonly observed with tomato leaf curl New Delhi virus (ToLCNDV). ToLCNDV is a bipartite begomovirus first identified in India on solanaceous crops (Khan et al. 2006). The inoculation of ToLCNDV on diverse solanaceous crops revealed that ToLCNDV isolate expressed disease symptoms readily in *Nicotiana benthamiana, N. tobaccum,* and Tomato while it did not result in infection of peppers (*C. annuum*) (Kushwaha et al. 2015). Similarly, ToLCNDV-ES strain in western Mediterranean basin was shown to readily infect cucumber but poorly infect tomato (Fortes et al. 2016). Given such variability in ToLCNDV pathogenesis on various host crops it is essential to establish host-virus susceptibility studies on diverse crop plants. Such studies would provide crucial insights toward the susceptible host range of ToLCNDV.

In tomato, typical ToLCNDV symptoms include curling and crinkling of leaves, vein clearing, yellow mottling, leaf puckering and blistering of leaves, and stunting leading to overall yield loss (Jyothsna et al. 2013), while in case of cucurbits prominent symptoms include leaf distortions and mosaic spread (Yazdani-Khameneh et al. 2016). However, in bitter gourd prominent symptoms include yellow mosaic, mild leaf curl and produce smaller fruits (Tiwari et al. 2010; Nagendran et al. 2014). The differential pathogenesis of ToLCNDV across different crops was related to differential expression of host defense factors in each plant species (Kushwaha et al. 2015). Therefore, molecular investigations involving ToLCNDV–Plant host interaction are important toward establishing the host range of ToLCNDV. In this study, we show that bitter gourd crop is highly susceptible for ToLCNDV and further by whitefly-mediated cross infectivity assays we demonstrate that ToLCNDV can move between tomato and bitter gourd crops. Overall, our studies suggest the cultivation of bitter gourd and tomato crops should be avoided in the close proximity.

The field study was conducted during summer 2020 in the research plot at Main Research Station (MRS), Hebbal, Bangalore, Karnataka (13.03° N, 77.5879° E, 916 m above mean sea level). Land was tilled in both directions and 15-cm-high raised beds were prepared. Tomato and chilli seedlings were raised in protrays under 50× nylon mesh net. One-month-old seedlings of chilli and tomato were transplanted on raised beds with a spacing of 90 × 60 cm each. Direct sowing of mungbean and bitter gourd was done on the same day with the spacing of 30 × 10 cm and 60 × 45 cm, respectively. Standard agronomic practices were followed for the entire season and observed for virus symptoms on crops. Plot size of mungbean, tomato, chilli, and bitter gourd were 90 m^2, 210 m^2, 190 m^2, and 190 m^2, respectively.

Three symptomatic and one non-symptomatic leaf sample were collected from each crop and brought to the laboratory on ice for further molecular analysis. The DNA was isolated from the four samples of each crop by CTAB method (Dellaporta et al. 1983). To detect begomovirus infection, these samples were subjected to polymerase chain reaction (PCR) using universal deng primers (Deng et al. 1994). PCR was performed in 30 µl reaction mixture containing standard PCR ingredients, i.e., DNA template (1 µl), 10 mm dNTP (2 µl), 10× PCR buffer (5 µl), Taq DNA polymerase (0.5 µl), (Thermo Scientific) 1 µl each of forward and reverse primers, and autoclaved double-distilled water to make up the volume. PCR conditions for amplification of the genome with deng primers were standardized as initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 7 min. MYMV CP specific primers (MYMVF–ATGGGKTCCGTTGTATGCTTG and MYMVR-GGCGTCATTAGCATAGGCAAT) (Naimuddin 2010), ChLCV CP specific primers (ChLCVF-CTC CAGACACTCTGGGGTAC and ChLCVR-GAATCTGGA CGACCTTACAGCC), and ToLCNDV CP specific primers (TNDF-CATGGTCTGAGGTGCATGC and TNDR-CAA GATCTTGACGGGCTTAC) were used to confirm the presence of MYMV, ChLCV and ToLCNDV, respectively. To avoid false-positive results, a negative control (No template) was used for each reaction. PCR amplicons were analyzed on 1% agarose gels and three PCR amplicons from each crop were directly sequenced in both the orientations. The sequence data obtained were compared with known nucleotide sequences at the NCBI database using the BLAST algorithm. Phylogenetic tree was constructed by a neighbor-joining method with 1000 bootstrap repetitions using Mega-X software (Kumar et al. 2018).

