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Cross amplification of 16S rRNA bacterial primer 27F/1492R on horticultural crop chloroplast genome

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ABSTRACT

Molecular techniques have been applied as fast and accurate detection methods of plant pathogenic bacteria. Bacteria-specific primer 27F paired with the universal primer 1492R was used in PCR to detect plant pathogenic bacteria in symptomatic leaves of tomato (Solanum lycopersicum L.), chili pepper (Capsicum annum L.), napa cabbage (Brassica rapa subsp. pekinensis L.), cabbage (Brassica oleracea var. capitata L.), and longan (Dimocarpus longan Lour.). The targeted single bands with length of 1400 bp were obtained but bidirectional sequencing of the PCR products recovered partial sequence of chloroplast instead of bacterial genomes. Thus, this result confirmed cross amplification of 27F/1492R primer pair against chloroplast genome of plants. Phylogenetic tree was constructed to separate the tested isolates according to their taxonomic relations: Order Sapindales, Solanales, and Brassicales. In-silico analysis on the new five and nineteen selected sequences in NCBI GenBank detected at least seven conserved regions with some single nucleotide polymorphisms. This report might be a cautionary advice for other researchers to avoid false positive results which could lead to misdiagnosis of bacterial plant diseases.

Keywords: Chloroplast, Molecular detection, Nucleotide polymorphisms, Phylogenetic tree, 16S ribosomal RNA

1. INTRODUCTION

Chloroplast is an important organelle in plant and algal cells where photosynthesis occurred. Just like mitochondria, chloroplast has its own DNA which was proposed in symbiognesis theory to be acquired by early eukaryotic cells from genome of photosynthesis performing endosymbiotic cyanobacterium (Ziehe et al., 2017).

Different molecular methods commonly targeted the sequences of 16S ribosomal RNA genes (16S) for detection and identification of bacteria, including those pathogenic to plants

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(Puspitasari et al., 2021). However, the highly homologous bacterial 16S to chloroplast 16S genes, as one of consequences of the chloroplast evolution, is causing difficulties to select an appropriate primer pair to be used in the molecular test. As example, the universal primer pairs 926F/1392R, 27F/338R, 27F/519R, 534F/1114R, and 926F/1114R targeting different regions of bacterial 16S were observed to be homologous to chloroplast 16S (Rastogi et al., 2010; Beckers et al., 2016). Efforts then developed other primers to minimalized the chloroplast contamination in conventional PCR and also in Next generation sequencing, with different efficiencies (Hanshew et al., 2013; Reigel et al., 2020; Thomas et al., 2020).

Primers 27F (covering 8 – 27 *Escherichia coli* rRNA positions) / 1492R (covering 1492 – 1507 *E. coli* rRNA positions) (Heuer et al., 1997) are among the most commonly used for species-level determination due to its ability to amplify the full length of bacterial 16S gene (Frank et al., 2008). The primer pair is also compatible with chloroplast 16S thus prone to mismatch with the gene (Yu et al., 2013). However, this information seems to be not well known among researchers working with molecular identification of phytopathogenic bacteria and might also those with other types of bacteria (Ibrahim et al., 2023).

In this study, sequences of chloroplast 16S gene of five symptomatic plant species collected in Ngablak, Magelang Regency, Indonesia was inadvertently obtained using PCR with 27F/1492R primers during attempts to detect bacterial plant pathogen. Therefore, this report served as a cautionary advice for other researchers to avoid similar mistake that could be costly in financial term, and even may lead to misdiagnosis of plant diseases. Importance of sequencing to confirm PCR results (Pruvost et al., 2022) was also emphasized here.

2. RESEARCH METHOD

Fields visit

Horticultural fields in Ngablak Subdistrict, Magelang Regency, Indonesia were surveyed on 16 September 2023 to observe bacterial diseases that may present and restrict production in the region.

