
Molecular identification of *Pseudoplagiostoma eucalypti* causing leaf spot and shoot blight diseases on eucalyptus in Thailand based on ITS rDNA sequence

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Abstract Leaf spot and shoot blight diseases are the main problems for eucalyptus forest plantations in Thailand. The diseases have been reported causing by *Cryptosporiopsis eucalypti* (anamorph). However, this fungus was re-evaluated and identified as *Pseudoplagiostoma eucalypti* (teleomorph) using molecular and morphological characteristics based identification. The aims of this study were to evaluate the genetic relationships among *Ps. eucalypti* isolated from Thailand and related species using ITS rDNA sequencing and to screen the restriction enzyme using *In silico* RFLP for distinguish between *Ps. eucalypti* and other related species. The disease samples were surveyed and collected from eucalyptus plantations in 10 provinces of Thailand. Fungal species were isolated and identified using morphological characteristics and colony growth measurements on synthetic media. The fungal isolates were identified as *Ps. eucalypti* which were of diverse morphologies especially for colony characteristics. Internal transcribed spacer (ITS) region of ribosomal DNA sequences of *Ps. eucalypti* were amplified and sequenced using ITS1 and ITS4 universal primers. The sequences were aligned and analyzed together with the sequences obtained from GenBank (DDBJ) using the CLC Main workbench. The UPGMA clustering showed that the eight sequences were in the same group with other sequences of *Ps. eucalypti* recorded in the database supporting by 100% bootstrap value. Results indicated that the eight isolates could be identified as *Ps. eucalypti* based on ITS region analyses, which corresponding to the morphological based identification. In addition, the screening of restriction enzymes for digestion of ITS region was conducted to distinguish between *Ps. eucalypti* and other related species using *In silico* RFLP in CLC Main Workbench. The screening of 10 enzymes showed that *AluI* and *HinCII* enzymes could differentiate *Ps. eucalypti* and *Ps. oldii* from *Ps. variabile*. While, three species of *Ps. eucalypti*, *Ps. oldii* and *Ps. variabile* could not be distinguished with *Ctr10I*, *EcoRI*, *HaeII*, *HaeIII*, *HinCII*, *MseI*, *StuI* and *TaqI* enzymes.

Keywords: Eucalyptus, Leaf spot and shoot blight disease, *Pseudoplagiostoma eucalypti*, *Cryptosporiopsis eucalypti*, ITS ribosomal DNA

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Introduction

Leaf spot and shoot blight diseases are the main problems for eucalyptus forest plantations in Thailand. The diseases were reported causing by a fungal pathogen, *Cryptosporiopsis eucalypti* Sankaran & B. Sutton. (Old *et al.*, 2003). This fungus can be associated with various symptom including leaf spot, leaf blight, canker, shoot blight on eucalyptus (*Eucalyptus camaldulensis*). The fungal pathogen causes severe leaf and shoot diseases which occurs commonly in *E. camaldulensis* and *E. urophylla* plantations in Thailand. The disease lesions are varies in shape, colour and size, however, it can be identified which based on the light cream-coloured, cupulate fungal fruiting bodies scattered on lesions on the both sides of leaves and conidial characteristic based identification. In moist warm environments, a cream-coloured conidial mass at the top of fruiting bodies can often be easily seen with the aid of a hand lens (Pongpanich, 1999). *Cryptosporiopsis eucalypti* was first reported by Sankaran *et al.* (1995) and have known as teleomorphs in *Pezicula* and *Neofabracea*. Although, *C. eucalypti* was different from the typical *Cryptosporiopsis*. *C. eucalypti* was accepted in *Cryptosporiopsis* by Verkley (1999) based on morphological characteristics.