Adult whiteflies collected from each replicate of infected bitter gourd, tomato, chilli, and mungbean fields were stored at − 20 °C in 70% alcohol. The DNA was isolated from a group of eight adult whiteflies of each replicate using the Chelex 100 resin method (Walsh et al. 1991). PCR was performed with universal*mtCOI* (mitochondrial cytochrome oxidase-1) primers (C1-J-2195F-5′TTGATTTTTTGGTCA TCCAGAAGT3′ and TL2-N-3014R- 5′TCCAATGCACTA ATCTGCCATATTA3′) (Frohlich et al. 1999) with following PCR conditions. Initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 1 min and 72 °C for 90 s, and a final extension at 72 °C for 7 min. PCR amplicons were analyzed on 1 percent agarose gels and PCR amplicons from each replicate of four crops were directly

sequenced in both orientations. The sequences obtained were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and consensus sequences were compared with the reference sequences of whitefly species available at NCBI database using BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Phylogenetic tree was established to derive the relationship using the ClustalW algorithm of the Mega-X software (Kumar et al. 2018).

In order to evaluate the disease movement pattern of ToLCNDV, a cross infectivity assay was set up. Whiteflies were reared on healthy cotton seedlings (*Gossypium hirsutum* L. cv. Varalaxmi) in glasshouse insectary at MRS, Hebbal, Bengaluru, Karnataka (Muniyappa et al. 1987; Czosnek et al. 1988). In this assay, young healthy crop plants (12–15-day-old seedlings) of mungbean, chilli, and bitter gourd were inoculated through viruliferous whitefly adults with 2 h starvation, 12 h AAP, and 24 h IAP. About 20 viruliferous whiteflies were released for inoculation in each insect proof cage. After that the inoculated seedlings were sprayed with 0.03% imidacloprid (17.8% SL) and kept separately in insect proof cages for symptom expression. The infection in the inoculated seedlings was further confirmed using ToLCNDV CP gene specific primer. Further to confirm the reverse movement of ToLCNDV to healthy tomato plants, symptomatic bitter gourd plants were used as a source of inoculum.

Tomato, chilli, mungbean, and bitter gourd crops susceptible to various species of begomovirus infection were co-cultivated to study the disease susceptibility and the disease spread pattern between the crops (Fig. S1). All the four crops grew well with a good crop canopy. The first incidence of begomovirus disease was noticed in mungbean. Mungbean yellow mosaic disease incidence was observed at 14 days after sowing (DAS) in mungbean. At 16 days after transplantation (DAT), first incidence of ToLCNDV was observed in tomato, whereas the incidence of chilli leaf curl virus (ChLCV) was first noticed in chilli at 25 DAT. Interestingly the leaf curl disease symptoms appeared in bitter gourd at about 35 days after sowing. Percentage of disease incidence in chilli, tomato, mungbean, and bitter gourd crops with respective viruses were 84%, 81%, 75%, and 79%, respectively. The disease symptoms observed in tomato, chilli, and mungbean were very evident of ToLCNDV, ChLCV, and mungbean yellow mosaic virus (MYMV), respectively. The typical symptoms of ChLCV in chilli include upward curling, reduction in size of the leaves, and stunting of the entire plant. Severely infected plants produce smaller and deformed fruits. The initial symptoms of MYMV in mungbean include small yellow specks followed by mottling on leaves. Later the entire plant turns yellow, stunted and pod size is reduced. However, the symptoms on bitter gourd crops were not distinguishable to relate to a particular type of begomovirus. Initial symptoms included just light yellow to green mottling but in the later stages severe mottling, curling, twisting of leaves, and yellowing of foliage were observed. In fact these plants appeared stunted with fewer twisted fruits (Fig. 1).

