Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci.
https://doi.org/10.1007/s40011-017-0960-y

RESEARCH ARTICLE

Detection, Characterization and In-Silico Analysis of *Candidatus Phytoplasma australasiae* Associated with Big Bud Disease of Tomato in India

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Received: 27 February 2017 / Revised: 19 December 2017 / Accepted: 27 December 2017
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Abstract Tomato plants showing witches broom symptoms were collected from different states of India. The presence of phytoplasma infection was confirmed by PCR using phytoplasma-specific primer of 16S rRNA and SecY gene. The sequence analysis of 16S rRNA and SecY gene of eight tomato big bud phytoplasmas showed maximum nucleotide (nt) identity of 95–100% with Peanut WB group (16SrII). Further in-silico RFLP analysis of 16S rRNA gene of TBB-Pun1, TBB-Ban, TBB-mal, TBB-Guj and TBB-Vns showed similarity coefficient of 0.68–0.95. Since threshold similarity coefficient for classifying the phytoplasma into new subgroup is set at 0.97, the strain under study significantly distinct from the representative strains in the subgroups of pea nut witches broom. Further, the phylogenetic analysis of tomato big bud phytoplasmas revealed that, they are closely clustered with peanut witches'-broom strains (16Sr II), specifically within the 16Sr II-D and 16Sr II-A subgroups. A comprehensive recombination analysis showed the evidence of both intra and inter-species recombination in seven tomato big bud isolates with most part of their 16Sr RNA F2nR2 fragments descending from Ca.P.brasiliense (16Sr XV) as major parent, except isolate TBB-Vns which had an intra species recombination with Cactus witches-broom-16Sr II-L as major parent. Similarly, in case of SecY gene, all the seven isolates have intra-species recombination with major portion descending from Vinca virescence-[16Sr VI-A] and Potato purple top wilt-[16Sr XVIII-B]. The genetic similarities and the potential threat of this new phytoplasma belonging to 16Sr II group of Peanut witches' broom' group infecting tomato in India are discussed.

Significance statement The phytoplasma associated with tomato big bud disease was characterized by Electron microscopy, PCR, in silico RFLP and recombination analysis showed that five new subgroup and three already existing tomato big bud phytoplasma were identified, which gives a good alarm signal to breed resistance varieties against big bud phytoplasma in India.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s40011-017-0960-y) contains supplementary material, which is available to authorized users.

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Keywords Tomato · Phytoplasma · India · Pea nut witches broom disease 16SrII · Polymerase reaction (PCR)

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the important vegetable crop grown throughout the country under diverse agro climatic conditions. The crop is prone to many fungal, bacterial and viral diseases. Among these, big bud disease of tomato caused by phytoplasma and transmitted by different phloem sap feeding insects, particularly leafhoppers,

Published online: 12 January 2018

planthoppers and psyllids is an emerging threat in almost all tomato growing states of India. In India, the disease was first reported on tomato [1]. Based on the expression of phenotypic symptoms on tomato plant, the phytoplasma is named as big bud or tomato dwarf or tomato stunt or stolbur phytoplasma. Researchers from different parts of world reported that, tomato big bud is caused by phytoplasmas belonged to different taxonomic groups of Omar and Foissac [2]. Among them phytoplasma related to peanut witches broom group (16SrII) known to infect many cultivated plants as well as weeds [3–6] is more prevalent on tomato. An effective approach to detect and classify phytoplasma targeting a highly conserved region of 16S rRNA, the spacer region between 16S rRNA and 23SrRNA is well documented [7–9]. Further, several phytoplasma strains have also been classified based by in-silico analysis of 16S rRNA restriction fragment length polymorphism (RFLP) pattern [10]. The phytoplasma belonging to the taxonomic group of 16SrII is known as *Candidatus Phytoplasma australasiae* [11] and is associated with diseases in economically important crops globally.

With this background, the survey was undertaken in the different tomato-growing regions of India during 2011–2013 for the incidence of tomato big bud disease and attempt was made to characterize the phytoplasmas associated with tomato big bud disease in India.

Material and Methods

Survey and Collection of Phytoplasma Isolates

The roving survey was conducted during 2011–2013 in tomato farmer fields of Punjab (Ludhiana), Karnataka (Bangalore and Malur), Gujarat (Junagadh), Tamil Nadu (Coimbatore) and Uttar Pradesh (Varanasi and Mirzapur) states, of India, to estimate the incidence of big bud disease of tomato. The incidence of big bud disease was estimated visually by counting the number infected plants moving diagonally across the field from one individual plant to the next.

Sample collection was restricted to plants showing typical big bud phytoplasma-like symptoms, since the aim of the study was to type the phytoplasmas present in tomato fields. The infected plant sample along with the non-symptomatic sample were collected from the tomato fields from each location. The isolates collected from some places were more than one as the infected plants were showing distinctive phytoplasma-like symptoms. The collected isolates were designated as TBB-Blr, TBB-Mal (from Karnataka), TBB-Coi (from Tamil Nadu), TBB-Pun1, TBB-Pun2 (from Punjab), TBB-Guj (from Gujarat), TBB-Mz and TBB-Vns (from Uttar Pradesh).

Transmission Electron Microscopy

To examine the presence of phytoplasma in the infected tissues of tomato plants, tissues were fixed in 4% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and washed with buffer several times. The tissues were dehydrated in a graded ethanol and thin sections (50 nm) were prepared using an Ultratome 2128 microtome (LKB) and stained with uranyl acetate and lead citrate as described by Amaral-Mello et al. [12]. The ultra-thin sections were examined by JEOL 2000 EX transmission electron microscope at 25 kV.