Cheewangkoon *et al.* (2010) mentioned for the taxonomy of *C. eucalypti* that was confused by its phylogenetically unrelated to the type species of *Cryptosporiopsis* (*Cryptosporiopsis nigra* = *C. scutellata*, *Helotiales*). Thus, the taxonomic position of *C. eucalypti* was resolved which based on morphology and phylogenetic analysis. DNA sequencing analysis was conducted in the 28 S nrDNA, the internal transcribed spacer (ITS), and beta-tubulin regions to determined the intra- and inter- species relationships (Cheewangkoon *et al.*, 2010). DNA sequence analysis indicated that conidial and ascospores of *C. eucalypti* revealed low intraspecific variation, two collections from Australia and one collection from Uruguay represented two novel taxa. Based on the phylogenetic analysis and teleomorphstage observation, *C. eucalypti* was shown to represent a new genus closely related to *Plagiostoma* (*Gnomoniaceae*, *Diaporthales*) for which the names *Pseudoplagiostoma* gen. nov. and *Pseudoplagiostomaceae* fam. Nev. (*Diaporthales*) were introduced (Cheewangkoon *et al.*, 2010).

In the previous study in Thailand, the DNA fingerprint analysis of *Ps. eucalypti* was conducted for genetic diversity observation using AFLP technique with four primer combinations. Results showed that there were 77 polymorphic bands and 178 total bands. All polymorphic bands were analyzed using NTSYS pc ver. 2.02 program. The similarity coefficient was obtained by using Dice's method and phenogram was then generated by UPGMA method. Results showed that fungal isolates that could be divided into ten groups with

cophenetic correlation of 0.955. It is indicated that there was genetic diversity among *Ps. eucalypti* isolates. However, these groups were not correlated to the localities and morphological characteristics (Lueangpraplut and Unartngam, 2011). The data earned from the previous study was useful for breeding improvement of eucalyptus resistance to leaf spot and leaf blight diseases. The aims of this study were to evaluate the genetic relationships among *Ps. eucalypti* isolated from Thailand and related species using ITS rDNA sequencing and to screen the restriction enzyme for distinguish between *Ps. eucalypti* and other related species using *In silico* RFLP.

Materials and methods

Fungal isolation

The disease samples were surveyed and collected from eucalyptus plantations in 10 provinces of Thailand including Kanchanaburi, Khon Kaen, Chachoengsao, Nakhon Pathom, Ratchaburi, Phetchabun, Nong Bua Lam Phu, Udon Thani, Prachin Buri and Uthai Thani. Fungal pathogen was isolated from leaf using tissue transplanting method. The disease leaf samples were cut into 0.5×0.5 cm. and then surface disinfected by 10% sodium hypochlorite for 5 min and washed through sterilized distilled water before transferred to potato dextrose agar (PDA). Then, the culture was incubated at 25°C under diurnal light. Single spore isolation was carried out for each to get pure culture isolate and maintain on PDA. Each fungal isolate was identified on the basis of morphological characters such as colony color, color and shape of conidia and conidiophores as described by Cheewangkoon *et al.* (2010).

DNA extraction

All fungal isolates were cultured in potato dextrose broth (PDB) with shaking for 2-3 days and harvested the mycelia on filter paper (Whatman No.1). Fungal mycelia were freeze-dried using Lyophilizer for 6-8 hours and stored at -20 °C until use. Freeze-dried mycelium was ground in the mortar using liquid nitrogen, then 50 mg. of grounded mycelium was suspended in 500 µl of extraction buffer (200 mM Tris HCl, pH 8.0; 250 mM NaCl; 25 mM EDTA and 0.5% SDS) and incubated at 65 °C for 30 min., then added with 500 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1). After centrifugation at 13,000 rpm. for 10 minutes, the upper aqueous phase was deproteinized by additional 1 vol. of Chloroform: Isoamyl alcohol. (24:1) and centrifuged at 13,000 rpm. for 10 minutes. The upper aqueous phase was transferred to 1.5 ml. microtube containing 2 µl of 10mg/ml RNAase and incubated at 37 °C for 30 min. Then

added with 500 µl. Chloroform: Isoamyl alcohol (24:1). After centrifugation at 13,000 rpm. for 10 minutes, the upper aqueous phase was added two volume absolute ethanol and stored at -20 °C for 1 hour. After the centrifugation at 13,000 g for 10 min., the precipitate DNA was washed with 200 µl. 70% ethanol and was centrifuged at 13,000 g for 10 minutes for two times. After the DNA drying or dissolved DNA with TE buffer (10 mM. Tris HCl, pH 8.0; 1 mM EDTA) was done by a modified method of Zimand *et al.* (1994).