Based on the phenotypic symptoms it was not distinguishable which type of begomovirus infected with bitter gourd as there was a standing inoculum source of MYMV, ChLCV, and ToLCNDV. Also, there was a possibility of mixed infection from different begomovirus types. Therefore, PCR amplifications using deng primers and coat protein gene primers specific to MYMV, ChLCV, and ToLCNDV were conducted. The PCR reaction with begomovirus specific deng primers resulted in an amplicon of 500 bp in the DNA samples of all the four crops (Fig. 2a). Further, the DNA samples of all four crops were subjected to PCR amplification using CP gene primers specific to MYMV, ChLCV, and ToLCNDV (Fig. 2b–d). Interestingly, only mungbean DNA samples amplified with MYMV specific CP gene primers and chilli DNA samples amplified with ChLCV specific CP gene primers yielding 900 and 1000 bp amplicons, respectively (Fig. 2b,c). While the DNA samples of both tomato and bitter gourd crops amplified with ToLCNDV specific CP gene primers with an expected amplicon of 1000 bp but interestingly no amplification was observed with chilli and mungbean DNA samples (Fig. 2d). These data clearly

Mosaic mottling with slight curling of leaves **Severe curling and reduction in leaf size**

Fig. 1 ToLCNDV infected bitter gourd plants showing leaf curl symptoms

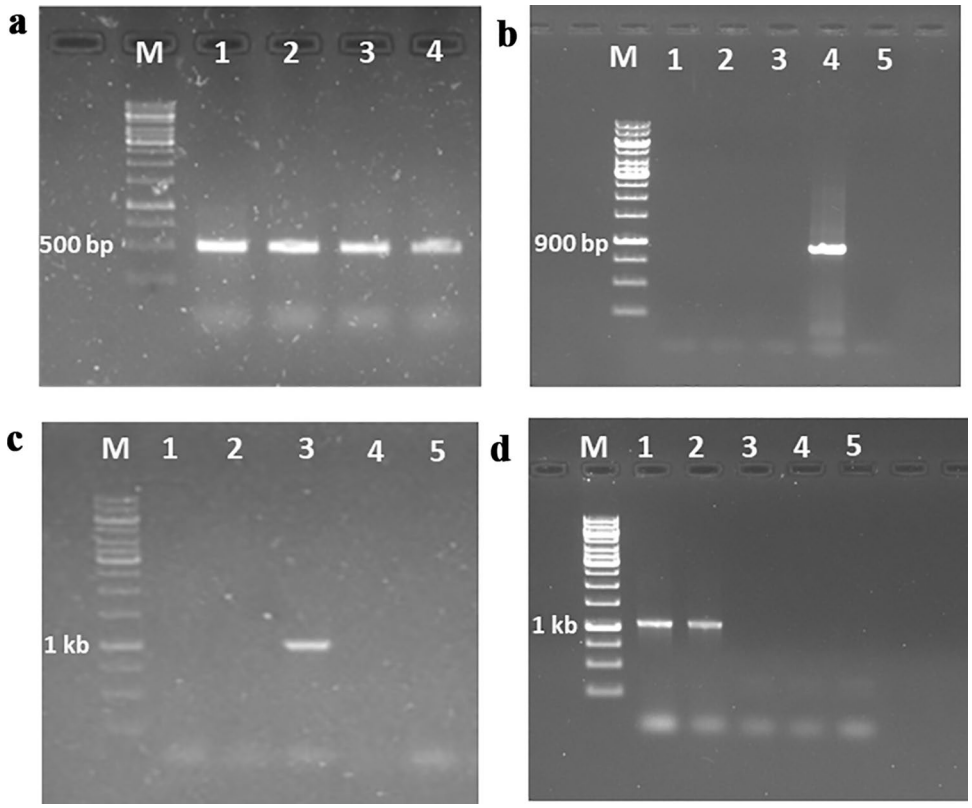

Fig. 2 PCR screening of leaf samples using **a** Deng primers, **b** MYMV specific CP gene primers, **c** ChLCV specific CP gene primers, and **d** ToLCNDV specific CP gene primers. Lanes, M: 1 kb marker, Lanes 1: Tomato, Lanes 2: Bitter gourd, Lanes 3: Chilli, Lanes 4: Mungbean and Lanes 5: Negative control

suggest that both tomato and bitter gourd crops were infected with ToLCNDV, while mungbean and chilli crops were not infected with ToLCNDV. Further, the PCR amplicons of bitter gourd, tomato, chilli, and mungbean were sequenced and compared with the sequences at NCBI database. BLASTn analysis of bitter gourd and tomato sequences showed the highest nucleotide identity of 98% with tomato leaf curl New Delhi virus, whereas chilli and mungbean sequences showed the highest nucleotide identity of 99% and 100% with chilli leaf curl virus and mungbean yellow mosaic virus, respectively. Phylogenetic analysis using Mega-X software revealed that 770 bp CP (AV1) gene amplified from bitter gourd and tomato DNA samples were closely clustered with CP (AV1) gene of tomato leaf curl New Delhi virus isolates (Fig. 3), whereas 770 bp CP and 774 bp CP in chilli and mungbean were closely clustered with chilli leaf curl virus and mungbean yellow mosaic virus, respectively (Fig. 3).

