Research Article

Biodiversity of endophytic bacteria isolated from duckweed (*Landoltia punctata*) and their IAA production

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Abstract

Endophytic bacteria are recognized for their plant growth promoting benefits. One of the mechanisms is the production of the phytohormone indole-3-acetic acid (IAA). In this study, duckweed isolates A1 and A2 were collected and characterized as *Landoltia punctata* based on their morphological characteristics and the phylogenetic analysis of the *atpF-atpH* intergenic region sequences. Seventy-one endophytic bacteria were obtained from both duckweed isolates. Molecular analyses of the 16S rRNA gene sequences revealed that the endophytic bacterial communities consisted of members in phyla *Actinobacteria* (10 isolates), *Deinococcus-Thermus* (1 isolate), *Firmicutes* (55 isolates) and *Proteobacteria* (5 isolates). Screening for IAA production yielded 27 positive strains. Quantification of the IAA content indicated variable efficiencies in IAA production by these endophytic bacteria. The highest IAA level was observed in *Deinococcus* sp. L2-88 (713.2±11.6 µg/mL). The results obtained from our study displayed the biodiversity of endophytic bacteria from *L. punctata* and suggest their potential role in plant growth promotion through IAA production.

Keywords: Duckweed; *Landoltia punctate*; 16S rRNA; Indole-3-acetic acid; endophytic bacteria.

1. Introduction

Duckweeds are small aquatic flowering plants that belong to the family *Lemnaceae* which consists of five different genera, namely *Lemna*, *Spirodela*, *Landoltia*, *Wolffia* and *Wolffiella.* Currently, 38 different species have been identified and characterized [1]. Duckweed plants generally float on the surface of or are slightly submerged under the water. Leaf-like fronds are modified leaves and stems that mainly function in photosynthesis and vegetative reproduction. Typically, morphological characteristics can be used for characterization of duckweeds at the genus level. However, identification at the species level is relatively difficult because of their small and highly reduced structures. Examination of these characteristics requires special expertise in plant morphology and anatomy [1]. A previous study has demonstrated the use of the *atpF-atpH*

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intergenic region as the DNA barcode specifically for the family *Lemnaceae*. The result showed that the sequence variations found in this region could be used to effectively differentiate 97 duckweed accessions representing 31 species at the species level. Thus, the region has been proposed as the DNA barcode marker for confirmation of duckweed species [1].

Bacteria that colonize intercellular spaces of a plant host without causing diseases are generally recognized as endophytic bacteria [2]. Endophytic bacterial communities have been studied in various monocotyledonous and dicotyledonous plant species [3]. Advances in sequencing technology and expansion of the 16S rRNA gene sequence database provide us a better understanding in the biodiversity of endophytic bacteria. This has led to discoveries of many bacterial endophytes that represent novel species in several genera [4-7]. Endophytic bacteria have been considered beneficial for the plant host. Several plant growth promotion activities have been reported in endophytic bacteria [3]. Production of the phytohormone IAA is one of the mechanisms through which endophytic bacteria promote plant growth. IAA is an auxin that is involved in virtually all aspects of plant growth and development [8]. A previous study showed the molecular responses of plant cells towards IAA that was produced by endophytic bacterium *Burkholderia kururiensis* [9]. Other studies have also demonstrated that inoculation of IAA-producing bacteria stimulated root hair and lateral root formation. This directly increased the water and mineral uptake ability of the plants and subsequently resulted in a higher growth rate [8]. The biosynthesis of IAA has been extensively studied in many bacterial species. Generally, the amino acid tryptophan is converted to IAA through five different biosynthetic pathways that resemble those found in plants [8]. In the present study, 71 isolates of endophytic bacteria of two *L. punctata* isolates were isolated and characterized. Their abilities in producing IAA were also examined.

2. Materials and methods

2.1 Plant materials

Duckweed isolates A1 and A2 were collected from natural ponds in Samutprakan and Bangkok provinces, respectively. Plants were examined for their morphological characteristics including frond morphology, the presence of roots and the number of roots on the lower side of each frond.