DNA extraction and PCR

Total DNA was extracted from the five plant samples using Genomic DNA Mini Kit (Plant) (Geneaid Biotech Ltd., Taiwan) with standard protocols. PCRs were performed with 27F/1492R universal primer pair for amplification of ± 1400 bp bacterial 16S rRNA region (Heuer et al., 1997). PCR mix was each prepared in a volume of 40 µl: 20 µl MyTaq HS Red Mix (Bioline, Germany), 2 µl (10 pmol/µl) each of reverse primer and forward primer, 4 µl of DNA or cDNA,

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and 12 µl of PCR-grade water. The thermal cycler program was 95 °C for 3 min as a pre-denaturation, 30 cycles of 95 °C for 1 min, 57 °C for 30 s, and 72 °C for 1.5 min, followed by 72 °C for 7 min as a final extension.

PCR products were loaded into 1% agarose gel stained with Florosafe DNA Staining (1st BASE, Malaysia) then electrophorized for 50 min at 50 V. Gel was then put on a UV transilluminator (Optima Inc., Japan) to observe band with the specific size. PCR products with single targeted band were send to 1st BASE biotechnological company in Malaysia to be sequenced bidirectionally using Sanger method. The recovered sequences were tested against NCBI GenBank database using nucleotide BLAST online software (https://blast.ncbi.nlm.nih.gov). Nucleotide sequences of the novel isolates were registered to NCBI GenBank to obtain accession numbers.

Phylogeny and polymorphisms studies

Sequences of NCBI GenBank isolates with high percentage identities and coverage of at least 99% to the newly obtained isolates from Ngablak Subdistrict were aligned using ClustalW suits in MEGA X freeware (Kumar et al., 2018), and then trimmed according to the lengths of sequences of new isolates (Praptana et al., 2023). For additional comparison and conserved region analysis, sequences from different organisms including plant pathogenic bacteria, plant growth promoting rhizobacteria (PGPR), microalgae, cyanobacteria, and vascular plant were added to the alignment (Table 1). Construction of phylogenetic tree was conducted by MEGA X using Maximum Likelihood (ML) statistical method with Tamura-Nei parameter model (Tamura and Nei, 1993). Significancy of the constructed branches was statistically tested using 1000 bootstrap replicates. Nucleotide polymorphisms among aligned sequences were observed using BioEdit freeware v.7.2.5 (Hall, 1999; Santosa et al., 2023).

Table 1. List of 16s rDNA and Chloroplast Sequences Used in This Study

No	Species	Order	Family	Domain	Accession number
1	*Pseudomonas fluorescens*	Pseudomonadales	Pseudomonadaceae	Bacteria	NZ_LT907842.1
2	*Pseudomonas syringae*	Pseudomonadales	Pseudomonadaceae	Bacteria	NZ_CP074578.1
3	*Xanthomonas vesicatoria*	Xanthomonadales	Xanthomonadaceae	Bacteria	NZ_CP018725.1
4	*Xanthomonas euvesicatoria*	Xanthomonadales	Xanthomonadaceae	Bacteria	NZ_CP018467.1
5	*Xanthomonas perforans*	Xanthomonadales	Xanthomonadaceae	Bacteria	NZ_CP116305.1
6	*Bacillus subtilis*	Bacillales	Bacillaceae	Bacteria	NZ_CP120681
7	*Bacillus pumilus_*	Bacillales	Bacillaceae	Bacteria	NZ_PTXV01000013.1
8	*Serratia marcescens*	Enterobacterales	Enterobacteriaceae	Bacteria	NZ_CP027798.1
9	*Zanthoxylum*	Sapindales	Rutaceae	Eukaryota	OP580971.1

