Candidatus phytoplasma malaysianum (16SrXXXII) associated with Elaeocarpus sylvestris decline in South Korea

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Introduction
Phytoplasmas are wall-less pleomorphic prokaryotes that were previously classified as mycoplasma-like organisms (MLOs) on account of their morphological similarity to mycoplasmas, first occurred in mulberry phloem with dwarf disease (Doi et al. 1967; McCoy et al. 1989; Bertaccini and Duduk 2009; Weintraub and Jones 2009; Pagliari and Musetti 2019). Phytoplasma chromosome size is about 600–880 kb, which is very small in different from other plant pathogens (Oshima et al. 2013).

Phytoplasma are pathogens that is the cause of over 1,000 diseases in different wild and cultivated plant species all over the world (Seemueller et al. 2002; Bertaccini 2007; Hoshi et al. 2007; Kumari et al. 2019; Namba 2019). In South Korea, such diseases have included jujube witches’ broom (Kim 1968), paulownia witches’ broom (La 1968), mulberry dwarf (Chang and Kim 1971), and sumac witches’ broom (Kim 1980) causing significant economic losses from a lot of trees affected throughout the country (Jung et al. 2012).

Currently, including crops, about 64 species of host plants infected with phytoplasma have been reported in Korea. (Chung et al. 2011; Jung et al. 2012; Lee et al. 2017). Plants infected with phytoplasma also show many symptoms, including witches’ broom, yellowing, phyllody, virescence, shortened internodes, and little leaf, as well as declining and vascular discoloration (Bertaccini and Duduk 2009; Kumari et al. 2019; Dermastia 2019). Meanwhile, with the development of molecular biology, PCR technique and sequence analysis on 16S ribosomal DNA, is in commonly used molecular diversity and taxonomy classification on phytoplasma (Lee et al. 1998; Bertaccini and Duduk 2009).

Elaeocarpus sylvestris var. ellipticus (Thunb.) H. Harra an evergreen tree belonging to Elaeocarpaceae, grown in southern China, south central Japan, Taiwan, Vietnam, and on Jeju Island in South Korea and it reproduces mostly by seed germination (Kim and Kim 2014). However since 2013, E. sylvestris trees on Jeju have shown decline, while some trees have died on streets, in parks or in their natural habitat (Lee et al. 2017). Since the 1980s, similar disease have also been reported in Japan named ELY (Elaeocarpus yellows), and E. zollingeri trees have been infected with phytoplasma and are in danger of decimation (Ikeda and Hashimoto 1996; Narazaki and Tsuda 2008; Kawabe 2010; Odawar City Cultural Property Protection Committee 2013; Kawabe et al. 2011; Satoh et al. 2014; Iwabuchi et al. 2018).

Until the present study, no research on the decline of E. sylvestris trees from phytoplasma has been conducted in Korea to date, even though the number of areas with damaged trees is increasing every year. The purpose of this study is to identify the cause of the decline in E. sylvestris trees using PCR assays, base sequences, and virtual-RFLP. In addition, we will determine whether the phytoplasma is found in the seeds of tree with decline symptoms.

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Table 1. List of E. sylvestris samples used in this study.

| No. | Isolate | Extraction part | Location | Symptoms | Strains |
|-----|---------|-----------------|----------|----------|---------|
| 1   | J0001   | Leaves          | Ido-Hanilberache Apt (jeju-si) | Yellows | ESDP-JJ1 |
| 2   | J0002   | Leaves          | Nohyeong-dong Golf Course (jeju-si) | Yellows | " |
| 3   | J0003   | Leaves          | Hwabuk industrial street trees (jeju-si) | Darks | ESDP-JJ2 |
| 4   | J0004   | Leaves          | Sindae-ro street trees (jeju-si) | Darks | " |
| 5   | J0005   | Leaves          | Halla- arboretum (jeju-si) | Yellows | " |
| 6   | J0006   | Seed and flesh  | Nohyeong-dong park (jeju-si) | Darks | ESDP-JJ3 |
| 7   | J0007   | Seed and flesh  | Western part police station (jeju-si) | Darks | " |
| 8   | J5001   | Leaves          | Ijuseo-ro street trees (seogwipo-si) | Yellows | ESDP-JS1 |
| 9   | J5002   | Leaves          | Jungmungwangwang-ro parking lot trees (seogwipo-si) | Darks | " |
| 10  | J5003   | Leaves          | Hyodon middle school (seogwipo-si) | Yellows | " |
| 11  | J5004   | Leaves          | Gangjeong-dong natural monument (seogwipo-si) | Yellows | " |
| 12  | J5005   | Leaves          | Cheonjiyeon falls (seogwipo-si) | Yellows | " |
| 13  | J5006   | Seed and flesh  | Gogeon mountain (seogwipo-si) | Darks | ESDP-JS2 |
| 14  | J5007   | Seed and flesh  | Yeraedong street trees (seogwipo-si) | Darks | " |

