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CHARACTERIZATION AND *IN VITRO* ANTIFUNGAL ASSAY AGAINST *FUSARIUM OXYSPORUM F.SP.PASSIFLORA* OF ENDOPHYTIC ACTINOMYCETES FROM PURPLE PASSION FRUIT PLANTS OF SOUTH SULAWESI, INDONESIA

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Abstract - Endophytic actinomycetes from purple passion fruit were screened and evaluated for their antifungal activity against of pathogenic fungi. A total of 32 isolates were obtained from people passion garden in Malino distric, South Sulawesi province, Indonesia. Morphological and chemotaxonomical analysis indicated that twenty-two isolates belonged to the streptomyces like-strain. This genus was the most dominant among the isolates (68.75%) of plant organs (roots, leaves and stem barks) of these showed inhibitory activity against *Fusarium oxysporum* f.sp. *passiflora*. Actinomycetes were most commonly recovered from leaves (50% of all isolates), followed by roots (43.75%) and stems (6.25%). Based on the phenotypic properties and phylogenetic and analysis of 16S rRNA gene, the selected strain EML-D1A5 was assigned to *Streptomyces durhamensis* strain CSSP538 (97% similarity).

INTRODUCTION

Association of endophytic microbe with their hosts are unique interaction because the microbe inhibits the inner organs and tissues of plants such as roots, stems and leaves without causing diseases (Coêlho *et al*. 2011). Endophytes with this capacity might profit from association with the plant, because colonization is enhanced. In turn, host plants benefit by biocontrol potential agent against fungal root and seed rots (Yuan *et al*. 1995). Althought the interaction between two organisms is not, yet, fully understood, over recent years they have been progressively extensively employed, either in agriculture (Ryan *et al*. 2008) or antimicrobe, anticancer (Li *et al*. 2008) and production of valuable pharmaceutical compounds (Stoble and Long. 1998). The presence of endophytic microbe in healthy plant crops has been demonstrated in the roots of plants such as maize (Araujo *et al*. 2000), banana (Cao *et al*. 2005) and some medicinal plants (Taechowisan and Lumyong 2003; Passari *et al.* 2015). Endophytic Actinomycetes have positive effect on host plant by producing phytohormone. Plant-associated Actinomycetes are rich sources of bioactive compounds including indole-derived molecules such as phytohormone indole-3-acetic acid (IAA) (Ting *et al*. 2008; Jasim *et al*. 2014), production auxin and gibberellins (Brown. 1972; Merckx *et al*. 1987). The metabolites of endopytic Actinomycetes inhibit a number of microbes (Gurney and Mantle. 1993; Yu *et al*. 2010), antibacterial activities (Castillo *et al*. 2006) and antifungal activities (Gupta *et al*. 1995; Ezra *et al*. 2004). Some endophytic bacteria exert several beneficial effect on host plants such as induction of resistances to plant pathogen (Chen *et al*. 1995; Sturz and Matheso. 1996), and nitrogen fixation (Kirchhorf *et al*. 1997). Numerous recent studies showed promising in endophytic Actinomycetes research. Application of microbial consortia that interact synergistically each other, they enhance the plant growth and protect from phyto-pathogens (Kurth *et al*. 2014). Therefore, studies on

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biological control of plant diseases used endophytic Actinomycetes origin to be an alternative to protect locally agricultural crop plants.

Purple passion fruit is one of the economically important fruits particulary in South Sulawesi, Indonesia for decades. Recently, these plants began to decrease due to a fungal diseases pathogen. *F. oxysporum* f. sp. *passiflora* is a strain which causing a vascular wilt plant of purple passion fruit. The fungal are the most destructive plant pathogens causing wilt fungal diseases in many crop plants. Fungal strain devastated a great number of passion fruit plantation. Various methods were applied to controlling the diseases such as application of fungicides and planting of fungal resistance varieties, but these method were uneffective. A cost-effective measure of control for the disease is still not available. At this time, many methods practically use to control of diseases along with the need to develop sustainable methods of diseases management has started the hunt for a suitable alternative control. Biological control agent may be used as an alternative management strategy because it is not only suppressing the diseases and increasing crop yield but also reducing the enviromental pollution due to chemical pesticides (Achari and Ramesh. 2014). Several studies have shown that endophytic bacteria isolated from crops such as tamato and chili (Amaresan *et al*. 2012), chickpea (Misk and Franco. 2011) and citrus (Araujo *et al*. 2002). However, the screening of antifungal endophytic actinomycetes of purple passion fruits has remained unexplored.