2.2 Amplification and sequencing of the *atpF-atpH* intergenic region

Duckweed fronds were ground into fine powder in liquid nitrogen in a microtube with a small pestle. DNA extraction, primers and conditions for amplification of the *atpF-atpH* intergenic region were performed according to the previously described method [1]. Sequencing of the amplified fragments was carried out with the *atpF-atpH* forward primer [1].

2.3 Phylogenetic analysis of the *atpF-atpH* intergenic region sequences

The sequences of the *atpF-atpH* intergenic region of various duckweed species were obtained from the GenBank database. Multiple alignment of the sequences of the *atpF-atpH* intergenic region of duckweed isolates A1 and A2 and those of other duckweed species was performed using CLUSTAL W, version 1.81 [10]. The alignment was manually adjusted to remove gaps and ambiguous bases. The phylogenetic tree was reconstructed using the unweighted pair group method with arithmetic means (UPGMA) using the MEGA program [11]. The bootstrap analysis [12] based on 1,000 resamplings was used to determine the confidence level of each cluster.

2.4 Isolation of endophytic bacteria from duckweed

Approximately one g of duckweed plants was rinsed with water to remove dirts on the outside and surface-sterilized with 10% sodium hypochlorite solution

supplemented with a few drops of Tween-20. Plants were washed five times in sterilized distilled water and ground with a mortar and a pestle. Ten-time dilution of the suspension was plated on 1/10 strength tryptic soy agar (TSA) and starch casein nitrate agar plates and incubated at 30°C for 7 days. Isolates with different colony characteristics were selected and purified by cross streaking on TSA plates. One hundred µl of the water used for the final rinse was plated on 1/10 strength TSA and starch casein nitrate agar and used as controls.

2.5 Bacterial DNA isolation and amplification of the 16S rRNA gene

Genomic DNA of endophytic bacteria was prepared according to the method previously described [13]. Amplification of the 16S rRNA gene was performed using universal primers 27F and 1492R [14]. The following temperature profile was used for amplification: initial denaturation at 94°C for 3 min; 40 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 90 sec; and final extension at 72°C for 5 min.

2.6 Amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of the 16S rRNA gene

The enzyme *Alu*I was used for the restriction analysis of amplified 16S rRNA gene fragments of each bacterial isolate. Five hundred ng of purified 16S rRNA gene fragments were used in digestion reactions. Digested products were examined by electrophoresis in 1.5% agarose gel. At least 30% of bacterial isolates of each ARDRA group were randomly chosen as representatives for partial sequencing of the 16S rRNA gene with the universal primer 27F. Pairwise analysis of the partial 16S rRNA gene sequences was performed using the EzTaxon server [15].

2.7 Screening for IAA-producing endophytic bacteria

Bacterial cells were grown in nutrient broth (NB) containing 5mM of L-tryptophan at 30°C for 48 hr. Bacterial cells were pelleted, and 200 µL of the culture supernatant was tested for the presence of IAA by adding 200 µL of the Salkowski's reagent [16]. The positive result was indicated by the solution turning pink.

2.8 Quantification of IAA production levels using spectrophotometry

Endophytic bacteria were grown on NA at 30°C for 48 hr and resuspended in 0.85% NaCl solution. Cell concentration was adjusted to McFarland standard No. 0.5. One hundred µl of bacterial suspension were inoculated into nutrient broth medium containing 5 mM of L-tryptophan and grown at 30°C for 48 hr. The culture supernatant was obtained by centrifugation. Two mL of the supernatant was added with 2 mL of Salkowski's reagent and quantified for light absorbance using the spectrophotometer at the wavelength 530 nm. The amount of IAA in the culture supernatant was determined using the IAA standard curve. All measurement was done in triplicate.