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	avicennae				
10	*Pometia tomentosa*	Sapindales	Sapindaceae	Eukaryota	NC_048999.1
11	*Toona sinensis*	Sapindales	Meliaceae	Eukaryota	OK638185.1
12	*Dimocarpus longan* isolate Ngablak	Sapindales	Sapindaceae	Eukaryota	This study (OR939686)
13	*Brassica rapa* subsp. pekinensis isolate Ngablak	Brassicales	Brassicaeae	Eukaryota	This study (OR939687)
14	*Brassica oleracea* var. capitata isolate Ngablak	Brassicales	Brassicaeae	Eukaryota	This study (OR939688)
15	*Capsella bursa-pastoris*	Brassicales	Brassicaeae	Eukaryota	OR941702.1
16	*Cardamine flexuosa*	Brassicales	Brassicaeae	Eukaryota	OY743223.1
17	*Begonia pedatifida*	Cucurbitales	Begoniaceae	Eukaryota	NC_071962.1
18	*Capsicum annuum* isolate Ngablak	Solanales	Solanaceae	Eukarya	This study (OR939689)
19	*Solanum lycopersicum* isolate Ngablak	Solanales	Solanaceae	Eukarya	This study (OR939690)
20	*Datura metel*	Solanales	Solanaceae	Eukarya	NC_069556.1
21	*Chlorella vulgaris*	Chlorellales	Chlorellaceae	Eukarya	MW900257.1
22	*Chlorella sorokiniana*	Chlorellales	Chlorellaceae	Eukarya	KJ742376.1
23	*Arthrospira platensis* NIES-39	Oscillatoriales	Microcoleaceae	Bacteria	AP026945.2
24	*Microcystis aeruginosa*	Chroococcales	Microcystaceae	Bacteria	NZ_JXYX01000013.1

3. RESULTS AND DISCUSSION

One of each bacterial symptomatic plants: chili pepper (*Capsicum annum* L.), tomato (*Solanum lycopersicum* L.), napa cabbage (*Brassica rapa* subsp. *Pekinensis* L.), cabbage (*Brassica oleracea* var. *capitata* L.), and longan (*Dimocarpus longan* Lour.) were collected then brought to Plant Pathology Laboratory, Universitas Gadjah Mada to be frozen at -4 °C until molecular test. These five plants representing 3 orders including Solanales, Brassicales, and Sapindales (Tambi et al., 2020) were tested in bacterial molecular detection using 27F/1492R primers.

Clear single bands of around 1400 bp in size appeared on agarose after electrophoresis (Figure 1). This initial result indicated presence of bacteria, either plant pathogenic or non-pathogenic, in the tested samples. However, BLAST analysis showed that the recovered nucleotide sequences from those PCR amplification results were actually small subunit ribosomal RNA region of chloroplast of plants. The isolates were then listed in GenBank with acc. nos. OR939686-90.

The currently accepted symbiognesis theory thought that plants obtained their chloroplasts from ancient endosymbiotic cyanobacterium, and thus the nucleotide sequences of their chloroplasts shared high identity with those of bacteria. Despite its versality in amplification of

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wide range of bacterial 16S regions in molecular tests, results of this study clearly showed that 27F/1492R universal primers made cross amplification to partial sequences of 16S ribosomal RNA of plant chloroplast, around 1400 bp in length. Thus, these results also confirmed previously reported non-specific amplifications (Hanshew et al., 2013; Yu et al., 2013).