Materials and methods

Diseased samples and extraction of DNA

Our investigation was conducted on E. sylvestris in Jeju island, where we collected 14 symptomatic samples which yellowing, darkening leaves and seeds near symptomatic leaves between April and September 2019 (Table 1). Samples were stored at 4°C, while to identify phytoplasma infection, DNA was extracted from 0.1 g of leaf midribs, petioles, and separating the flesh and seeds of the drupe, using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

PCR

PCR with the phytoplasma universal primers P1 (5'-AAGAGTTTGATCCTGGCTCAGGATT-3') and P7 (5'-CGTCCTTCATCGGCTCTT-3') was used to amplify 16S rRNA, the 16S-23S intergenic spacer (ITS) region, tRNA-Ile, and partial 23S rRNA (Deng and Hiruki 1991; Schneider et al. 1995). Nested PCR analysis was conducted using universal primer R16F2n (5'-GAAAAGCAGCTTAAAGCTTACG-3') and R16R2 (5'-TGACGGGGCTTGTGTACAAACCGG-3') (Lee et al. 1993; Gundersen and Lee 1996). Reactions were carried out using a 25 µl volume reaction mixture containing 100 ng/µl DNA, Emerald- Amp GT PCR Master Mix [2 X Premix] (Takara, Shiga, Japan) for 10 pmol of each primer, and the rest was filled with distilled water. Conditions for PCR amplification were as follows: one cycle for denaturation at 94°C for 7 min, 35 cycles denaturation at 94°C for 1 min, annealing at 55°C (nested PCR 58°C) for 2 min, and extension at 72°C for 3 min, with a final extension step of 72°C for 10 min (Lee et al. 1993; Gundersen and Lee 1996; Lee et al. 1998; Han 2005; Kamala-Kannan et al. 2011). The secA gene was amplified using the primers SecAfor1 (5'-GARATGAAAACCTGGRAGAAGG-3') and SecArev3 (5'-GTTTTRGCAGGTCATGCATCNC-3'). PCR conditions with the primer pair SecAfor1/SecArev3 were 94°C for 2 min followed by 35 cycles of 94°C for 30s, 53°C for 60s and 72°C for 90s, with a final extension step of 72°C for 15 min (Hodgetts et al. 2008).

Comparison of sequencing analysis

To find taxonomic lineage of pathogen that caused the diseased E. sylvestris trees, we used the iPhyClassifier software from the 16SrRNA gene (Zhao et al. 2009). In addition, we using 17 key restriction enzymes: AluI, BamHI, BfaI, BstU1, DraI, EcoRI, HaeIII, HhaI, HinfI, HpaI, HpaII, KpnI, Sau3AI, MseI, Rsal, Spal and TaqI were used to compare the virtual-RFLP analysis results.

Amplified PCR products were purified using a PCR Purification Kit (Bioneer, Seoul, South Korea) and cloned to transform Escherichia coli JM109 cells according to manufacturer’s instructions (Qiagen, Valencia, CA, USA). The sequencing was performed by Macrogen Co. (Daejeon, Korea). Sequence alignments were analyzed using GENETYX-WIN 4.0 (GENETYX, Tokyo, Japan), and phylogenetic analysis was performed with MEGA ver. 10.0 software using the Neighbor-joining method, with the default values and 10,000 replications for bootstrap analysis.

Results

Symptomatology

The most of diseased E. sylvestris trees were observed in various locations including streets, parks, around the apartment and natural habitats on Jeju. In some street trees, symptomatic trees appeared to decline symptoms collectively, more than 70% trees showed that decline symptom from April to October. Early symptoms were as the previous year’s leaves began yellowing and darkening. A number of leaves fall sharply, the leafless branches becoming weak. After years of repetition of these symptoms the tree eventually dries up and dies over time (Figure 1).

Detection of phytoplasma and molecular diversity

Polymerase chain reaction with phytoplasma universal primers (P1/P7) amplified the expected DNA fragment of 1.8 kb and targeted 16S rRNA, The 16S–23S ITS region, and partial 23S rRNA. Nested PCR with R16F2n/R16R2 primers resulted in the predicted 1.2 kb amplicon in leaves, both flesh and seeds detected with phytoplasma (Figure 2). The phytoplasma universal primers (SecAfor1/SecArev3) also amplified the expected size of 0.84 kb and targeted the partial secA gene (Figure 2). The nucleotide sequences of the 16S rRNA and secA genes of infected leaves and seeds on
Table 2. Classification of phytoplasma species reference based on 16S rDNA gene.