The DNA samples of adult whiteflies collected from the bitter gourd, tomato, chilli, and mungbean fields resulted in PCR amplicons of 900 bps with *mtCO1* primers (Fig. 4a). The *mtCO1* PCR amplicons were sequence determined and compared with the reported sequences of whitefly at global level. BLASTn analysis of *mtCOI* sequences suggests the highest nucleotide identity of 99% with Asia-1 genetic group and phylogenetic analysis (Fig. 4b) suggests a close clustering with whitefly sequences belonging to the Asia-1 genetic group.

Since the leaf curl disease symptoms are observed in bitter gourd crop alone only after tomato crop being infected in the field. Based on this we hypothesized that possibly ToLCNDV from tomato plants could have served as a source inoculum for transmission to bitter gourd by whiteflies. To validate this hypothesis, a cross infectivity assay was conducted. In this assay, whiteflies were starved for 2 h and were separately fed for acquisition of leaf curl virus on symptomatic tomato plants, 20 viruliferous whiteflies (12 h AAP) were allowed to feed on 12–15-day-old healthy seedlings of bitter gourd, chilli and mungbean separately (24 h IAP). Out of the three crops, inoculated bitter gourd plants only showed light yellow to green mottling, slight upward curling of leaves (Table 1) within 15 days of inoculation with 66.66 per cent transmission. Chilli and mungbean plants remain healthy even after 30 days of inoculation. These observations clearly suggest that ToLCNDV moved from tomato plants to bitter gourd in the field (Fig. 5a). Further, to verify if the virus could move from infected bitter gourd plants to healthy tomato plants, symptomatic bitter gourd plants were used as a source of virus for whiteflies (Fig. 5a). Interestingly, within 15 days of inoculation the tomato plants showed ToLCNDV symptoms, such as yellowing of top leaves, upward curling, and crinkling of leaves. Thus, these observations clearly suggest that ToLCNDV could move from tomato to bitter gourd crop and vice versa. The movement of ToLCNDV