PCR amplification of ITS rDNA region

DNA specimens were amplified in ITS region of rDNA using PCR amplification. Amplification of ITS region was done using 40 µl PCR reaction each containing 2 µM of each primer, 2.5 units of TaKaRa Ex Taq DNA polymerase (Takara, Japan), and the supplied dNTP mixture (containing 2.5 nM of each dNTP) and Ex Taq reaction buffer (containing 2 mM Mg 2+X. Polymerase Chain Reaction was carried out using T professional Standard Gradient (Biometra) under the following condition: 95°C for 3 min, then 35 cycles of 95 °C for 30 S, 55 °C for 1 min, and 72 °C for 1 min, and final step of 72 °C for 10 min. The PCR amplification of the ITS regions was amplified using primers ITS1 (5'TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1990). After amplification, 3 µl of the reaction product was electrophoresed on 1 % (w/v) agarose gels containing 0.1 µl/ml GelStar (Nucleic Acid Gel Stain, 10000X concentrate in DMSO) in TBE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 8.0). PCR products were purified using Illustra™ Micro Spin Columns (GE Health care UK Limited). The purified PCR products were sequenced at BioDesign Company Limited, Thailand.

Sequence alignment and data analysis

Multiple alignment was performed using Clustal W (Thompson *et al.*, 1994). Sequences were analyzed together with sequences obtained from GenBank including *Ps. eucalypti* (GU973532, GU973530, GU973529, GU973519, GU973518, GU973515) *Plagiostoma devexum* (EU255001) and *Gnomonia rhododendri* (EU255044). Phylogenetic analysis of the data were done by distance method using CLC Main workbench (version 5.5). The distance matrix for the aligned sequences was clustered using UPGMA method. Reliability of the inferred tree was estimated by 1000 bootstrap resampling using the same program.

Screening of restriction enzyme using in silico RFLP

The sequences of ITS rDNA of *Ps. eucalypti* and closely related species were digested for screening restriction enzyme using *In silico* RFLP in CLC Main work bench (version 5.5). Ten restriction enzymes including *Alu I*, *Ctr 10I*, *Eco RI*, *Hae II*, *Hae III*, *HinC II*, *Hinf I*, *Mse I*, *Stu I* and *Taq I* were screened.

Results and discussions

Morphological observation

The disease samples were surveyed and collected from eucalyptus plantations in 10 provinces of Thailand including Kanchanaburi, Khon Kaen, Chachoengsao, Nakhon Pathom, Ratchaburi, Phetchabun, Nong Bua Lam Phu, Udon Thani, Prachin Buri and Uthai Thani. The disease symptoms were spot and blight on leaves. The conidial masses occurred on the leaf with pale yellow drops of exuding conidia after incubation in the moist chamber (Fig. 1). Fungal samples were isolated and identified using morphological characteristics and colony growth measurements on synthetic media (PDA). The morphological characteristics of conidia were ellipsoid to elongate-ellipsoid in shape, hyaline and 4.63-6.71 x 13.33-20.96 μm . in size which they were similar to the species description in the previous study (Cheewangkoon *et al.*, 2010; Pongpanich, 1999; Old *et al.*, 2003). The fungal isolates were identified as *Ps. eucalypti* which diverse morphologies especially for colony characteristics (Fig.2). Cheewangkoon *et al.* (2010) observed fungal pathogens from Eucalyptus in plantations on 4 continents and from 10 countries. The 39 isolates were cultured on many synthetic media including OA, PDA, MEA and PNA. The three identified species including *Ps. eucalypti*, *Ps. oldii* and *Ps. variabile* were shown very similar on OA, PDA and PNA. However, they could be distinguished in culture on MEA. Result showed that feature could be used to distinguish these fungal species (also on PNA and OA, but not on PDA). Moreover, it is correlated to the sequence data that all isolates delineated three distinct species within a monophyletic lineage.