3. Results and Discussions

3.1 Characterization of duckweed isolates A1 and A2

Morphological characteristics including frond morphology, the presence and the number of roots of duckweed isolates A1 and A2 were examined. Fronds of both isolates were oval-shaped with dark and light green colors on the upper and lower sides of the fronds, respectively (Figure 1A). Accumulation of red pigments and multiple roots were observed on the lower side of each frond (Figure 1B). Characterization of members in the family *Lemnaceae* traditionally relied on morphological and anatomical characteristics [17]. The presence of roots can be used to differentiate *Landoltia, Lemna* and *Spirodela* from *Wolffia* and *Wolfiella* [17]. Members in genera *Landoltia* and *Spirodela* produce several roots on the lower side of each frond, while those in the genus *Lemna* produce one root per frond. Reddish pigments are accumulated in vegetative fronds of

Landoltia and *Spirodella* species but absent in *Lemna* species. Based on our observation on frond and root characteristics, duckweed isolates A1 and A2 were likely members of either the genera *Landoltia* or *Spirodela*.

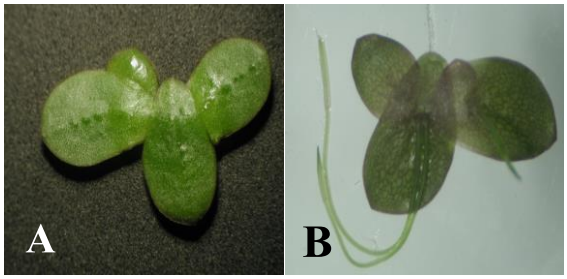

Fig.1. Morphological characteristics of duckweed isolates A1. The upper and lower sides of the sample are shown in (A) and (B), respectively.

For molecular characterization, the plastid *atpF-atpH* intergenic region of duckweed isolates A1 and A2 were examined. The phylogenetic analysis was performed between the sequences of the duckweed isolates A1 and A2 and those available in the GenBank database. The similarity levels between duckweed isolates A1 and A2 and other isolates of *L. punctata* were 100%. Consistently, the phylogenetic tree reconstructed by the UPGMA method showed that isolates A1 and A2 formed a clade with *L. punctata* isolates 7248, 7487, 7260, 8721, 7449 and 9278 (Figure 2). This was significantly supported by the bootstrap value of 100%. DNA barcoding is a molecular method that has been widely used in identification of living organisms. For example, the mitochondrial *cytochrome c oxidase subunit I* (*COI*) gene has been successfully used as the DNA barcode marker for identification of animals at the species level [18-19]. However, the application of *COI* for DNA barcoding in the plant kingdom has proved difficult [1]. Instead, the use of multiple loci for plant identification has been proposed [20]. However, a previous study demonstrated that the *atpF-atpH* intergenic region can be effectively used as a single universal DNA barcode marker at the species levels in the family *Lemnaceae* [1]. Taken together with the morphological characteristics, the results obtained from the phylogenetic analysis indicated that duckweed isolates A1 and A2 are *L. punctata.*

3.2 Diversity of bacterial endophytes from *L. punctata*

Seventy-one bacterial isolates were obtained from *L. punctata* isolates A1 and A2. Genomic DNA of bacterial endophytes was isolated and used for amplification of the 16S rRNA gene. ARDRA analysis of amplified 16S rRNA fragments was used to assign isolates to ARDRA groups. The restriction analysis yielded 21 different ARDRA groups. At least 30% of the isolates from each group were randomly selected for partial sequencing of the 16S rRNA gene. ARDRA has been described as a tool for the differentiation of bacteria [21]. The method relies on dissimilarities of 16S rRNA gene sequences between different bacterial species. Amplified 16S rRNA gene fragments are digested with a restriction enzyme to generate restriction patterns. Bacterial strains with similar restriction patterns are placed into the same ARDRA group. Subsequently, representatives of each ARDRA group are selected for 16S rRNA gene sequencing. The identity of non-sequenced strains could be inferred from those of the sequenced strains of the same ARDRA group. A previous report showed that 169 endophytic bacteria isolated from soybean plants were divided into eleven ARDRA groups. For characterization of all isolates at the genus level, at least 20% of the bacteria from each ARDRA group were chosen for 16S rRNA gene sequencing [22]. Another study showed that 148 isolates of mycobacteria obtained from clinical samples could be effectively identified by comparing their restriction patterns with the reference patterns of known *Mycobacterium* species.