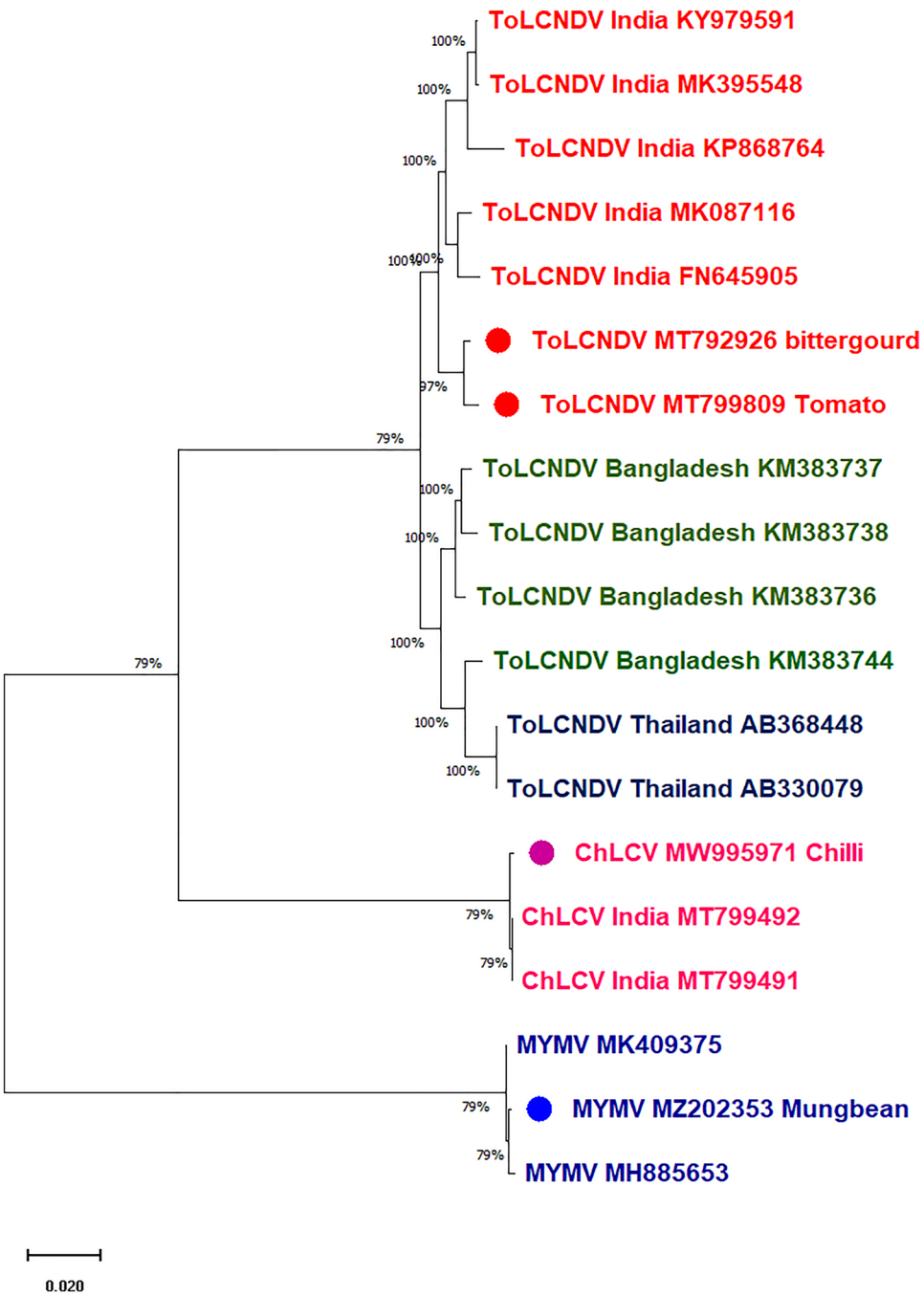

Fig. 3 Phylogenetic dendrogram showing the relationship of bitter gourd, tomato, chilli, and mungbean amplicon sequences with other isolates of ToLCNDV, ChLCV, MYMV available at NCBI database. ToLCNDV, tomato leaf curl New Delhi virus; ChLCV, chilli leaf curl virus; MYMV, mungbean yellow mosaic virus

between tomato and bitter gourd plants was further confirmed using ToLCNDV specific primers (Fig. 5b).

Begomoviruses constitute the largest genus in the entire virosphere showing a wide host range and the associated crop diseases (Zerbini et al. 2017; Walker et al. 2019). Crop loss due to *Begomovirus* infection is common in several crops, including tomato, chilli, okra, gourds, watermelon, legumes, and papaya (Varma and Malathi 2003; Kumar et al. 2017; Venkataravanappa et al. 2020). Incidentally, in most of the Asian countries where the land availability per capita is minimum, most of these crops are cultivated in small cultivable plots (Bisht et al. 2020). Therefore, the knowledge of the disease movement pattern under small plot cultivation scheme may provide insights toward appropriate crop selection. In this study, tomato, chilli, mungbean, and bitter gourd crops were cultivated in adjacent microplots (Fig. S1) in a begomovirus disease prone hotspot region. The disease incidence data were recorded to note the first appearance of the disease in each crop. Despite MYMV incidence in mungbean first, MYMV did not spread to other three crops which was confirmed with MYMV specific CP gene primers. ToLCNDV in tomato was noticed secondly followed