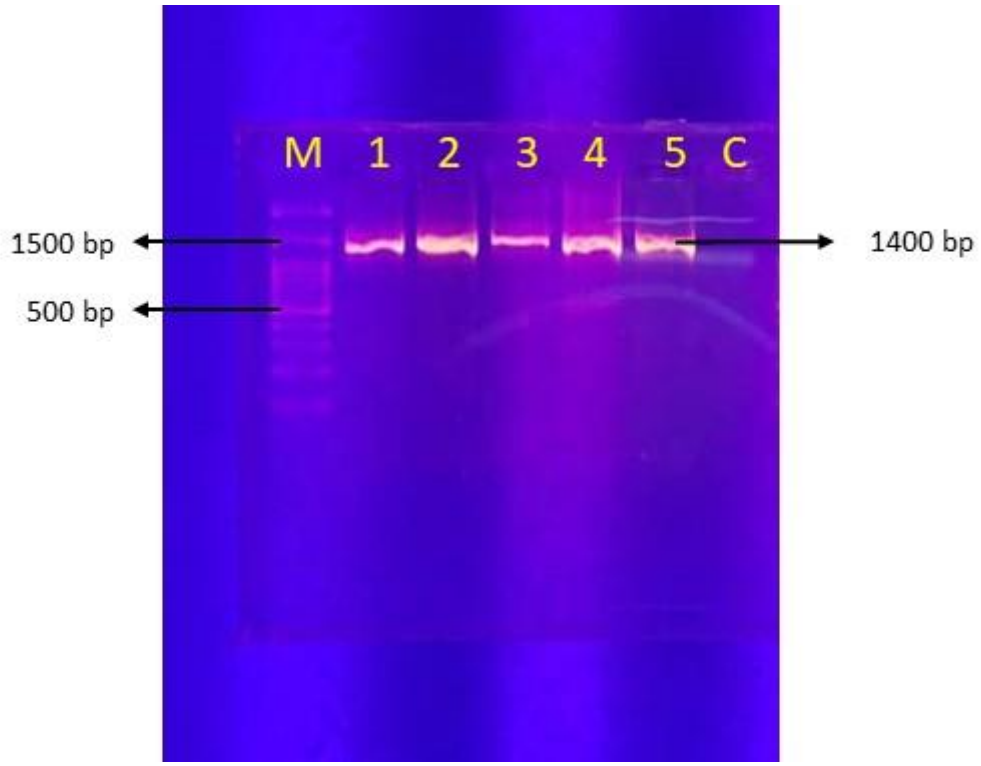

Figure 1. PCR amplification using 27F/1492R primers on DNA templates directly extracted from plants generated the expected single bands of around 1400 bp in size. 1) Longan isolates no. OR939686; 2) Napa cabbage isolate no. OR939687; 3) Cabbage isolate no. OR939688; 4) Chili pepper isolate no. OR939689; 5) Tomato isolate no. OR939690; M) Ladder; C) Negative control with sterile double distilled water as template

Phylogenetic tree was constructed based on the sequence alignment of 16S rRNA. The phylogenetic tree showed the evolutionary relation of selected organisms which can be grouped into three main cluster including cluster I belongs to order Sapindales, Solanales, Brassicales, Chlorellales, Oscillatoriales, and Chroococcales, cluster II belongs to order Bacilles, and cluster II belongs to order Pseudomonadales, Xanthomonadales, and Enterobacteriales. However, each order further grouped into several sub-clusters (Figure 2).

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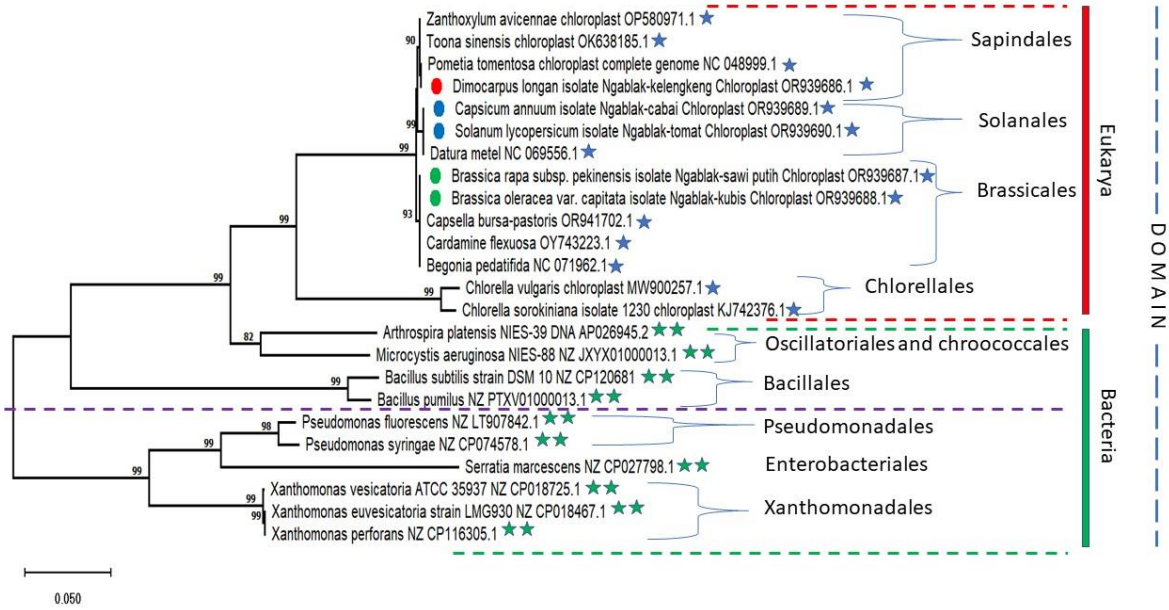