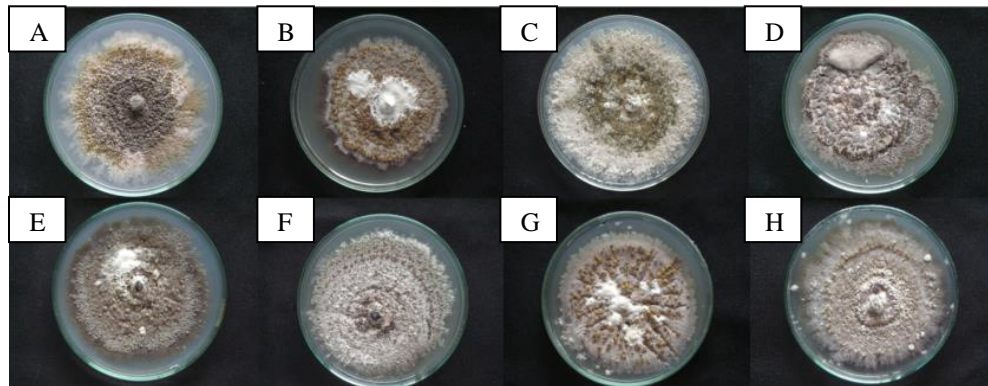


Fig. 1. Colonial diversity of *Pseudoplagiostoma eucalypti* on PDA

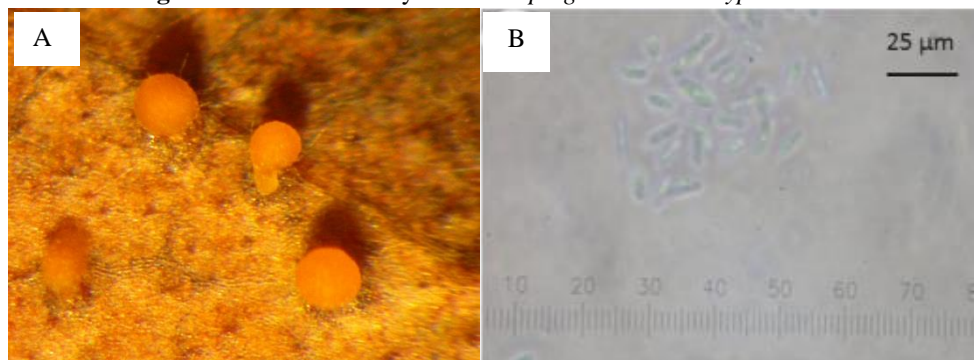


Fig. 2. Conidial masses of *Pseudoplagiostoma eucalypti* oozing from pycnidia onto the eucalyptus leaf surface (A) and characteristic of conidia observed under the microscope(B)

Molecular identification based on ITS rDNA sequence

Eight isolates of *Ps. eucalypti* were amplified and sequenced in the region of ITS using ITS1 and ITS4 universal primers, then all sequences were aligned and analyzed together with the sequences obtained from GenBank (DDBJ) including the sequences of *Ps. eucalypti*, *Gnomonia rhododendri* and *Plagiostoma devexum* (out group). According to the results of Cheewangkoon *et al.* (2010), the ITS region and beta-tubulin gene sequences could be used to distinguish the three species of *Ps. eucalypti*, *Ps. oldii* and *Ps. variabile*. Thus, in this study, the closely related species as *Ps. oldii* and *Ps. variabile* were not analyzed together while *G. rhododendri* was used as the outgroup including with *P. devexum*, because within the *Diaporthales*, *Pseudoplagiostoma* that more similar to the species of the *Gnomoniaceae* based on morphological characteristics of teleomorph (Barr,1978; Castlebury *et al.*, 2002; Cheewangkoon *et al.*, 2010). All sequences of *Ps. eucalypti* in the present study were 566-582 bp in length, and 503-514 bp when aligned together with other sequences from database. The similarity coefficient among eight sequences of