PCR Amplification of 16S rRNA and SecY Gene of Tomato Big Bud Phytoplasma

DNA Extraction

Total nucleic acid was extracted from eight tomato big bud phytoplasmas infected and healthy tomato samples by CTAB method [13]. PCR amplification of 16S rRNA gene was done by using universal primer pairs P1/P7 [7, 14] followed by nested PCR with R16F2n/R2 primers [9]. Further, SecY gene of big bud phytoplasm was amplified by SecYF2 (II) and SecYR1 (II) [15]. DNA amplification was performed [16] and amplified products 16S rRNA and SecY gene were purified, cloned and selected clones were sequenced with automated sequencing ABI PRISM 3730 (Applied Biosystems) from Eurofins Genomics India Pvt. Ltd (Karnataka, India).

Sequence Analysis

To assess the taxonomic positions of tomato big bud phytoplasma isolates, full length 16Sr RNA and SecY gene sequences derived were queried using iPhyClassifier online tool [17]. Searches for sequence similarity to the available sequences in the database were performed using BlastN (http://www.ncbi.nlm.nib.gov/). The sequences showing highest scores with the present isolates were obtained from database and aligned using SEAVIEW program [18]. The sequence identity matrixes for the big bud phytoplasmas were generated using Bioedit Sequence Alignment Editor (version 5.0.9) and phylogenetic tree was generated by MEGA7 software [19] using the neighbour joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously. Split-decomposition trees were constructed with 1000 bootstrap replicates based on parsimony splits implemented in SplitsTree version 4.11.3 with default settings [20]. Recombination analysis was carried out using the Recombination detection program (RDP4), GENECOV, Bootscan,

Max Chi, Chimara, Si Scan, 3Seq which are integrated in RDP4 [21].

In-Silico Enzyme Digestion

In-silico restriction enzyme digestion of F2n/R2 fragment and virtual gel plotting was done using online iPhyClassifier tool [22]. The restriction enzymes prescribed for the classification of phytoplasma 16Sr RNA gene into different groups and subgroups on the basis of RFLP analysis were employed [10]. After in silico restriction digestion, a virtual 3.0% agarose gel electrophoresis image was generated and these virtual PCR–RFLP patterns were used for finer differentiation of tomato phytoplsama isolates from the existing members of the peanut witches-broom group (16SrII).

Results and Discussion

Disease Incidence

The field survey was undertaken to know the incidence of big bud phytoplasma disease in tomato at five locations comprising different states (Punjab, Karnataka, Gujarat, Tamil Nadu and Uttar Pradesh) of India. During survey, the tomato plants showing the distinctive phytoplasma-like symptoms such as bushy appearance, intense proliferation of laterals buds and plant apices generally lacking leaves and very small, thick and distorted youngest leaves was recorded (Fig. 1). Similar symptoms caused by phytoplasma on fruits, vegetables, cereals and trees crops were reported in earlier studies as well [22]. Further, the disease incidence was calculated by counting the number plants infected over healthy plants in each filed and expressed as Per cent disease Index. The disease incidence varied between fields at different locations and ranged between 10 and 15% in Punjab, 15–20% in Karnataka, 12–15% in Gujarat, 18–20% in Tamil Nadu and 10–15% in Uttar Pradesh. Infected samples along with the non-symptomatic samples from the tomato fields in each location were collected and used for further characterization.

Fig. 1 Tomato plant showing bid bud disease symptoms under natural conditions

Electron Microscopy

Ultra thin sections of petiole tissues from phytoplasma infected tomato plant samples under electron microscopy showed presence of the phytoplsama in all infected samples. The numerous pleomorphic bodies (phytoplasma) in the sieve elements of xylem cells, phloem parenchyma cells and companion cells were present. Phytoplasma units lacked cell wall and were bounded by unit membrane. These bodies looked like rounded, elongated or pleomorphic structures that contained ribosome-like granules measuring about 200–400 nm in size. The phloem cells were completely filled with phytoplasma (Fig. 2). No phytoplasma-like corpuscles were observed in the phloem vessels of leaf samples from healthy plants.

PCR Amplification

The classification of phytoplasmas based on the highly conserved 16S rRNA gene does not always provide the molecular distinction. Therefore, for the finer differentiation of phytoplasma group, the most useful and reliable taxonomic information is provided by the sequencing of both 16S ribosomal gene with SecY gene. Phytoplasma 16S rRNA gene ($\sim$ 1.8 kb size) was amplified from eight tomato big bud diseased samples collected from five different geographical locations of India, using universal primer pairs P1/P7 specific to 16S rRNA region of phytoplasma [8, 9]. The PCR products obtained were re-amplified in the nested PCR using primers R16F2n/R16R2, which yielded a strong PCR amplicon of approximately 1.2 kb DNA fragment (Fig. 3a). Similarly, the SecY gene

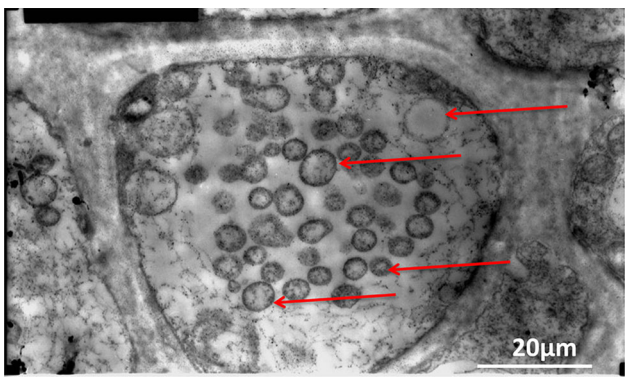


Fig. 2 Transmission electron micro graph of tomato phloem tissues infected with tomato big bud plants containing phytoplasma having shape of pleomorphic units scattering inside the phloem elements