The present study was carried out to isolate Actinomycetes from roots, stem bark and leaves of passion fruits, widely cultivated in South Sulawesi, Indonesia. The microbes were screened for antifungal activities for the purpose of this study, endophytic Actinomycetes were isolated and screened *in vitro* for their abilities to inhibit fungal pathogens.

MATERIALS AND METHODS

Plant Material. Samples (roots, leaves, stem bark) of purple passion fruits were collected from passion fruits of farm garden at Malino district, South Sulawesi province, Indonesia. Plant samples were kept in sterilized plastic bags and stored at 4°C until isolation. Isolation of strain was carried out immediately after samples were sent back to the laboratory.

Isolation and selection of Actinobacteria. All the collected samples of the purple passion fruits were washed with running tap water to remove soils and debris. Isolation of endophytic actinomycetes was done by cutted the tissues into small pieces (0.2 x 4 cm^2) and subjected to surface sterilization. The excised roots, leaf and bark stems were surface- sterilized using by serial treatment of etanol 70% (v/v) for 10 min, 1% sodium hypochlorite for 2 min. Finally, root tissue, leaf and stem bark were washed in sterilized distilated water for a 5 minutes, then dried using sterile filter paper. Approximately 0.1 x 1 cm^2 of plant tissue were cutted and transferred onto starch casein (SC) agar supplemented with nystatin 100µg/mL. The surface sterilization process was confirmed by spreading aliquots of the sterile distilled water from the final rinse on SC agar medium, followed by incubation at 35°C, and observation of microbial growth. If there was no visible growth of microbial colony on the surface of agar plates, the sterilization was assumed completed. Colonies of endophytic actinomycetes appeared surrounding of plant sample tissue after incubation at 35°C for 2-3 weeks. Purified of endophytic actinomycetes cells was done by transferred of colonies onto freshly mannitol-soy agar medium plate until a single colony showed purity. The pure culture was maintained at a 15% sterile glycerol suspension at -80°C for long-term preservation.

Screening for antifungal antagonism. *In vitro* antifungal activities of endophytic actinomycetes isolates were assesed by using dual assay antagonistic method against the *Fusarium oxysporum* f. sp. *passiflora* (Barakate *et al*. 2002). Each of actinomycetes isolated obtained from isolation procces was spreaded onto SC agar medium and incubated 35°C for 7 days. A plug agar of isolates (6 mm in diameter) was tranferred onto the edgen of sabaroud agar plate, while the tested fungi inoculated at the edge of the 9 cm plate on the same media. The plates were incubated 30°C for 7 days. Tested fungal plugs were also placed on uninoculated actinomycetes used as control. The endophytic actinomycetes was showed inhibition growth against to inhibited growth fungi considered as endophytic actinomycetes producing antifungal. The fungal inhibition was calculated from the equation:

$$\mathrm{R} = \frac{(R1 - R2)}{R1}\ x\ 100$$

Where: R = fungal inhibition (%), R1 = the fungal growth radius of control culture, R2 = the distance of fungal growth in the direction of actinomycete colony.

Morphological and physiological characterization To study cultural caharacteristic of selected isolate, the strain EML-D1A5 was grown on several media, namely yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4),(ISP7). Aerial and substrate mycelia were determined by comparison with colour chips from the Color Harmony Manual (Jacobson *et al*. 1958). In order to examine morphological characteristics, the selected strains were grown on SC agar by using slide culture method and spore morphology were assesed by light microscopy.

Physiological caharacteristics of strain EML-D1A5 were determined as follow. Hydrolysis of starch was determined as described by Gordon *et al*. (1974). Utilization of carbohydrates and nitrogen as sole carbon and nitrogen sources was tested by using medium (ISP9), respectively as described by Shirling and Gottlieb. (1966). The temperature range for growth was determined on ISP2 in a temperature gradient incubator.