Ps. eucalypti were 89.58-99.05% and 95.16-98.83% when compared with the sequences of *Ps. eucalypti* obtained from database. The UPGMA clustering showed that eight sequences of *Ps. eucalypti* were in the same group with other sequences of *Ps. eucalypti* recorded in the database supporting by 100% bootstrap value (Figs. 3,4) and separated from other species, *G. rhododendri* and *P. devexum*. Results indicated that the eight isolates could be identified as *Ps. eucalypti* based on ITS sequence, which correlated to the morphological based identification. It is suggested that *Ps. eucalypti* sequences were in the monophyletic clade which should be developed the DNA marker or specific primer for distinguish *Ps. eucalypti* and other closely related species. As the research of Pandey *et al.* (2010) that developed the specific primer for identification of *Cylindrocladium quinqueseptatum* causing agent of leaf and seedling blight diseases of Eucalyptus. The specific primer was designed for amplification in the region of ITS rDNA which was specific to the species of *C. quinqueseptatum*. The desined primers were ITSFCQ-1f (5'ATCTCTTGGTTCTGGCATGC3') and ITSFCQ-1r (5'GAGACTCCAGAGCGAGGTGT3') and the size of amplified frangment was 245 bp. Moreover, *C. quinqueseptatum* can be detected after 6 days inoculation with ITSFCQ-1f and ITSFCQ-1r primers although the disease symptom was not developed.

In the present study, the screening of restriction enzymes for digestion of ITS region was conducted to distinguish between *Ps. eucalypti* and other closed related species, *Ps. oldie* and *Ps. variable* using CLC Main Workbench. The screening of 10 enzymes showed that *Alu* I and *HinC* II enzymes could differentiate *Ps. eucalypti* and *Ps. oldii* from *Ps. variable*. While, *Ctr1* 0I, *EcoR* I *Hae* II *Hae* III *HinC* II *Mse* I *Stu* I and *Taq* I enzymes could not distinguish three species, *Ps. eucalypti*, *Ps. oldii* and *Ps. variable* (Fig. 5). Result suggested that the RFLP based identification was not suitable to use for distinguish these species. In the contrast, Maharaj and Rampersa (2011) used the restriction enzymes to differentiate *Collectotrichum gloeosporioides* and *C. truncatum* which causing agents of chilli and papaya anthracnose. Fourty-eight isolates of *Collectotrichum* were amplified in the ITS1-5.8S-ITS2 regions of rDNA, the amplified fragementes were digested with *Alu*I, *Hae*III, *Pvu*II, *Rsa*I and *Sau*3A. Results indicated that these restriction enzymes can be used to distinguish between *C. gloeosporioides* and *C. truncatum* causing agents of papaya anthracnose. Moreover, *C. gloeosporioides* and *C. truncatum* isolated from chilli could be differentiated using *Alu*I, *Apa*I, *Pvu*II, *Rsa*I and *Sma*I restriction enzymes. As the previous report, Krupa (1999) identified the ectomycorrhiza using PCR-RFLP with *Alu* I, *Bsur* I, *Hinf* I, *Hpa* II and *Taq* I

restriction enzymes. Results showed the band patterns which specific to *Hebeloma crustuliniforme*.

In the present study, it is indicated that ITS sequence analysis based identification could be used to distinguish among *Ps. eucalypti* and other closely related species. Moreover, the nested primer should be developed for molecular identification of these species in the future.

Table 1. The specimens of *Pseudoplagiostoma eucalypti* used for ITS sequence analysis

Isolate	Location	GenBank accession number	Species
KAN3	Thailand	AB627948	<i>Ps. eucalypti</i>
KHO2	Thailand	AB630954	<i>Ps. eucalypti</i>
CHA1	Thailand	AB630955	<i>Ps. eucalypti</i>
CHA2	Thailand	AB630956	<i>Ps. eucalypti</i>
CHA3	Thailand	AB630957	<i>Ps. eucalypti</i>
CHA4	Thailand	AB630958	<i>Ps. eucalypti</i>
NAK1	Thailand	AB630959	<i>Ps. eucalypti</i>
NAK2	Thailand	AB630960	<i>Ps. eucalypti</i>
CBS115788	Thailand	GU973532	<i>Ps. eucalypti</i>
CPC13473	Thailand	GU973530	<i>Ps. eucalypti</i>
CPC13471	Thailand	GU973529	<i>Ps. eucalypti</i>
CPC14075	China	GU973519	<i>Ps. eucalypti</i>
CPC14163	Uruguay	GU973518	<i>Ps. eucalypti</i>
CPC12292	Bhutan	GU973515	<i>Ps. eucalypti</i>
		EU255001	<i>Plagiostoma devexum</i>
		EU255044	<i>Gnomonia rhododendri</i>