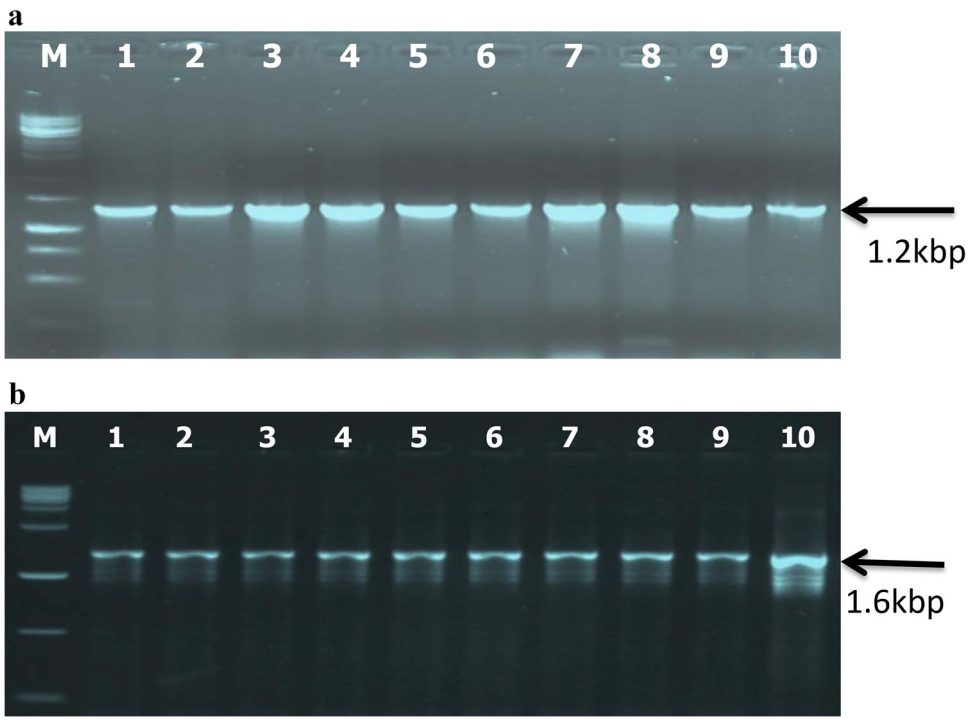


Fig. 3 Amplification of phytoplasma 16S rRNA gene in tomato big bud samples by **a** N-PCR using the primers R16F2n/R16R2 (1.2 kb products) and **b** SecY gene primers SecYF2 (II)/SecYR1 (II) (1.6 kb products) from infected plants. M 1 kb marker (MBI Fermentas Life Sciences, Germany), Lane 1—TBB-BLR, lane 2—TBB-Mal, lane 3—TBB-coi, lane 4—TBB-Pun1, lane 5—TBB-Pun2, lane 6—TBB-Guj, lane 7—TBB-MZ, lane 8—TBB-Vns, lane 9 positive sesame sample; lane 10 positive brinjal little leaf sample. Phylogenetic tree based on sequences of **c** 16S rRNA and **d** SecY gene from Tomato big bud phytoplasma with other phytoplasma strains using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches

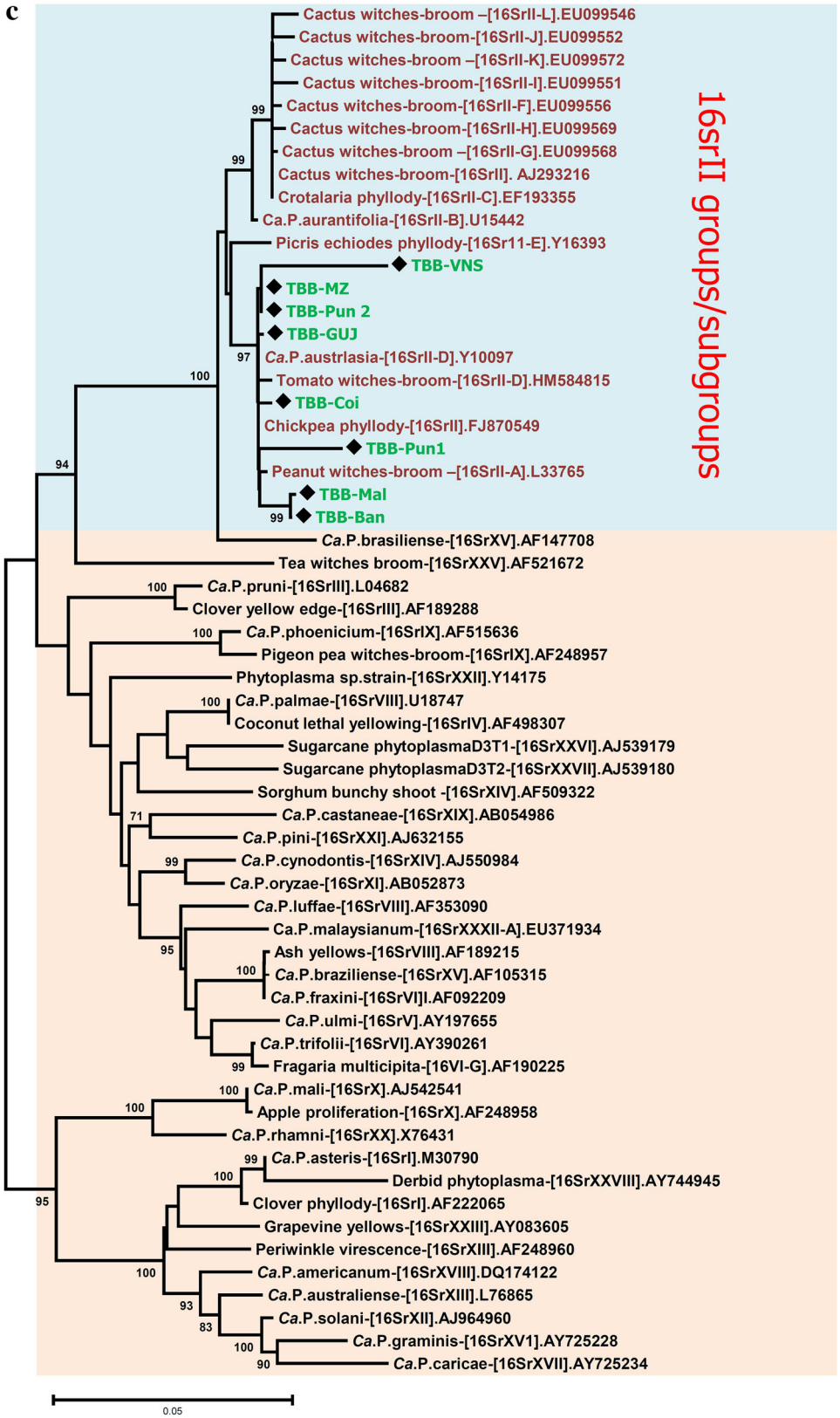
of tomato big bud phytoplasma isolates was amplified by using the primer pair SecYF1/SecYR1 [15]. The resulted PCR amplicon of $\sim$ 1.6 kbp size was corresponding to the SecY region of phytoplasma (Fig. 3b). There was no amplification in water and the healthy tomato samples which served as negative controls. Sesame and brinjal phyllody phytoplasma DNA was used as positive control. The amplified PCR products of phytoplasma isolates from infected tomato plants were subsequently cloned and sequenced.