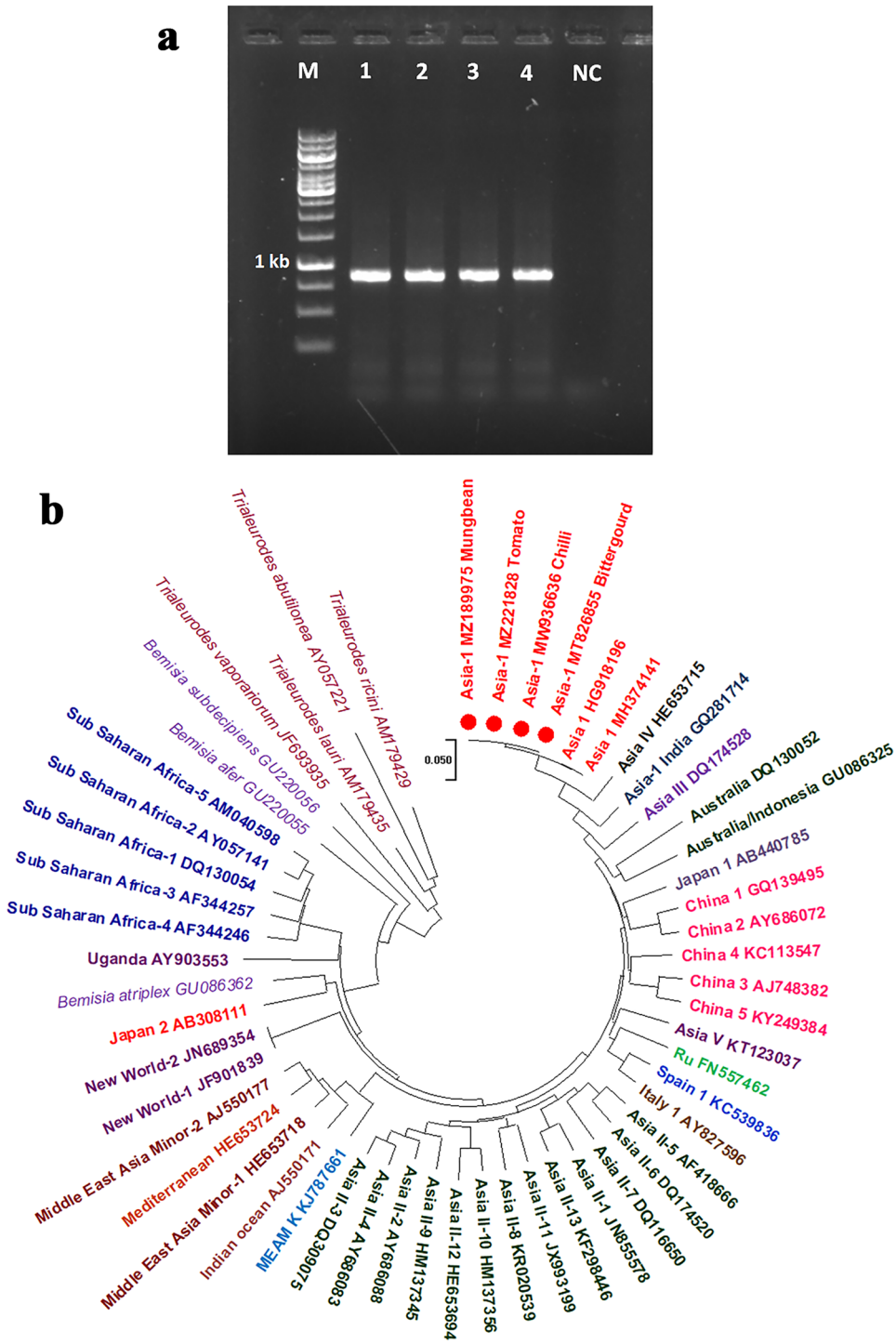

Fig. 4 Molecular detection and analysis of cryptic species of *B. tabaci* (Asia-1) (**a**). *mtCOI* amplicon analysis on 1% agarose gel. Lanes, M: 1 kb marker; Lane 1: *B. tabaci* (Bitter gourd), Lane 2: *B. tabaci* (Tomato), Lane 3: *B. tabaci* (Chilli), Lane 4: *B. tabaci* (Mungbean), Lane, NC-Negative control. **b** Neighbor-joining phylogenetic dendrogram based on *mtCOI* amplicon sequence indicating the relationship of *B. tabaci* with other isolates at NCBI database

by ChLCV in chilli. Curiously, the begomovirus symptoms appeared in bitter gourd lastly. Although the symptoms were indicative of begomovirus infection in bitter gourd it was not clear whether the symptoms were due to infection of MYMV or ToLCNDV or ChLCV. However, the molecular detection of bitter gourd DNA samples using CP gene primers specific to MYMV, ToLCNDV, and ChLCV clearly indicated the presence of ToLCNDV in bitter gourd (Fig. 2).

