SUMMARY AND CONCLUSION

The present study is reporting the molecular detection and sequence analysis of ‘Ca. L. asiaticus’ associated with citrus greening and citrus witches’ broom diseases in three citrus growing Indian states viz., Maharashtra, Uttar Pradesh and Assam. Although the geographical areas were well known for citrus cultivation, the recent estimates revealed the noticeable decline in citrus production of these states. The present survey reflects that the conditions of citrus orchards in the regions are becoming poor day by day and most of them are already at its devastating stage. Based on symptoms observations, HLB incidences in all three states were in range of 2.5 – 54.3 per cent. The disease was predominated in all citrus cultivars, with highest incidences in Mosambi sweet orange cultivar whereas least incidences in acid lime. Variable symptoms including mottling, severe chlorosis with green veins, pale-green coloring of young leaves, zinc deficiency like symptoms, vein yellowing, general yellowing and chlorotic leaves with scattered green spots i.e., green island in severe cases were observed in different citrus cultivars. However it was difficult to visually screen HLB symptomatic plants in the field as many of the surveyed orchards were highly neglected and most of the plants were confused with nutrient deficiency symptoms. The existence of insect vectors of psyllid (Diaphorina citri Kuwayama) was very common especially in sweet orange orchard; which must be the cause for high disease incidence in the all surveyed regions.

The study revealed existence of ‘Ca. L. asiaticus’ in all surveyed geographical states of India as PCR amplification was obtained with the primer set OI1/OI2c and not with OA1/OI2c and GB1/GB3, which were previously reported for detection of ‘Ca. L. africanus’ and ‘Ca. L. americanus’, respectively. Similarly, ‘Ca. L. asiaticus’ specific fragment (703 bp) on amplification of rplA-rplJ gene with primer set A2/J5 supported the findings; otherwise the primer amplifies 669 bp size fragments for ‘Ca. L. africanus’ strain. Also the maximum identity on BLAST analysis of amplified 16S rDNA and rplA-rplJ gene sequences proved the existence of ‘Ca. L. asiaticus’ only. Unlike to these two genes, 16S/23S intergenic region could not been able to distinguish between ‘Ca. L. asiaticus’ and ‘Ca. L. americanus’ strains. Beside its same size amplified fragment for all strains, the sequence similarity between ‘Ca. L. asiaticus’ and ‘Ca. L. americanus’ strains was the major concerned for the region. Additionally, the phylogenetic study of the intergenic region was even confusing, as all ‘Ca. L. africanus’ could
not been formed a separate clade (Figure 4.23). Although the intergenic 16S/23S rRNA spacer region was known to be highly variable at both the interspecies level and the intra species level; the results signifies that the 16S/23S region in the genus “Candidatus Liberibacter” does not vary much within a given species (Figure 4.20, 4.23).

In present analysis, two new lineages: 16Sr-XV and 16Sr-XVI have been added in to the fourteen 16Sr SNP lineages (16Sr-I to16Sr-XIV) (Table 4.5). Although the new lineages have been claimed based on single nucleotide variation at one position for each lineage, respective SNPs have been detected in two distinct ‘Ca. L. asiaticus’ strains for separate lineage, i.e. 16Sr-XV in ‘ALD-AN’ and ‘AN4’, while 16Sr-XVI in ‘AN3’ and ‘AN4’. Additionally, the phylogenetic analysis supported our claim as both the new lineages appeared in separate subclade (Figure 4.21), whereas ‘MA3’ clubbed in 16Sr-VI lineage. The 16S region was able to clearly differentiate among the three ‘Ca. L. asiaticus’, ‘Ca. L. africanus’ and ‘Ca. L. americanus’ subgroups and should be a clear target to distinguish between the different HLB isolates from all over the world. Likewise in rplA-rplJ gene, ‘Ca. L. asiaticus’ strains ‘MA3’, ‘AN4’ and ‘AN5’ grouped in β-rp SNP lineage II, whereas ‘ALD-AN’ and ‘AN3’ were claimed in new lineage IV with single nucleotide variation at two positions (Table 4.6). Here again, distinct subclade for ‘Ca. L. asiaticus’ strains ‘ALD-AN’ and ‘AN3’ in phylogeny authenticated the reported new lineage in the study (Figure 4.22).

The extent of genetic variations among twenty six HLB isolates at the loci CLIBASIA_01645 a hyper variable region of the pathogen genome was found comparatively much better than same at the loci 16S rDNA, 16S/23S intergenic region, and rplA-rplJ gene. The availability of first complete genome of Ca. L. asiaticus strain Psy62 allowed researchers to study the TRN variation at the region for first time. In the course of time few more complete genomes from ‘Ca. L. asiaticus’ strains has been reported, however the CLIBASIA_01645 was tagged different as ‘WSI_01555’ in strain ‘gxpsy’ (CP004005) and ‘CGUJ_01645’ in ‘Ishi-1’ strain (AP014595), with the same characteristic TRN variability. In present study, our Indian ‘Ca. L. asiaticus’ isolates were compared with these three available ‘Ca. L. asiaticus’ complete genomes (Figure 4.18). Based on TRNs Ca. L. asiaticus has been classified as Class I of TRNs ≤5, Class II of TRNs >5 but ≤ 10, Class III of TRNs > 10 but ≤ 15, and Class IV of TRNs > 15. It was observed that the Class II genotype was most predominant followed by the Class I
genotype, and the least predominant was Class IV genotype. Maharashtra state, particularly the northern Maharashtra region, was the only state with Class IV genotype (Figure 4.17, Table 4.7). Moreover, the representative isolates from different citrus species from the same region of western Maharashtra were considered for in silico RFLP which have also shown noticeable variations in analysis.