16S rRNA and SecY Gene Sequence Analysis of Tomato Big Bud Phytoplasma Isolates

The alignment of nucleotide sequences (16Sr RNA and SecY gene) of eight tomato big bud phytoplasma isolates (TBB-Blr, TBB-Mal, TBB-coi, TBB-Pun1, TBB-Pun2, TBB-Guj, TBB-Mz, TBB-Vns) collected from different locations of India revealed that, they shared the nt identity of 94.2–99.8% in 16Sr RNA and 97.8–99.5% in SecY gene among themselves and the sequences are available in the database under Accession Numbers of KF700075-82 (16S rRNA) and KT970078-84, KF700077 (TBB-BLR) (SecY gene).

Comparison of 16S rRNA and SecY Gene of Tomato Big Bud Phytoplasma Isolates with Other Phytoplsama

F2nR2 primed fragment of 16Sr RNA gene sequences of eight tomato big bud phytoplasma isolates under study were compared with the corresponding region of 51 different groups of phytoplasma retrieved from the database (Table S1a). The eight big bud phytoplasma isolats showed maximum nt identity of 95–100% with the Peanut WB group (16Sr II) and subgroups namely *Ca. P. australasiae* (Y10097), Chickpea phyllody (FJ870549), Tomato witches-broom (HM584815), Peanut witches-broom (L33765), Picris echiodes phyllody (Y16393), Cactus witches-broom (EU099552) and Ca.P.aurantifolia (U15442) (Table S2a). In contrast, big bud phytoplasma under investigation showed 89–90% identity with the other different groups of phytoplasma. These results were well supported by a phylogenetic analysis showing the big bud



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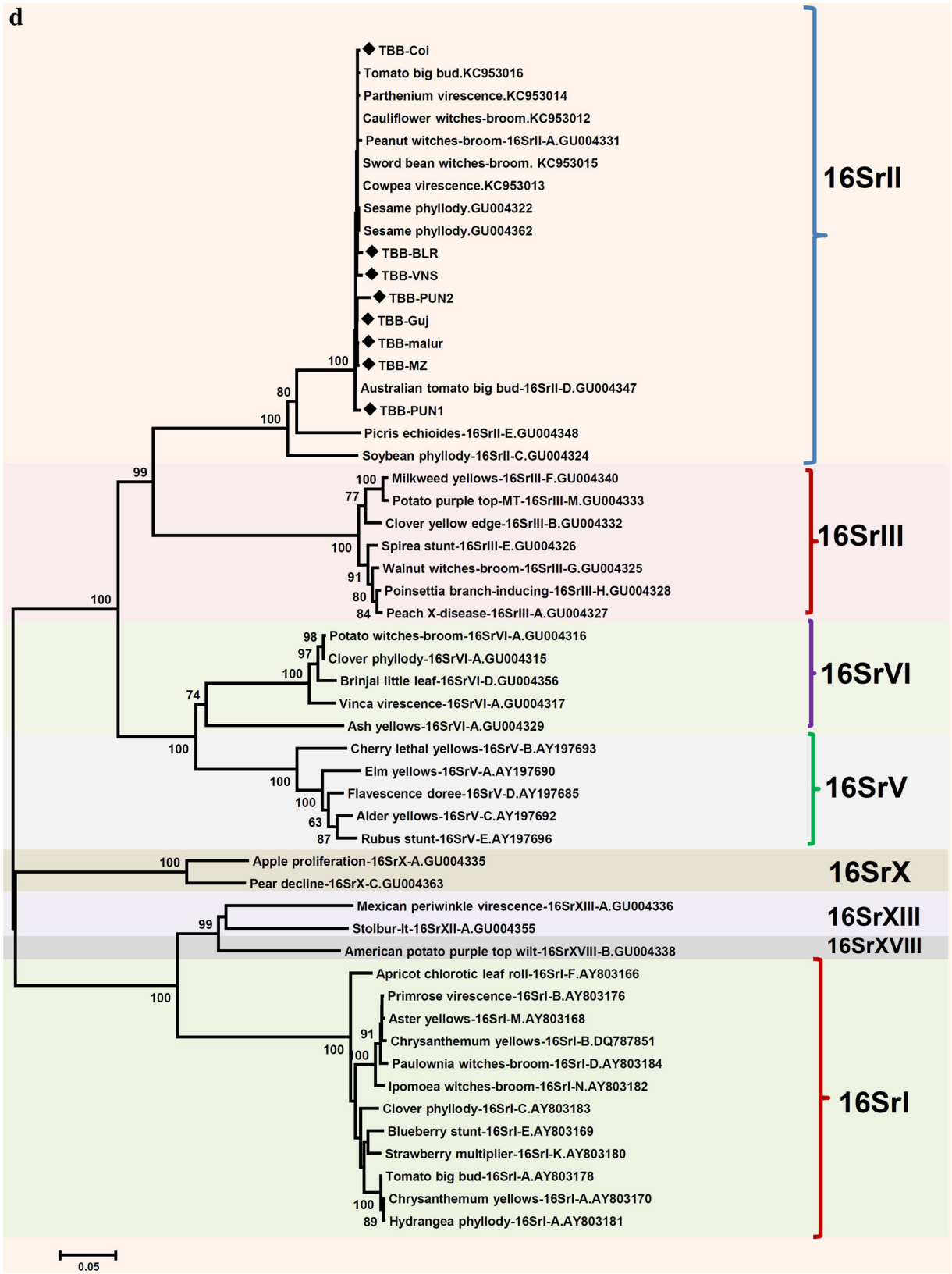