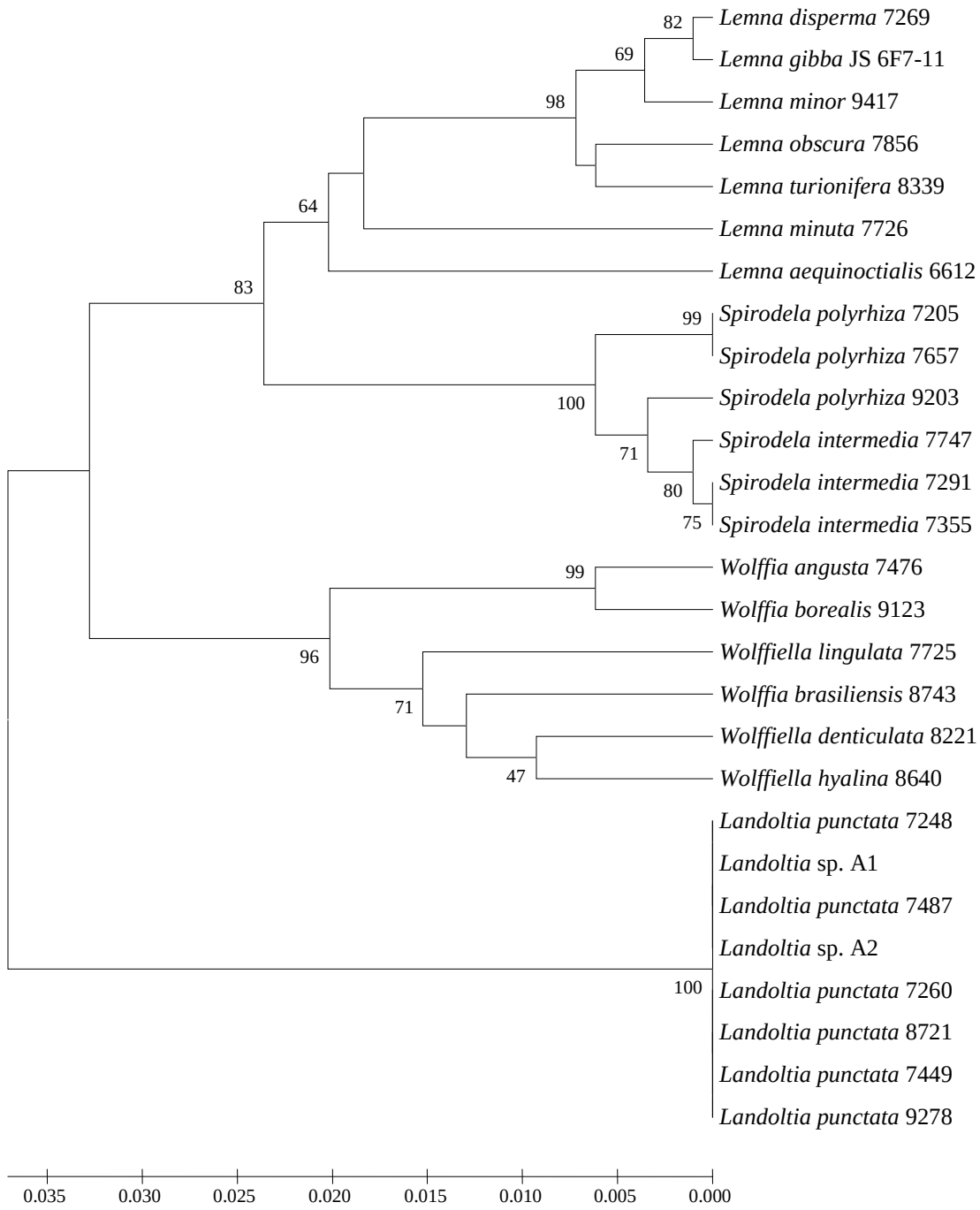

Fig.2. Phylogenetic tree demonstrating the phylogenetic relationship between *Landoltia* sp. isolates A1 and A2 and other members in the family *Lemnaceae*. The tree was reconstructed using the UPGMA method and based on the *atpF-atpH* intergenic region sequences. The numbers of the bootstrap value are present at tree nodes as the percentage of 1,000 replicates. Only values higher than 50% are shown.