| Phytoplasma strain | Associated plant disease | Genbank Accession No. | Percentage of similarity |
|--------------------|--------------------------|-----------------------|--------------------------|
| ELY-BN1            | Elaeocarpus zollingeri yellows phytoplasma | LC227960             | 99.6                     |
| MYD                | Malaysian yellow dwarf coconut phytoplasma | EU498727             | 99.6                     |
| MOP                | Malaysian periwinkle virescence phytoplasma | EU371934             | 99.6                     |
| SCW81              | Salt cedar witches'-broom phytoplasma | FJ432664             | 88.0                     |
| CaWB-Emp-YNws1     | 'Camptotheca acuminata' witches'-broom phytoplasma | MH141802             | 99.6                     |
| TrWB-hn            | 'Trema tomentosa' witches'-broom phytoplasma | MW138004             | 99.7                     |
| Bangi 4            | 'Wodyetia bifurcata' yellow decline phytoplasma | KY073856             | 87.0                     |
| SHK03              | Sugarcane phytoplasma | MK351909             | 94.0                     |
| Pin127S            | Candidatus Phytoplasma pini | A1632155             | 93.8                     |
| RhCa               | 'Rhamnus cathartica' stunt phytoplasma | JQ868449             | 89.8                     |
| 21419156           | 'Corylus avellana' proliferation phytoplasma | KP407881             | 88.2                     |
| PPT-PEPPER-        | Mexican potato purple top phytoplasma | JQ745314             | 86.2                     |
| ESFY-Ch            | 'Prunus armeniaca' apple proliferation phytoplasma | MG748694             | 88.2                     |
| CP                 | Candidatus Phytoplasma trifoli | AY390261             | 96.3                     |
| fasa1              | Sesame phyllody phytoplasma | JX566571             | 92.1                     |
| WBDB               | Candidatus Phytoplasma aurantifolia | U15442              | 84.2                     |
| AY-WB              | Aster yellows phytoplasma | AY389828             | 87.8                     |
| EY1                | Elm yellows phytoplasma | AY197655             | 95.2                     |
| WPWB               | Wax leaf privet witches'-broom | AB249322             | 95.1                     |
| JRWB               | Japanese raisin witches'-broom | AB442218             | 95.1                     |
| JBW                | Jujube witches'-broom | AB052879              | 94.9                     |
| Acholeplasma palmae |                        | L33734             | 81.6                     |

Figure 1. Decline symptoms of Elaeocarpus sylvestris tree. (A) Yellowing leaves, (B) darkening leaves, (C) seeds near symptomatic leaves, (D) asymptomatic leaves, (E) decline symptoms tree in the park.

Figure 2. Agarose gel electrophoresis patterns of PCR products 16S rRNA gene and secA gene amplified by nested PCR using primer pair R16F2n/R16R2 and Direct SecAfor1/SecArev3 from E. sylvestris with decline symptoms in Jeju island. (Lane: M. molecular weight marker (100 bp), 1 ~ 14: the symptomatic leaves and seeds, 15: jujube witches' broom phytoplasma, 16: sumac witches' broom phytoplasma).
Jeju showed a 100.0% identity to strains ESDP-JJ1, JJ2, JJ3, JS1, and JS2. The pairwise alignment of the 16S rRNA gene sequence with available sequences in the NCBI database showed that the phytoplasma was more than 99.5% correlated with Malaysian periwinkle virescence group strain ELY-BN1, MaPV, MOP, MYD, and TtWB (Figure 4, Table 2). The 16S rRNA and 16/23S ITS sequences of *E. sylvestris* decline phytoplasma

![Image](image-url)
(ESDP) were deposited in GenBank accession numbers: OL689203, OL689204, OL689205, OL689206 and OL689207. The 16S rRNA phytoplasma sequences were insufficient for identification of phytoplasmas belonging to the same group. Pairwise alignment of the secA gene sequence with sequences obtained from the NCBI database revealed that the phytoplasma was more than 98.2% associated with Malaysian periwinkle virescence group (Figure 5). The secA gene sequence of *E. sylvestris* decline phytoplasma (ESDP) were deposited in GenBank accession numbers: OL689198, OL689199, OL689200, OL689201 and OL689202.

The virtual-RFLP patterns derived from the query 16S rDNA gene (F2nR2 fragment) of strain *Elaeocarpus sylvestris* decline phytoplasma (ESDP) is clearly different RFLP band pattern reported Malaysian periwinkle virescence (MaPV), Malayan Oil Palm (MOP), Malayan Yellow Dwarf (MYD) (Figure 3).