Figure 2. A neighbor joining phylogenetic tree based on 1451 bp of 16S ribosomal RNA gene of plant chloroplasts (blue star) and chromosomal genome (green star) of eukaryote and bacteria. Dot signs highlighted the isolates of five plant species observed in this study, which were grouped into three subclusters according to their respective Order: Sapindales, Solanales, and Brassicales

Twenty-four sequences of partial 16S rDNA from chromosomal DNA and chloroplast were aligned (Table 1). Sequence comparison showed relative high similarity in range 1 – 20 bp; 300 – 373 bp; 912 – 992 bp; 1051 – 1080 bp; 1090 – 1122 bp; 1378 – 1429 bp; 1493 – 1522 bp (Figure 3).

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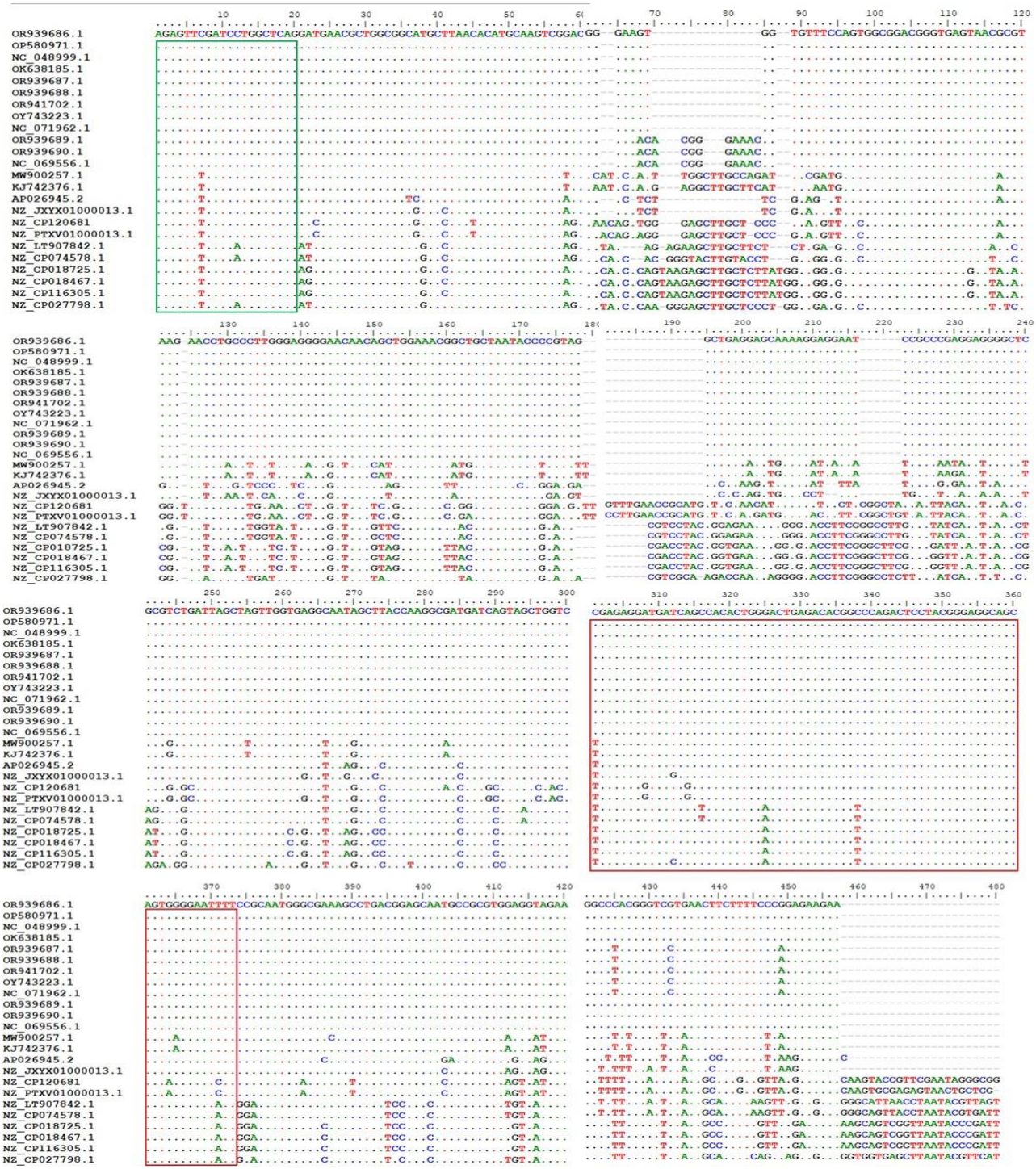