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phytoplasma isolates forming separate group with the above mentioned phytoplasma within the 16SrII Peanut WB group. The tomato big bud phytoplasmas isolate TBB-Coi (Tamil Nadu), TBB-Guj (Gujarat), Pun1 (Punjab), TBB-Mz (Mirzapur) and TBB-Vns (Varanasi) are in the clade of *Ca. P. australasiae* (Y10097) and Tomato witches broom (HM584815) specifically within the subgroup 16SrII-D. The other phytoplasmas isolates TBB-Pun2 (Punjab), TBB-BLR and TBB-Mal (Karnataka) were closely clustered with peanut witches'-broom strains (16SrII), specifically within the subgroup 16SrII-A (Fig. 3c).

Similarly, SecY gene sequences of eight tomato big bud phytoplasma isolates were compared with the corresponding region of 47 different groups of phytoplasma [20] (Table S1b). The analysis showed that eight big bud phytoplasmas isolates in the current study (TBB-Blr, TBB-Mal, TBB-coi, TBB-Pun1, TBB-Pun2, TBB-Guj, TBB-Mz, TBB-Vns) showed highest nucleotide identity of 98.2–99.4% with Tomato big bud (KC953016), 93.1–94.5% with Sesame phyllody (GU004362) and 93.1–94.1% with Australian tomato big bud (GU004347) phytoplasms belong to 16SrII Peanut WB group (Table S2b). In contrast, eight big bud phytoplasma isolates showed less than 82.9% identity with the other members of different groups. These results were well supported by phylogenetic analysis, showing the SecY gene of eight phytoplasma isolates (TBB-Blr, TBB-Mal, TBB-coi, TBB-Pun1, TBB-Pun2, TBB-Guj, TBB-Mz, TBB-Vns) closely clustering with Tomato big bud (KC953016), Sword bean witches broom (KC953015), Sesame phyllody (GU004362, GU004322) and Australian tomato big bud (GU004347) phytoplasmas belonging to 16SrII Peanut WB group (Fig. 3d). The analysis showed Indian tomato infecting phytoplasmas forming a monophyletic cluster with Asian-Australasian origin phytoplasmas and thereby establishing a close relationship between 16SrII-A and 16SrII-D.

A consensus for naming novel phytoplasmas was recommended by the IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group that a 'Candidatus Phytoplasma' species description should refer to a single, unique 16S rRNA gene sequence that has 97.5% similarity to that of any previously described 'Ca. Phytoplasma' species' [23] and [24]. Based on the analysis of 16S rRNA and SecY gene, the identified tomato big bud phytoplasma isolates in the present study belonged to the Peanut WB group especially with subgroups 16SrII-A and 16SrII-D. This is the first report of subgroup 16SrII-A and 16SrII-D of tomato big bud phytoplasma belonging to the Peanut witches-broom group from India. Similarly, diverse groups of phytoplasma associated with tomato big bud disease have been identified and they belonged to 16SrI, 16SrII, 16SrV, 16SrXII and 16SrVI groups/sub groups [2, 25–27].