Table 1. Diversity of endophytic bacteria. Isolates designated with L1 and L2 were obtained from *L. punctata* A1 and A2, respectively. Isolates indicated in bold were used for the analysis of the partial 16S rRNA gene sequences.

ARDRA	Phylum	Genus	Isolates
1	*Actinobacteria*	*Janibacter*	L2-71, L2-79, L2-89, **L2-90**, L2-92, **L2-97**
2	*Actinobacteria*	*Janibacter*	**L2-95**, L2-96
3	*Actinobacteria*	*Starkeya*	L2-27, **L2-35**
4	*Deinococcus-Thermus*	*Deinococcus*	**L2-88**
5	*Firmicutes*	*Bacillus*	**L2-21**, L2-40, **L2-49**, L2-50, L2-60, L2-62, **L2-75**
6	*Firmicutes*	*Bacillus*	**L2-67**
7	*Firmicutes*	*Bacillus*	**L2-48**
8	*Firmicutes*	*Bacillus*	**L1-14**, L1-21, L1-24, L1-25, **L1-S11**, L1-S26
9	*Firmicutes*	*Bacillus*	**L2-2**
10	*Firmicutes*	*Bacillus*	**L1-1**, **L1-3**, L1-4, L1-5, L1-6, L1-7, L1-11, L1-12, **L1-13**, **L1-17**, L1-26, L1-27, L1-28, **L1-S1**, **L1-S4**, L1-S5, L1-S8, L1-S13, L1-S16, **L1-S17**, L1-S19, **L1-S20**, L1-S23, L1-S28, L1-S29, **L1-S31**, L1-S34, L1-S35
11	*Firmicutes*	*Bacillus*	**L1-S3**, L1-S10, **L1-S18**, L1-S27, L2-11, L2-80
12	*Firmicutes*	*Bacillus*	**L1-S22**
13	*Firmicutes*	*Bacillus*	**L1-2**
14	*Firmicutes*	*Bacillus*	**L1-10**
15	*Firmicutes*	*Halobacillus*	**L2-73**
16	*Firmicutes*	*Paenibacillus*	**L2-32**
17	*Proteobacteria*	*Acinetobacter*	**L2-24**
18	*Proteobacteria*	*Acinetobacter*	**L2-70**
19	*Proteobacteria*	*Brevundimonas*	**L2-72**
20	*Proteobacteria*	*Caulobacter*	**L1-S9**
21	*Proteobacteria*	*Rhizobium*	**L2-30**

Additionally, the average time for the analysis was 36 hours [23]. These examples demonstrate the advantages of ARDRA in terms of the cost reduction for DNA sequencing, the effectiveness and the rapidness of the technique. The pairwise alignment analysis of the partial 16S rRNA gene sequences using the EzTaxon server revealed the diversity of the endophytic bacterial community of *L. punctata* (Table 1). The majority of the endopytic bacteria of *L. punctata* in this study belonged to those of *Firmicutes*. They represented 77.4% (55 isolates) of the population, and members of the genus *Bacillus* were mostly found. *Actinobacteria* was the second largest group (14.1%; 10 isolates), and *Janibacter* species were dominant in this group. Five isolates (7.1%) of bacterial endophytes were in the phylum *Proteobacteria* and were classified as members of the genera *Acinetobacter*, *Brevundimonas*, *Caulobacter* and *Rhizobium*. Only one isolate (1.4%) was identified as *Deinococcus* sp. of the phylum *Deinococcus-Thermus.*Very little is known about endophytic bacterial communities of *L. punctata*. Previously, the diversity of endophytic bacteria was reported in some