Figure 3. Multiple alignment of five new and 19 partial sequences of 16SR DNA from chromosomal and chloroplast genomes listed in NCBI GenBank. Green boxes indicate the position of 27F and 1492R primer. The red boxes indicate conserved regions which showed relative high similarity among the sequences and few polymorphisms

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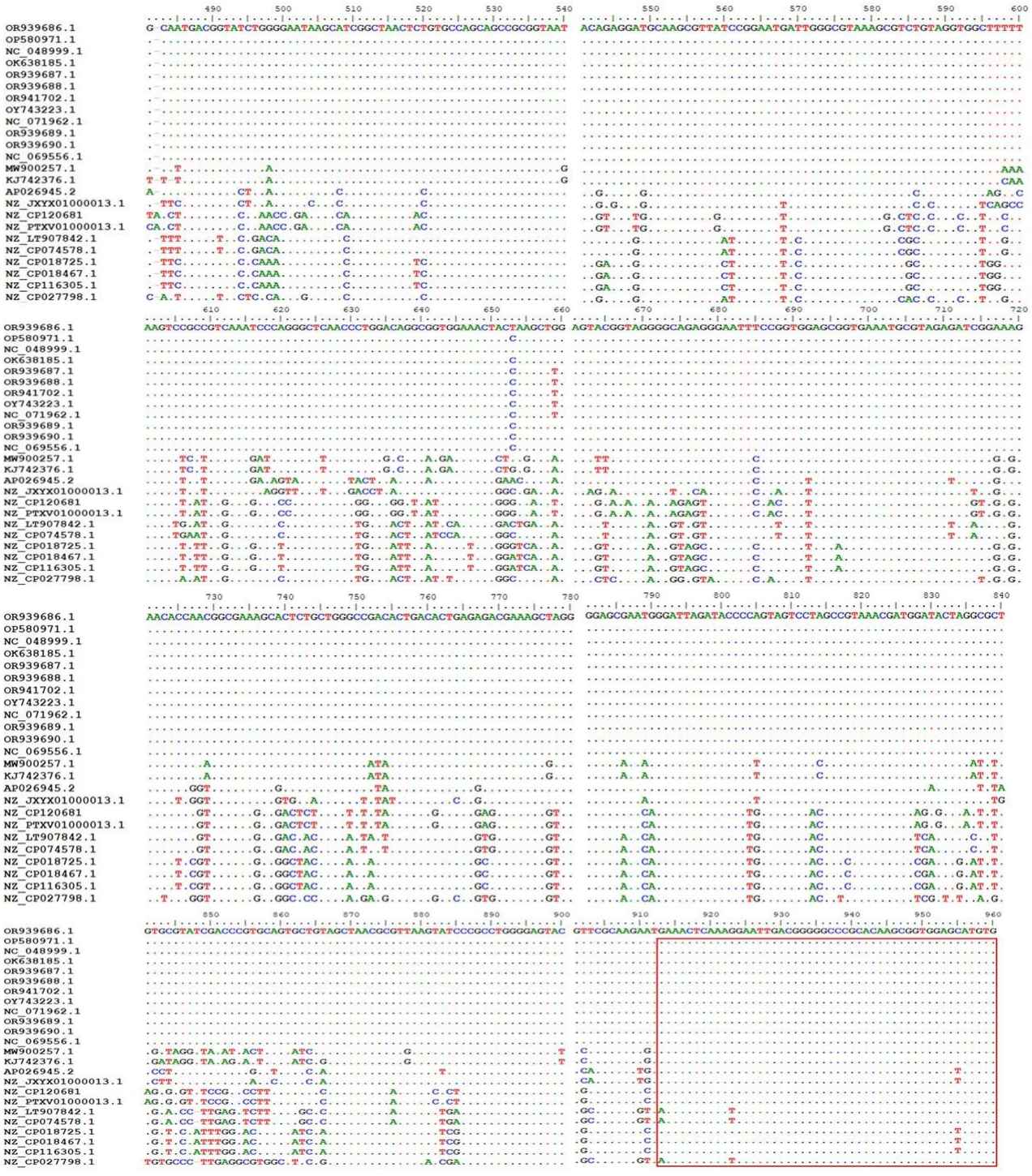