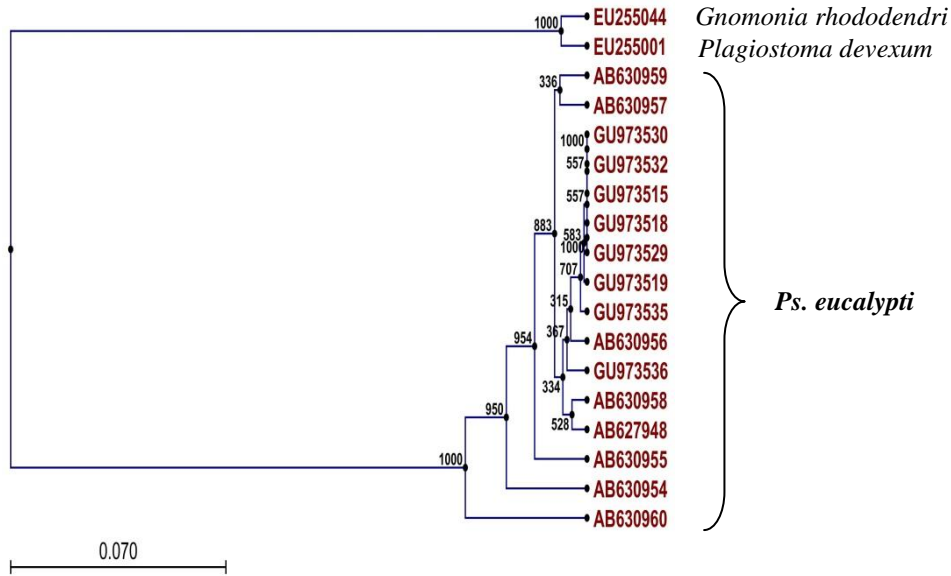


Fig. 3. UPGMA phenogram showing the phylogenetic relationship within the species of *Ps. eucalypti* based on ITS rDNA sequences. Bootstrap values are indicated on the branches (1000 replication). Bar, 7 nt substitutions per 100 nt.

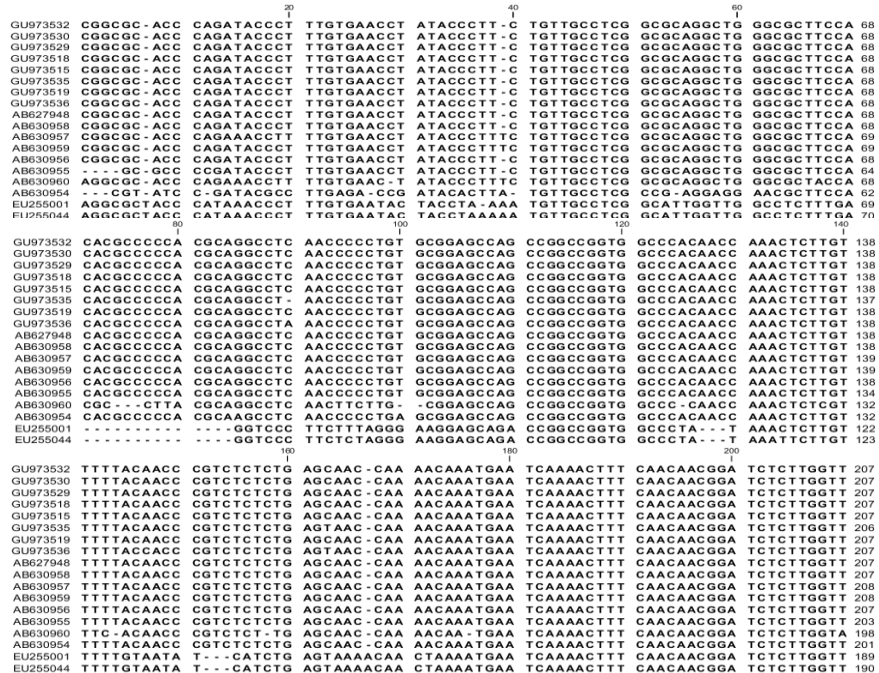


Fig. 4. Multiple alignment of ITS sequence of *Ps. eucalypti* with sequences obtained from GenBank (DDBJ) using CLC Main workbench (version 5.5).