Molecular characteristics and Phylogenetic analysis. The 16S rRNA gene from selected strain was amplified and sequenced. The DNA genome of strain EML-D1A5 extraction and purification was carried out using the Pure link genomic DNA kit (In-vitrogen, Carlsbad, CA, USA) according to the manufacturer instructions. The 16S rRNA gene was amplified using the following primers following primers: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) to amplify 1500bp fragment. The PCR cycling was conducted as follow: pre-denaturation of the target DNA at 96^{o}C for 3 min followed by 30 cycles at 95^{o}C for 1 min, primer annealing at 56^{o}C for 1 min, and primer extension at 72^{o}C for 5 min, the reaction mixture was held at 72^{o}C for 5 min. The resulting PCR products were visualized by electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide (Petrosyan *et al.* 2003). The amplicon of DNA fragments was sequenced using sequencer model ABI 3100 sequencer according to manufacturers' instructions (ABI PRISMA 3100 Genetic Analyzer User's Manual).

The nucleotides obtained were subjected to BLAST analysis using by the NCBI database deposited in NCBI GenBank. The 16S rRNA gene sequence of strain EML-D1A5 was aligned with those of representatif strain members of other 39 Streptomycete genera using CLUSTAL-X. Phylogenetic trees were deduced by using the neighbor-joining method using the Phylip version 3.5 (Saitou and Nei. 1987) with bootstrap values based on 1000 replication (Felsenstein. 1985).

RESULTS

Evaluation of the surface sterilization protocol

A total of 32 of Actinobacteria endophytic strain were collected from purple passion plant health at five sampling locations of passion fruit plants of farming garden in Malino distric, South Sulawesi, Indonesia. Evaluation of samples from all actinomycetes cultured with the surface sterilization protocol showed no Actinomycetes growth on SC agar. It showed that epiphytic Actinomycetes were eliminated, so the strain were obtained indeed endophytic. The strains were classified into streptomycete-like strain (characterized by abundant aerial mycelium with powdery spores) and non-streptomycetes strain (slimy colonies coloured red, oranges and brown to black). The strains were grouped into different plant organs such as root, leaves and stem bark on agar starch casein agar media. In vitro antagonistic assay was used to select Actinomycetes which produce antifungal against phytopathogenic fungi. There are five isolates obtained from roots and leaves inhibited the growth of fungal tested. However, no isolate from stems bark inhibites fungi (Table 1).

Screening for antifungal antagonistic

Five of 23 isolates obtained showed strongs antagonisms against to *Fusarium oxysporum* f.sp.

Table 1. Endophytic actinomycetes was obtained from plant organs of purple passion fruit plantation in South Sulawesi, Indonesia.

Group	No. of isolates		
	Roots	Leaves	Stem bark
Streptomycete-like strain	8 (3)	13 (2)	1
Non-streptomycetes strain	6 (1)	3	1
Total	14	16	2
Percentage	43.75	50	6.25

Number in brackets indicate that the number of isolates that inhibit of *Fusarium oxysporum* f.sp. *passiflora* by testing *in vitro* *passiflora* (Fig. 1). Morever, the selected strain has been demonstrated for a variety of plant pathogen and showing inhibitory activities against many species of tested fungi such as *Phytophthora* sp, *Aspergillus* sp, and *Trichoderma* sp (data not shown). The percentage of inhibitory of selected actinomycetes strain showed that the five selected actinomycets strains have inhibitory potency pathogenic fungi with inhibitory level more than 60 % at the end of incubation (Fig. 2). Strain EML-D1A5 showed the highest inhibitory among selected strains (83.6%), whereas the the lowest is EML –A2P1(69 %).

Preliminary identification of selectes endophytic strain

Morphological and physiological properties of strain EML-D1A5 shown in Figure 3. According to microscopic morphology observation (100x magnification) showed that selected strain has a typical character as the *Streptomyces* genera. The strain showed the structure of spore chains opened spiral.