The molecular studies indicated that, in the field, ToLCNDV would have moved from tomato to bitter gourd crop but not to either mungbean or chilli crops (Fig. 2). This may possibly be due to the presence of MYMV in mungbean and ChLCV in chilli crops already. However, to validate this observation, a cross infectivity assay was conducted wherein whiteflies carrying ToLCNDV inoculum were exposed to seedlings of mungbean, chilli, and bitter

Table 1 Vector transmission of ToLCNDV through *Bemisia tabaci*[a] for cross infectivity assay

Source of inoculum	Test plants	% Transmission[b]			Mean[c]	Symptoms observed
		R1	R2	R3		
Tomato	Bitter gourd	70	60	70	66.66	Initially light yellow to green mottling, upward curling of leaves, great reduction in leaf size
Bitter gourd	Tomato	60	60	60	60.00	Yellowing of top leaves, rolling and crinkling of leaves

[a]Experiment conducted thrice

[b]Starvation: 2 h; AAP: 12 h; IAP: 24 h; 20 viruliferous whiteflies

[c]Out of 10 plants inoculated

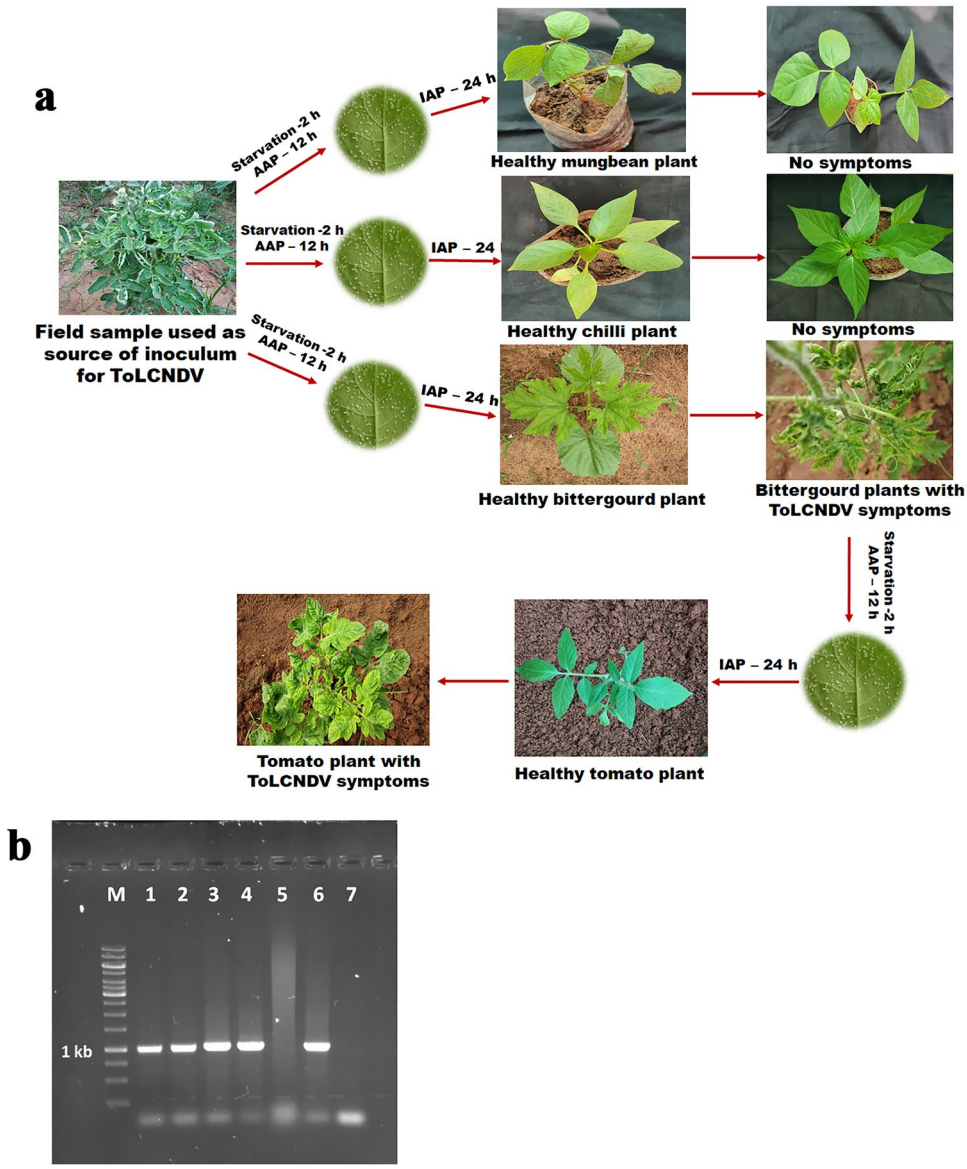

Fig. 5 Cross infectivity and characterization of disease movement **a** cross infectivity assay, **b** PCR products analysis on 1% agarose gel. ToLCNDV specific CP primers were used in the PCR reactions. Lane, M: 1 kb marker; Lanes 1–4: symptomatic bitter gourd samples, 5: healthy bitter gourd sample 6: symptomatic tomato sample, 7: healthy tomato sample

gourd crops (Fig. 5). Only bitter gourd plants expressed typical ToLCNDV symptoms, while mungbean and chilli plants did not show symptoms of ToLCNDV. This clearly demonstrates that the bitter gourd crop is vulnerable to ToLCNDV than mungbean and chilli crops. Thus, it is possible that ToLCNDV readily infects bitter gourd crop over mungbean or chilli crops. Differential host expression of ToLCNDV has been adequately reported. ToLCNDV

infection of chilli was reported from India (Khan et al. 2006) and Pakistan (Hussain et al. 2004), while Kushwaha et al. (2015) were unsuccessful in inoculating chilli with ToLCNDV. Similarly, ToLCNDV-ES strain in western Mediterranean basin were shown to readily infect cucumber but poorly infect tomato (Fortes et al. 2016).

The whitefly is the only known vector for transmission of begomoviruses. However, several previous studies suggest many begomoviruses are differentially transmitted by different species of the *B. tabaci* cryptic species complex (Bedford et al. 1994; Chowda-Reddy et al. 2012; Hidayat and Rahmayani 2007; Sanchez-Camphos et al. 1999; Venkataravanappa et al. 2017; Fiallo-Olive et al. 2020). Our analysis of the whitefly population using mitochondrial cytochrome oxidase-1 (*mtCOI*) sequence comparison reveals that the whitefly belongs to Asia type-I (Fig. 4). Asia type-I vectors being polyphagous we expected the transmission of ToLCNDV to mungbean and chilli plants in cross infectivity assays. Surprisingly, only bitter gourd plants expressed symptoms, while mungbean and chilli plants were asymptomatic (Fig. 5). This clearly demonstrates that bitter gourd crop is a more susceptible natural host for ToLCNDV in Southern Karnataka, while mungbean and chilli are not. The non-transmission of ToLCNDV to mungbean and chilli plants may be possibly due to certain non-host resistance mechanisms in these crops (Baruah et al. 2020). Similarly, in the field trials, despite MYMV incidence occurred first it did not spread to other crops. This may also be due to certain non-host resistance mechanisms against MYMV in tomato, chilli, and bitter gourd crops. Non-host resistance mechanisms have been extensively reported previously (Baruah et al. 2020). Tomato was found to be a non-natural host plant of tobacco mild green mosaic virus (TMGMV) due to the presence of Tm-1 resistance gene that inhibits virus replication (Ishibashi et al. 2009). Similarly, certain Brassica species were found to be resistant to cauliflower mosaic virus (CaMV) due to lack of specific transcription factors (Covey et al. 1990).

Diverse crop cultivation is indispensable in small- and medium-scale farming (Bisht et al. 2020). This is to ensure the household food security and also to maximize the agricultural income. However, growing multiple crops alongside poses a threat of sudden crop loss due to the movement of disease from one crop to the other. Therefore, the success of diverse crop cultivation mainly depends on the selection of the crops. The insights from diverse crop cultivation studies, such as the one demonstrated in the current study, could prove valuable in selecting the crop plants with lower disease susceptibility and disease movement between the crops. Our findings discourage mixed cropping system with solanaceous and cucurbitaceous crops.

Genbank accession numbers

MT792926, MT799809, MW995971, MZ202353, MT826855, MW936636, MZ189975, MZ221828.

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Authors contribution GVNSMK conducted all the experiments, analyzed data, and wrote manuscript draft. ABN assisted GVNSMK for conducting experiments. NN designed the experiments, supervised data analysis and contributed to manuscript writing. TRG supervised the data analysis and wrote the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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