Regarding witches’ broom disease incidences, the disease was found only in Maharashtra region and highest incidence was reported in Nagpur area. It was observed that the disease is restricted to only acid lime cultivar. Two types of symptoms were common in the surveyed area, *via* typical witches’ broom symptoms, and rubbery wood like symptoms (Figure 4.11 and 4.13). Moreover, PCR based molecular detection of phytoplasma – induced witches’ broom disease of citrus was done using phytoplasma universal nested PCR primer sets R16F2n/R16R2, R16mF2/R16mR1 and fU5/rU3. Consistent and reproducible amplifications observed with R16F2n/R16R2 and fU5/rU3 primer sets suggest that these primer sets can be routinely used for the diagnosis of citrus phytoplasma in India. BLAST analysis of JQ808143 sequence confirmed the *Candidatus* Phytoplasma spp. as a causal organism for the witches’ broom disease of acid lime in India. Thus the present study reports rapid and sensitive molecular diagnosis by PCR of causal *Candidatus* Phytoplasma infecting acid lime surveyed region. Further molecular evaluation to identify the nature phytoplasma to the species and group has to be confirmed.

Summing up, major citrus growing areas in three different states were surveyed for HLB and WBDL incidences. For genetic evaluation of HLB four genomic loci of selected ‘*Ca. L. asiaticus*’ isolates were analyzed. The 16S rDNA and *rplA-rplJ* gene were found suitable for detection of *Ca. Liberibacter* subgroup, while 16S/23S intergenic region does not vary much among the subgroups and should not be considered for characterization. The loci ‘CLIBASIA_01645’ was comparatively more variable and hence should be the choice to study genetic variability among ‘*Ca. L. asiaticus*’. The single nucleotide variations at 16S rDNA and *rplA-rplJ* genes of north Indian ‘Ca. L. asiaticus’ has added two 16Sr SNP lineages and one β-rp SNP lineage to earlier reported respective lineages. In case of rapid molecular diagnosis of WBDL, conventional PCR was not recommended and separate nested PCR with three different primer sets were carried out. Out of these three primer sets R16F2n/R16R2 and fU5/rU3 found suitable and was recommended for routine WBDL detection of Indian isolates.
Conclusion

On concluding remark, nine districts from three different states viz. Maharashtra, Uttar Pradesh and Assam were surveyed for the incidences of citrus greening (HLB) and citrus witches’ broom disease. The HLB incidences in all surveyed region were in the range of 2.5 to 54.3 per cent, with highest incidences in Mosambi sweet orange cultivar. Variable characteristic HLB-like disease symptoms were observed in different citrus cultivars. The pathogen was also observed in insect vector psyllid *Diaphorina citri* Kuwayama. Comparatively, citrus witches’ broom disease incidences were less and found only in Maharashtra region. The percentage disease incidence of the disease was ranged between 2.9 to 6 per cent. The different isolate cultures of both the diseases were separately maintained in the polyhouse.

The HLB isolates were subsequently tested by PCR amplification with different primer sets targeting 16S rDNA, 16S/23S rDNA intergenic regions, *rplA*-rplJ gene and CLIBASIA_01645 loci of HLB pathogen. The PCR products were cloned and sequenced in both the orientations. WBDL isolate cultures established in glass house were also subsequently screened using different set of phytoplasma specific universal primers by nested PCR. The presence of phytoplasma was confirmed in all six established cultures.

With the help of different bioinformatics tool, molecular evolution of 16S rDNA, 16S/23S rDNA intergenic regions, *rplA*-rplJ gene and CLIBASIA_01645 loci of HLB pathogen was done. The region 16S rDNA was found to be best to detect citrus greening casual pathogen and can be used to distinguished at species level, whereas genomic regions 16S rDNA, *rplA*-rplJ and CLIBASIA_01645 can be used to distinguish ‘*Candidatus Liberibacter asiaticus*’ at strain level.

Future prospects

Citrus greening disease and citrus witches’ broom disease are two important graft and vector transmissible diseases responsible for low citrus production and productivity in the country. The present study documents about the recent status of HLB, a serious century old problem and WBDL, a newly emerging threat to the Indian citrus industry. It also provides a robust framework for understanding the pathogens and their variations at its genomic level. However, more similar work has to be carried out for the isolates from other citrus growing belts.
of India to get a better picture of the situation at national level in future. Till date, limited information about the causative pathogens of citrus greening disease and citrus witches’ broom disease from India is available. Complete genome sequencing of Indian strains of ‘Ca. L. asiaticus’ would be an important task for future research. Similarly, work should also be focused on control strategies targeting genetic manipulation of both the pathogens and insect vectors that are responsible for field spread of these diseases. After proper diagnosis, infected plants in the orchard need to be removed and destroyed so that the pathogen may not spread to nearby healthy plants. Similarly, PCR based molecular diagnostic tools should be used for implementing citrus budwood certification program in all major citrus growing belts of the country to produce and distribute virus-free certified planting materials to the growers.