Figure 3 continue

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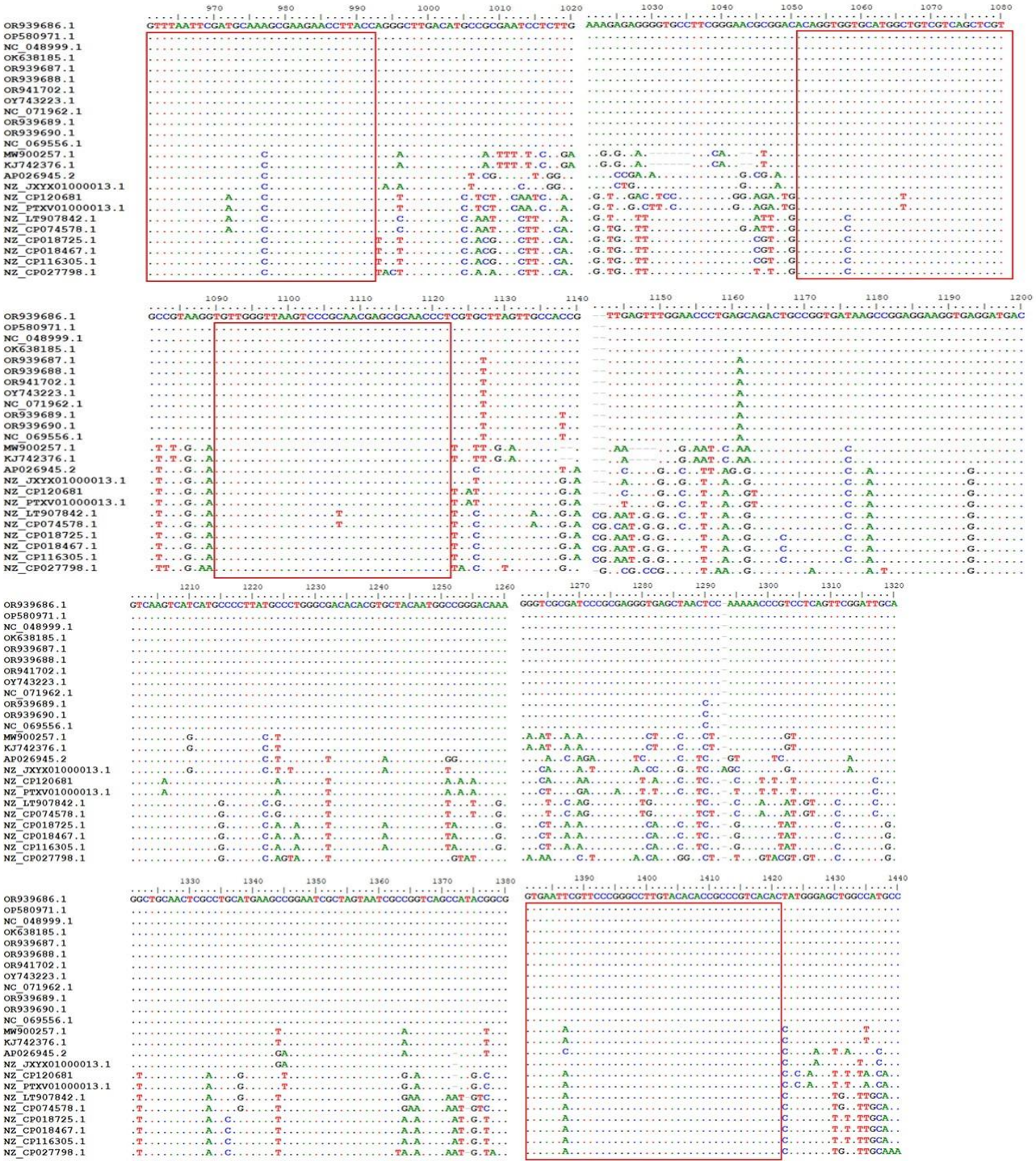