In-Silico Analysis of 16Sr RNA Sequences

Results obtained from iPhyClassifier analysis of virtual RFLP patterns of the 16S rRNA gene of eight tomato big bud phytoplasma isolates revealed that TBB-Pun1 (coefficient of similarity 0.87), TBB-Blr (0.94), TBB-Guj (0.95) and TBB-Vns (0.68) represents a previously un-described subgroup in the group 16SrII. They exhibit restriction patterns different from (Fig. 4a–c, e), that of the 16S rRNA gene from subgroup 16SrII-D (Accession No. Y10097, *Ca. P. australasiae*). However, the isolate TBB-Mal (0.92) exhibited restriction pattern different from that of the 16S rRNA gene from subgroup 16SrII-A (Accession No. L33765, Peanut witches' broom), while isolates TBB-Coi, TBB-Mz and TBB-Pun2 exhibited similar restriction pattern to that of *Ca. P. australasiae* (Accession No. Y10097, similarity coefficient of 1.00) (Fig. 4d). Based on the threshold similarity coefficient for new subgroup, delineation should be set at 0.97 [10, 27]. Therefore, tomato big bud phytoplasma isolates (TBB-Pun1, TBB-Ban, TBB-Mal TBB-Guj and TBB-Vns) showing similarity coefficient less than 0.97 may be considered as new subgroup under 16SrII Peanut WB group. Enzymes which distinguish the different tomato big bud phytoplasma isolates are HaeIII and MseI (TBB-Pun1), HaeIII and HpaI (TBB-BLR and TBB-mal), HpaII and MseI (TBB-Guj) (Fig. 4a–c) and AluI, BfaI, BstUI, HpaI, MseI, RsaI TaqI (TBB-Vns) (Fig. 4e). The RFLP patterns of each phytoplasma were highly conserved. The unknown phytoplasmas can be identified by comparing the patterns with the available RFLP patterns of known phytoplasmas [10, 24]. Generally, it has been accepted that even one restriction site difference (within the 16S rRNA gene F2nR2 region) between a phytoplasma strains from previously established subgroups may be considered as new subgroup [28]. Therefore the TBB-Pun1, TBB-Ban, TBB-Mal, TBB-Guj and TBB-Vns may be considered as new subgroups under 16SrII Peanut WB group.

Neighbor-Net and Recombination Analysis of 16S rRNA and SecY Gene

The neighbor-net analysis (using split tree program) of 16S rRNA and SecY gene of phytoplasma isolates under the present study with other groups of phytoplasma revealed the extensive network of evolution in 16SrII groups/subgroups, indicating the occurrence of recombination (data not shown). The split decomposition analysis showed a "rectangular" network structure, thereby exhibiting distant relationship of big bud phytoplsama belonging to 16SrII groups/subgroups and SecY gene with all other groups of phytoplasmas. It was also evidenced by the phylogenetic analysis and the split graph.