DISCUSSIONS

In the present study, there were 23 Actinomycetes strains obtained from the purple passion fruit plants. The result showed that endophytic Actinomycetes can be isolated amongs inside the roots, leaves and stem bark of purple passion fruits. Majority of the strain from roots and leaves were Streptomyces like strain indicating that mainly Streptomyces spp genera are existing as endophytic in purple passion fruits. Endophytic streptomycete actinomycetes (SA) and nonstreptomycete actinomycetes (NSA) have been isolated from within live tissues of various plant species (Coombs and Franco 2003; Rosenblueth and Martinez-Romero 2006). Streptomyces is also the most dominant endophytic genus in the roots of plants such as tomato (Coa *et al*. 2004; Sardi *et al*. 1992), wheat (Justin and Christopher. 2003) and some medicinal plants (Qin *et al*. 2009).

Predominance of streptomycetes in any habitat is well known both in rhizosphere area and or in plant tissue. The presence of endophytic Actinobacteria assosiated with plant tissues may play important roles in plant both health and development. Many studies reported that bacteria (such as Actinobacteria) protect plant against pathogens like fungal diseases (Zhao *et al*. 2012). Moreover, the endophytic bacteria may influences of their metabolic product for plant growth and physiologic (Gupta *et al*. 1995). Endophytes with this capacity might profit from association with the plant, because colonization is enhanced.

Antagonistic activities of microbe have been reported against several fungal plant pathogens fungal such as bacteria, actinomycetes and fungi. Here we report the antifungal properties of endophytic actinomycetes isolates of passion fruit against *Fusarium oxysporum* f.sp. *passiflorae*. Inhibiting capacity of pathogenic fungi beside passion fruits-Fusarium wilt fungi showed that strain EML-D1A5 has high potency as fungal biocontrol agent. The capabilities of the strain to inhibit of passion fruit-Fusarium wilt pathogen and other genera of fungal show that this strain most prospect applied for pathogen fungal controlling particulary on agricultural crop plant. Some endophytic bacteria direct the potential benefit on the host plant, such as antagonist against fungal pathogen including including *Fusarium oxysporum* (Coa *et al*. 2005), *Rhizoctonia solani* (Sadeghi *et al*. 2006) and *Verticillium dahliae* (Meschke and Schrempf. 2010), *Gaeumannomyces graminis* var. *tritici* and *R. solani* (Coombs *et al*. 2004). In addition, endophytic Actinomycetes has been reported the stimulation of plant growth through the formation of growth hormones (Sturz *et al*. 1997; Stoltzfus *et al*. 1997; Reinhold-Hurek and Hurek 1998) and induction of plant resistance to pathogens (Liu *et al*. 1995). Mechanisms of action of endophytic actinomycetes in suppressing pathogen are production of substances particulary secondary metabolite (antibiotics), cell wall degrading enzymes (chitinase) and competition for nutrient (sideropore)(El-Tarabily and Sivasithamparam 2006).

Strain EML-D1A5 was observed to grow well on some of the media tested, including ISP1, ISP 2, ISP 3 (oatmeal agar), ISP 5 and Bennet but it has no growth on ISP4 (inorganic salts-starch). The aerial and substrate mycelia were white on all tested agar media. Utilization on of carbon sources was determined on ISP 9 medium supplemented with sterile carbon sources. The strain utilized glucose, galactose, manitol, xylose, raffinose, sucrose and dextrose but not lactose, rhamnose, fructose, sorbitol. Besides the different of genotypic character, there are many phenotypic differences between strain EML-D1A5 and the most closely related species of the genus Streptomyces (Table 5). The phylogenetic tree (Fig.3) reconstructed on

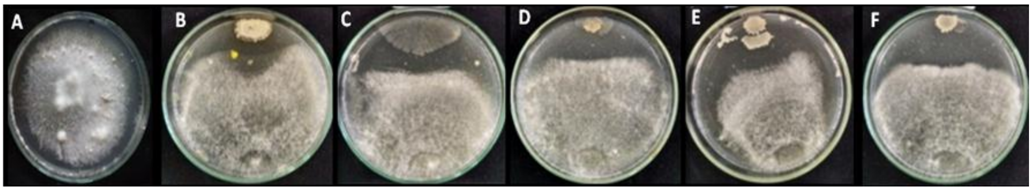

Fig 1. *In vitro* inhibition activity of selected endophytic actinomceyes strain against test fungi A). *Fusarium oxysporum* f. sp. *passiflorae*, B); Strain EML-A_2P_1; C). Strain EML-A_1L_1; D). Strain EML-A_1N_3; E). Strain EML-D_1A_3 and F). Strain EML-D_1A_5