**Discussion**

The results of the pairwise alignment of the 16SrRNA and the secA gene sequence revealed that the phytoplasma in each was 99.5%, 98.2% linked to others with *Candidatus* phytoplasma malaysianum (16SrXXXII). In the virtual-RFLP results, it was different from the previously reported 16SrXXXII-A (MaPV), 16SrXXXII-B (MYD), 16SrXXXII-C (MOP) group (Nejat et al. 2013). Among the 17 restriction enzymes, in BstUI enzyme case, 16S rDNA gene of strain ESDP were distinguished from 16SrXXXII-A (MaPV), 16SrXXXII-B (MYD), 16SrXXXII-C (MOP), and restriction enzyme Bfal can be distinguish 16SrXXXII-C (MYD). In addition, *Hind* enzyme also distinguish 16S rDNA gene of strain ESDP were distinguished from 16SrXXXII-A (MaPV), 16SrXXXII-B (MOP), the other enzymes showed the same patterns. As a result of this, we propose that ESDP strain a new subgroup within the 16SrXXII group. Associated with Malaysian periwinkle virescence group (16S rDNA gene of strain ESDP were distinguished from 16SrXXXII-A (MaPV), 16SrXXXII-B (MYD), 16SrXXXII-C (MOP), and restriction enzyme Bfal can be distinguish 16SrXXXII-C (MYD). In addition, *Hind* enzyme also distinguish 16SrXXXII-A (MaPV), 16SrXXXII-B (MOP), the other enzymes showed the same patterns. As a result of this, we propose that ESDP strain a new subgroup within the 16SrXXII group. Associated with Malaysian periwinkle virescence group (16SrXXXII) Strains including Malaysian periwinkle virescence (MaPV), Malaysian Oil Palm (MOP), Malayan Yellow Dwarf (MYD), *Camptotheca acuminate* witches-broom (CAWB), *Trema tomentosa* witches-broom (TrWB), and *Elaeocarpus zollingeri* yellows (ELY) (Nejat et al. 2009, 2013; Iwabuchi et al. 2018; Yu et al. 2020), among these, *E. zollingeri* yellows (ELY) closely hosts related and symptoms are similar to *E. sylvestris* decline in Korea. PCR-RFLP and virtual RFLP analysis also showed that the korean and Japanese diseased *E. zollingeri* yellows (ELY) displayed a band pattern that was similar to the analysis results with the restriction enzymes *RsaI*, *HpaI*, and *MsiI* (Satoh et al. 2014). However, using the *AluI* enzyme, it was showed that different band pattern from *E. sylvestris* decline in Korea. Japanese studies of *E. zollingeri* yellows (ELY) reported phytoplasma infection by types other than Ca.
phytoplasma malaysianum, including Ca. phytoplasma asteris (Kawabe et al. 2001, 2010). In our studies, however, only Ca. phytoplasma malaysianum was detected. Further research is needed on this.

In Japan, insect vectors of ELY has not yet to be determined (Satoh et al. 2014; Iwabuchi et al. 2018). Likewise, it is still unknown whether Leafhoppers carry phytoplasma disease in Korea. Preventing the spread of phytoplasma disease requires identifying the insect hosts associated with it.

As for the reports about phytoplasma transmission in seeds, including apricot, peach, corn, and carrot (Necas et al. 2008; Çağlar et al. 2019; Carminati et al. 2019). Theoretically speaking, Phytoplasma transmission via seeds has not yet been reported in Korea (Shin 1980). However, seeds of diseased E. sylvestris, phytoplasma was detected by the PCR analysis performed in this study. This trees mostly reproduces by seed germination, but the phytoplasma was detected in the seeds. There are a risk of the spread of phytoplasma in the end.

Phytoplasma were present in trees displaying decline symptoms. The present study is the first confirmed report of Ca. phytoplasma malaysianum (16SrXXII) in E. sylvestris leaves and seeds on Jeju Island in South Korea. In response to this, considering that various symptoms and signs of decline are evident, we provisionally propose to name it “Elaeocarpus sylvestris decline phytoplasma (ESDP).”

Conclusions

This study was conducted to discover phytoplasma in Elaeocarpus sylvestris growing in Jeju Island, various decline symptoms observed including yellowing, darkening and branch dieback. Trees infected with phytoplasma die progressing rapidly and a number of trees are being cut down. As a result of PCR using phytoplasma universal primer P1/P7, R16F2n/R2, and SecAfor1/SecArev3 phytoplasma detected in leaves and seeds. We analyzed that E. sylvestris decline sequences, and found partial 16S rRNA sequences to be 99.5%, and secA genes 98.2% similarities with Ca. phytoplasma malaysianum (16SrXXII). As a result of virtual-RFLF analysis with 17 key restriction enzymes with 16S rRNA gene, ESDP strain was presumed a new subgroup within the 16SrXXII group.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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