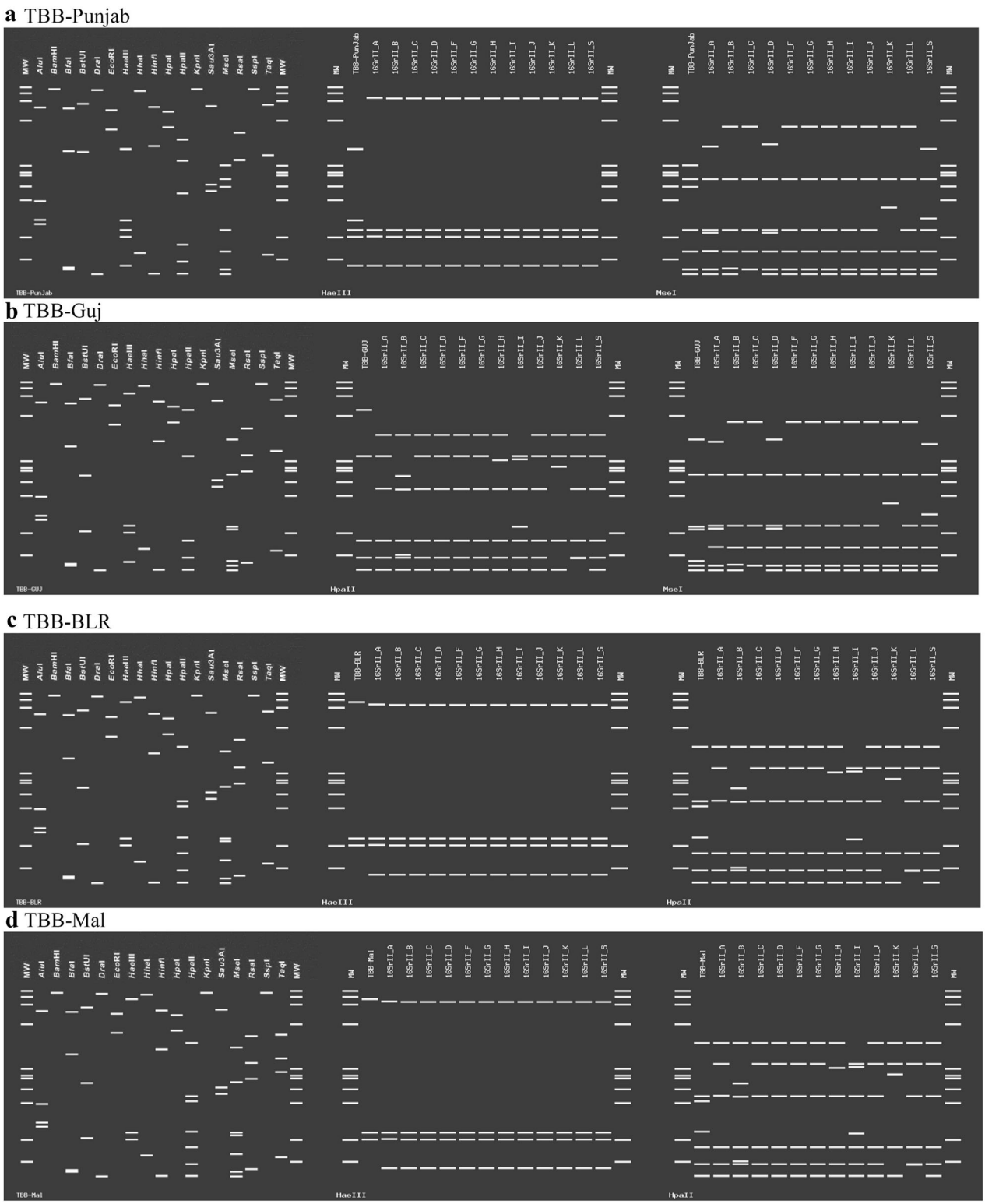

Fig. 4 Virtual RFLP patterns derived from in silico digestions, using iPhyClassifier, of F2n/R2 fragments of 16S rRNA gene from strains of **a** TBB-Pun1 (Accession No. KF700075), **b** TBB-Guj (Accession No. KF700078), **c** TBB-Blr (Accession No. KF700077), **d** TBB-Mal (Accession No. KF700082), **e** TBB-Vns (Accession No. KF700080) using 17 restriction endonuclease enzymes (left): AluI, BamHI, BfaI, BstUI, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, RsaI, SspI, and TaqI. Virtual RFLP patterns of TBB-Pun and TBB-Guj (HaeIII and MseI), TBB-Blr and TBB-Mal (HaeIII and HpaI) and TBB-Vns (AluI, BfaI, BstUI, HpaI, MseI, RsaI TaqI) to distinguishing strain Indian phytoplasmas from other strains in group 16SrII. The restriction fragments were resolved by in silico electrophoresis through 3% agarose gel. MW, WX174 DNA-HaeIII digest

e TBB-VNS

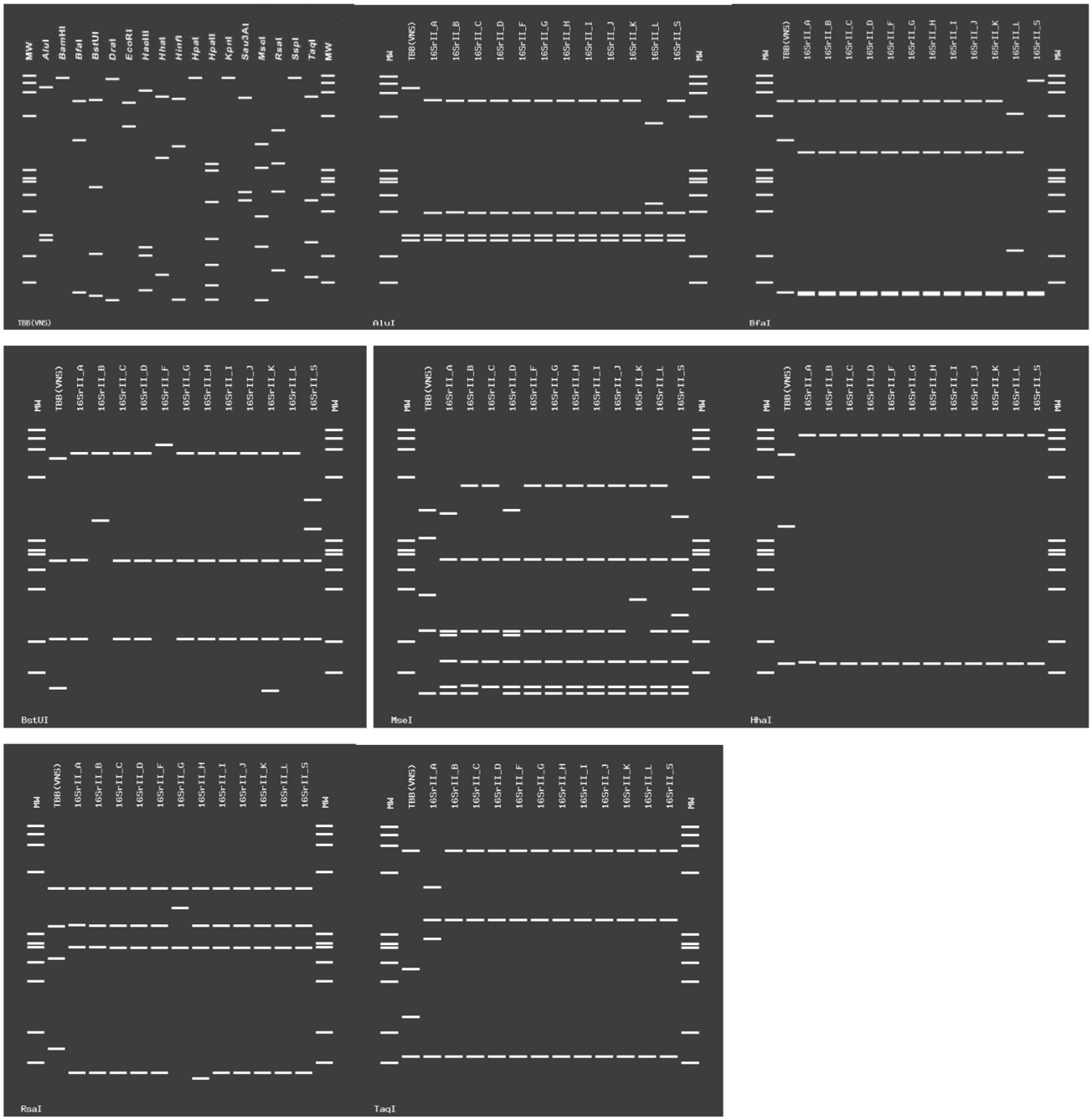

Fig. 4 continued

A comprehensive analysis of recombination using RDP4 showed the evidence of inter as well as intra species recombination in eight tomato phytoplasma isolates with most part of their 16S rRNA F2nR2 fragments being descendent from Ca.P.brasiliense (AF147708), except in TBB-VNS isolate which had an intra-species recombination with Cactus witches-broom (EU099546) (Fig. 5a, Table S2a). Similarly, in case of SecY gene, all the seven isolates had intra-species recombination with other phytoplasmas with major portion of their SecY gene being descendent from Vinca virescence (GU004317) and Potato purple top wilt (GU004338) (Fig. 5b, Table S2b). The recombination analysis suggested that tomato big bud phytoplasma isolates have obtained at least some of its sequence by recombination from Ca.P.brasiliense (16SrXV), Cactus witches-broom (16SrII) for 16S rRNA, Vinca virescence-[16SrVI] and Potato purple top wilt-[16SrXVIII] for SecY like ancestors, which had only been