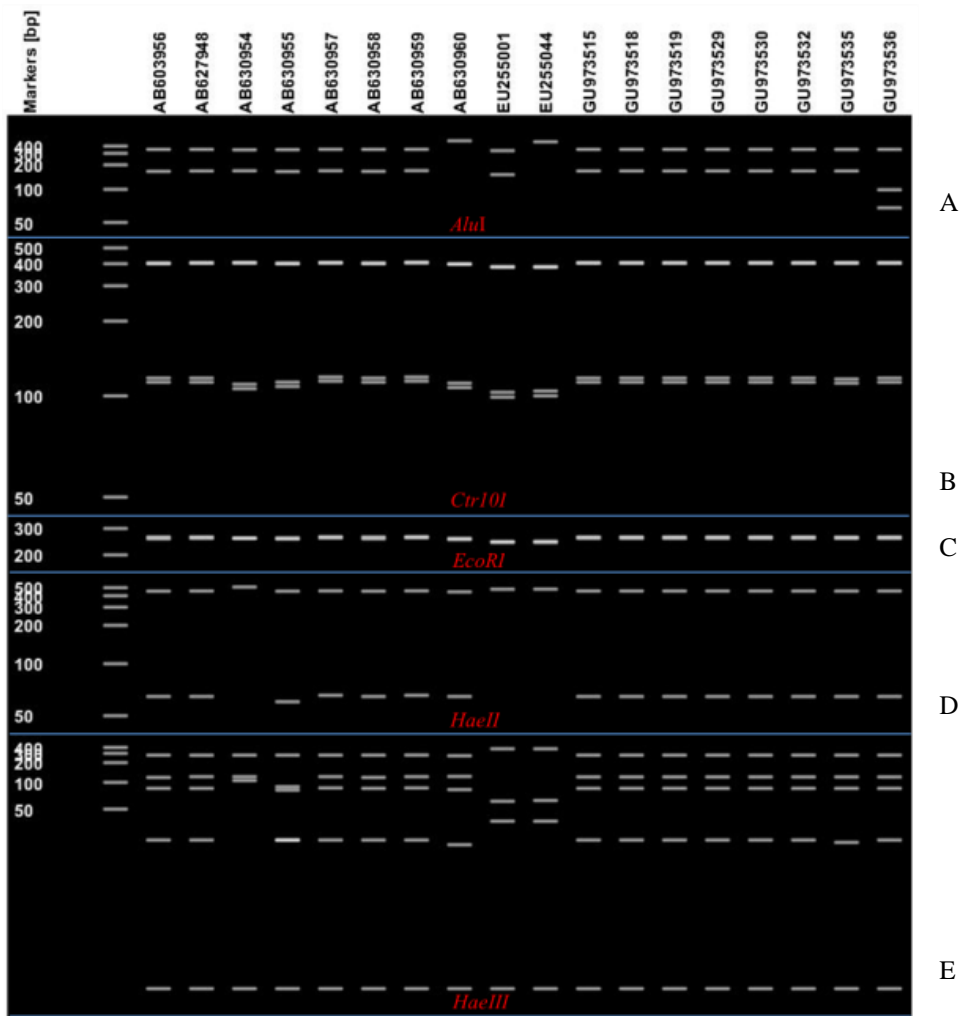


Fig. 5. *In silico* RFLP band pattern obtained from digestion of ITS rDNA of *Ps. eucalypti* and other related species with *Alu* I (A) *Ctr1* 0I (B) *Eco* RI (C) *Hae* II (D) and *Hae* III (E) restriction enzymes using CLC Main workbench (version 5.5).

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