Table 2. Cultural characteristics of strain EML-D1A5 on different culture medium

Agar Medium	Aerial Mycelium		Substrate mycelium		Growth	Soluble pigment
	HCM	Colour	HCM	Colour		
ISP1	RAL 1013	Oyster white	RAL 1014	Ivory	Good growth	-
ISP2	RAL 1013	Oyster white	RAL 1014	Ivory	Good growth	-
ISP3	RAL 1015	Light ivory	RAL 1015	Light ivory	Moderate growth	
ISP4	-	-	-	-	No growth	-
ISP5	RAL 1015	Light ivory	RAL 1014	Ivory	Good growth	-
BENNET	RAL 1013	Oyster white	RAL 1013	Oyster white	Moderate growth	-

HCM, Harmony Colour Manual

Table 3. Pysiological properties of strain EML-D1A5 on physico-chemical factor

pH		NaCl (%)		Temperature (°C)	
3	-	0	+	4	-
4	-	3	++	25	++
5	+	4	+	30	++
6	+	5	-	35	++
7	+	6	-	37	++
8	++	7	-	40	-
9	+	8	-	45	-
10	+/-	9	-		
11	+/-	10	-		
12	-	11	-		

++= good growth ( ), += moderate growth ( ), +/- = weak growth, - = no growth

Table 4. Utilization of nitrogen and carbon sources of strain EML-D1A5

No	Nitrogen sources utilization		Carbon sources utilization	
1	$(NH_4)_2SO_4$	++	D-glucose	+
2	$AgNO_3$	-	D-lactose	-
3	$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	-	D-galactose	+
4	$C_2H_8N_2O_4$	++	D- mannitol	+
5	$(NH_4)(NO_3)$	++	D-xylose	+
6	$NaNO_3$	-	Rhamnose	-
7	KNO_3	++	Raffinose	+
8	$Fe(NO_3)_3$	-	Sucrose	+
9	$Ba(NO_3)_2$	-	L-fructose	-
10	L-valine	+	Sorbitol	-
11	L-glutamic acid	+	Cellobiose	-
12	L-asparagine	++	Maltose	-
13	L-arginine	+	Dextrose	+
14	Iso leucine	+	Myo-Inositol	-
15	DL α-amino n- butyric acid	-	Arabinose	+

++ : growth, well utilized , +: growth, moderate utilized, +/- : growth, weakly utilized, -: not utilized

Table 5. Phenotype and pysiological properties of strain EML-D1A5 and related species

Characteristic	Strain EML-D1A5	*Streptomyces durhamensis* AS 4.1699	*Streptomyces puniciscabiei* KACC 20253	*Streptomyces filipinensis* AS 4.1452
Morphology and pigmentation				
Aerial mass colour	W	GR	WO	G
Substrate mycelium	W	WB	WB	Y
Spore chain morphology	Spiral	Spiral	Rectiflexuous	Spiral
Soluble pigment on ISP3	-	-	-	-
Carbon utilization				
Raffinose	+	+	+	+
D-xylose	+	W	+	+
Rhamnose	-	-	+	-
D-Manitol	+	+	+	+
Lactose	-	-	ND	ND
L-Arginine	+	+	+	-
pH4	-	-	+	+
7% NaCl	-	-	+	-

+, Well utilized/ present; W, weakly utilized; 2, not utilized/absent; ND, not determined. W, white; GR, grey–red; WO, white–orange; G, grey; WP, white–purple; WB, white–brown; Y, yellow; BR, brown–red.