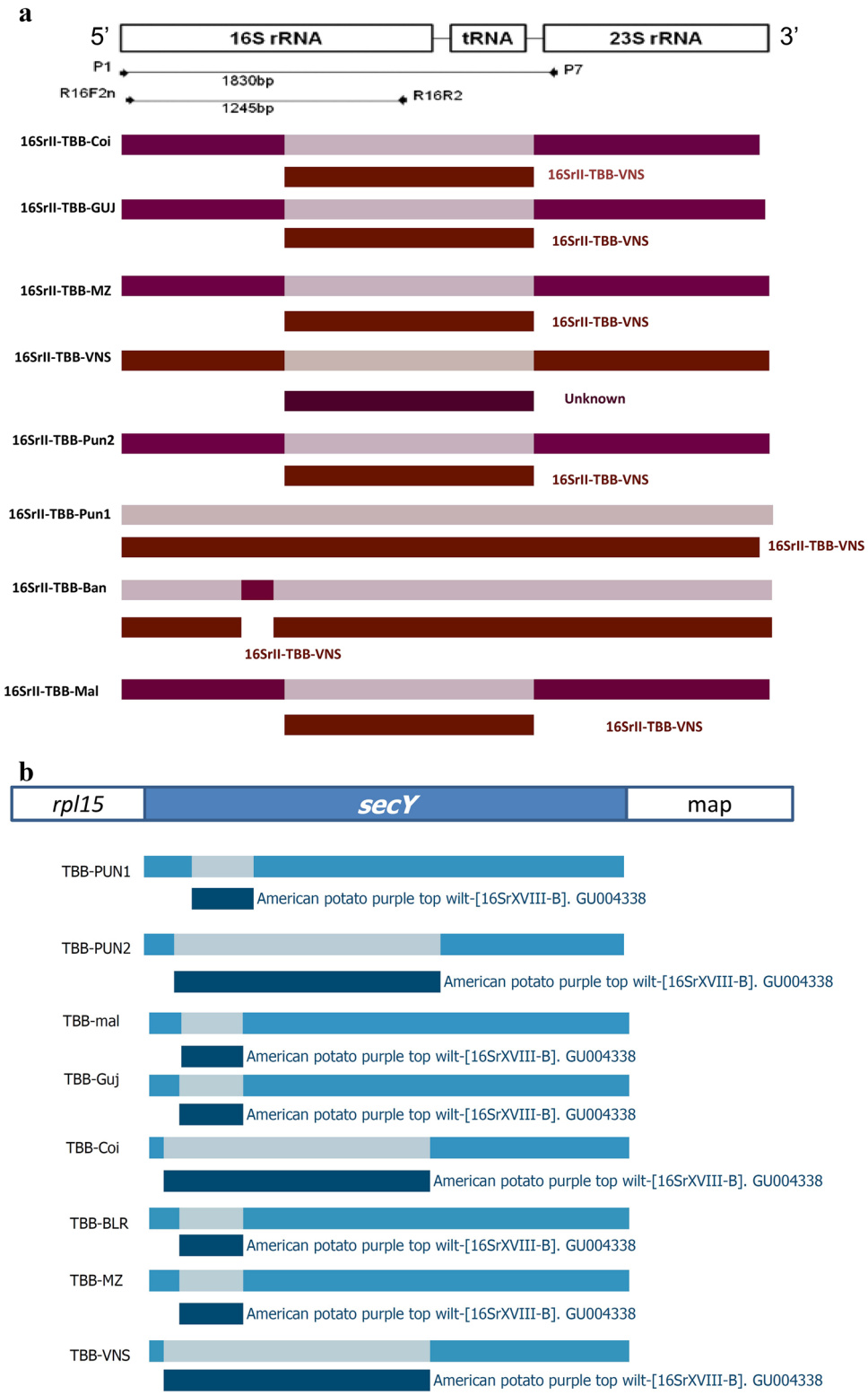

Fig. 5 Analysis of recombination of 16S rRNA (**a**) and SecY gene (**b**) of phytoplasma isolates (TBB-Pun1, TBB-Ban, TBB-Guj, TBB-Mal, TBB-Vns, TBB-Coi, TBB-MZ and TBB-Pun2) from tomato: The phytoplasma acronyms given are: Tomato big bud Phytoplasma (TBB), American potato purple top wilt-[16SrXVIII-B]. Sequence of indeterminate origin is indicated as "unknown". The bars below the isolate name indicate their genome and the boxes below this with phytoplasma acronyms indicate the approximate position at which recombination has occurred in the genome of phytoplasma isolates

reported from China, Brazil and USA [21] but not from India. This suggested that recombination between the parents of tomato big bud phytoplasma isolates either occurred before introduction to India or tomato big bud phytoplasma isolates are present in the country but is yet to be identified. Recombination is a major mechanism in creating genetic diversity in phytoplasmas and has played a key role in the evolution of wild-type line (OY-W) and mild-symptom line (OY-M) of onion yellows phytoplasma [28].

Conclusion

The study highlights the identification of new strains of tomato big bud phytoplasma from different locations of India. In order to provide a better picture of the pathogenic as well as genetic divergence among tomato big bud phytoplasma from India, there is a need to conduct similar holistic investigation among higher number of tomato big bud phytoplasma isolates which could be helpful to generate resistant material against tomato big bud phytoplasma in tomato.

Acknowledgements The research was supported by the project "*Consortium platform on Vaccines and diagnostics*" funded by Indian Council of Agricultural Research (Grant No. F.No.AS/2/8/2016-ASR-IV), Government of India, New Delhi, India.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest to publish this manuscript.

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