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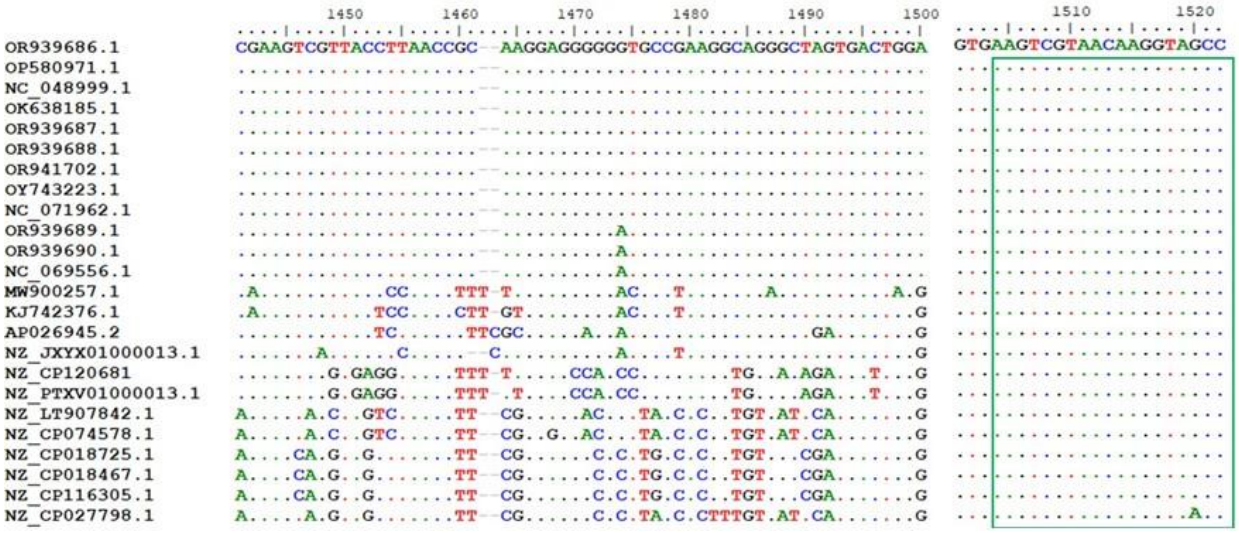

Figure 3 continue

Researchers have proposed new primer pairs to reduce chloroplast contamination in PCR for amplification of bacterial 16S region (Hanshew et al., 2013; Reigel et al., 2020; Thomas et al., 2020). However, the new primers might not be efficient, as also was suggested by our in-silico study that the region amplified by 27F/1492R has only seven conserved regions (Figure 2). This could be one of reasons that 27F/1492R primers are still often used by many researchers to detect and identify bacteria for various purposes (Yuwantiningsih et al., 2015; Indraswari et al., 2021; Rodiansyah et al., 2021), including plant diseases diagnosis (Puspitasari et al., 2021).

4. CONCLUSIONS

Result of current study cautioned the application of 27F/1492R primers in molecular detection using total DNA extracted directly from plants as it may lead to misdetection of bacteria. Researchers dealing with plant pathogenic bacteria were advised to obtain DNA from pure bacterial culture when using 27F/1492R. If DNA extraction from plant become essential, such as during dealing with obligate bacteria, it can be suggested to use other primers that are less likely to anneal to chloroplast sequences. Result of this study also strongly underlined the need of sequencing to confirm PCR amplification results.

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