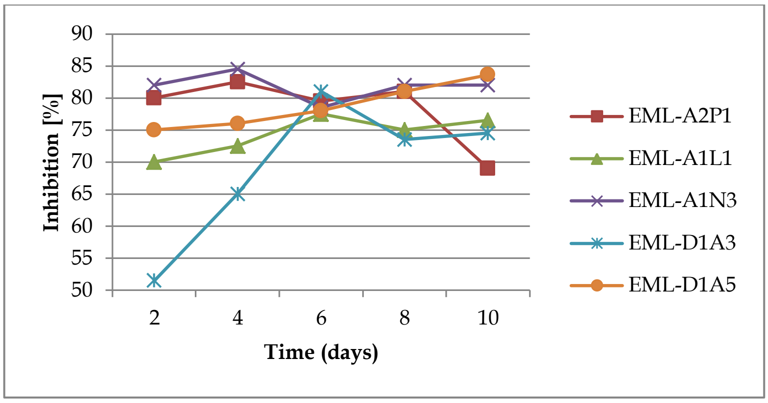

Fig 2. Inhibition of tested fungal of *Fusarium oxysporum* f.sp. *passiflorae* by selected endophytic actinomycetes strain on Starch Casein agar for different incubation periods

the basis of 16S rRNA gene sequences indicated that strain EML-D1A5 was phylogenetically most closely affiliated to the genus Streptomyces Strain EML-D1A5 shared similarity to *Streptomyces durhamensis* ATCC 23194 (97 % similarity). Based on the phenotypic properties and phylogegetic and analysis of 16S rRNA gene, the strain EML-D1A5 was assigned to *Streptomyces durhamensis* strain CSSP538 clade (NCBI Genbank).

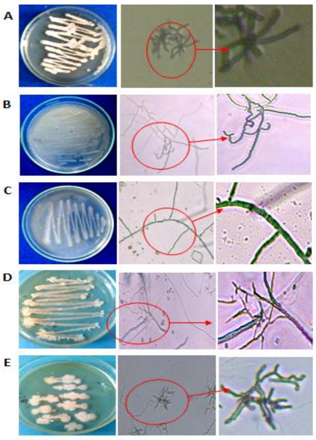

Fig 3. Morphology of colony and mycelium structure of endophytic selected strain (magnification 400X). A Strain EML-A_2P_1; B). Strain EML-A_1L_1; C). Strain EML-A_1N_3; D). Strain EML-D_1A_3 and E). Strain EML-D_1A_5

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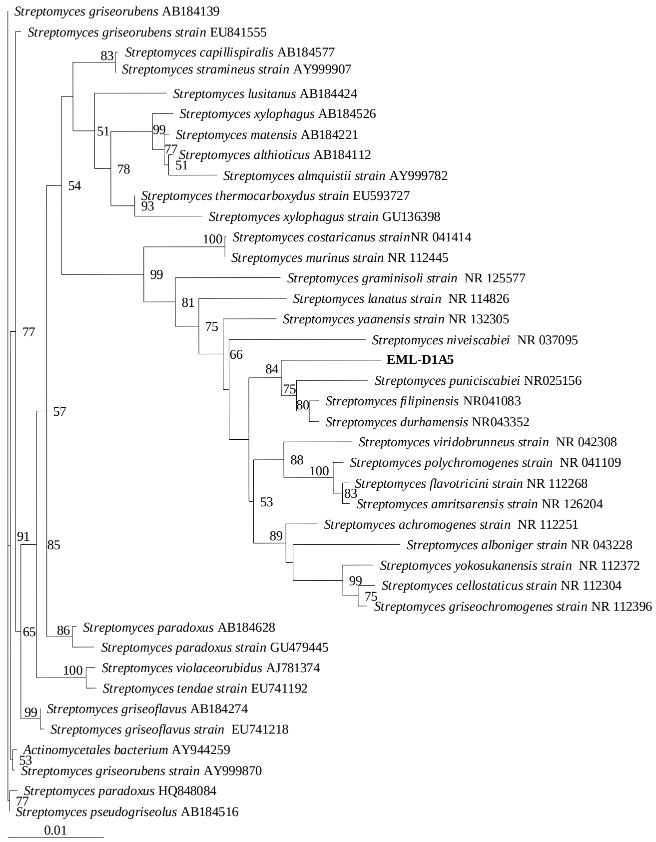

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Fig 3. Neighbor-joining phylogenetic tree inferred from 16S rRNA gene sequences. The phylogenetic tree shows the phylogenetic relationship of endophytic bacteria strain EML-D1A5 with related genera. Bootstrap values are expressed as percentages of 1000 replications. Bootstrap values, ≥50% are shown at branch points. Score bar represents 1 nucleotide substitution per 100 nucleotides**.**

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