aquatic plant species including *Phragmites communis*, *Potamogeton cripus*, *Nymphaea crispus* and *Najas marina*, and the largest group of endophytic bacteria was members of the phylum *Proteobacteria* [24]. This is in contrast to the result obtained in our study where members of the phylum *Firmicutes* were dominant. Additionally, the distinctions in endophytic bacterial communities obtained from two duckweed isolates were also observed. All isolates that belonged to the phyla *Actinobacteria* and *Deinococcus-Thermus* were obtained from *L. punctata* isolate A2. In contrast, 74.5% of the isolates that were classified as members of the phylum *Firmicutes* were from *L. punctata* isolate A1, while 80% of the isolates in the phylum *Proteobacteria* were from *L. punctata* isolate A2. Several factors that influence the structures of endophytic bacterial communities have been proposed. These included environmental conditions, the presence of phytopathogens, plant tissues, plant cultivars and plant species [13, 22, 24-27]. Because the two isolates of *L. punctata* were collected from different locations, our result suggests that environmental factors play an important role in the diversity of endophytic bacteria of *L. punctata* isolates A1 and A2. A previous report studied the variation of root-associated bacterial communities that were caused by soil types, maize cultivars and root localization. The results indicated that, among the three factors, the type of soil is the major cause that influenced the diversity of the bacteria [27].

3.3 Production of the phytohormone IAA by endophytic bacteria

All 71 endophytic bacteria were screened for their ability to produce the plant hormone IAA. The result showed that 27 isolates (38%) produced IAA in the test medium. Twenty-one IAA-producing isolates belonged to the genus *Bacillus.* Other isolates that were also tested positive for IAA production consisted of members in the genera *Deinococcus* (1 isolate), *Janibacter* (1 isolate), *Starkeya* (1 isolate), *Paenibacillus* (1 isolate), *Acinetobacter* (1 isolate) and *Brevundimonas* (1 isolate). IAA production levels of these isolates were quantified using spectrophotometry. The result showed that the IAA levels were relatively different between genera (Table 2). The highest IAA level (713.2$\pm$11.6 µg/ml) was observed in *Deinococcus* sp. L2-88. IAA production varied between *Bacillus* isolates. It ranged from 14.9$\pm$1.1 µg/mL in *Bacillus* sp. L1-14 to 225.7$\pm$28.7 µg/mL in *Bacillus* sp. L1-4. The IAA production level of *Paenibacillus* sp. L2-32 was also found at 124.1$\pm$13.9 µg/mL. In contrast, *Janibacter* sp. L2-71 (37.1$\pm$10.7 µg/mL), *Starkeya* sp. L2-35 (18.7$\pm$0.9 µg/mL), *Acinetobacter* sp. L2-70 (29.2$\pm$8.7 µg/mL) and *Brevundimonas* sp. L2-72 (46.2$\pm$3.4 µg/mL) were less efficient in producing IAA.

The results obtained from our study was consistent with previous reports. Various *Bacillus* species have been demonstrated as IAA producers, for example, *Bacillus amyloliquefaciens*, *Bacillus subtilis* and *Bacillus megaterium* [28-30]. IAA production was also demonstrated in members of genera *Janibacter*, *Paenibacillus*, *Acinetobacter* and *Brevundimonas* [31-34]. In contrast, there was no report on IAA production by *Deinococcus* and *Starkeya* species. However, a search in the Uniprot database (http://www.uniprot.org) yielded one result that was indicated as an auxin efflux carrier protein of *Deinococcus geothermalis* DSM 11300^{T}. This suggests that *Deinococcus* bacteria are likely another group of IAA producers.

The plant hormone IAA has been recognized as a critical phytohormone required for cell division, cell extension, cell differentiation and organ development [35]. As a result, IAA production is considered one of the direct mechanisms in plant growth promotion by endophytic bacteria [35]. A previous study demonstrated that Indian mustard plants that were inoculated with IAA-producing strains of *Bacillus megaterium* and *Pseudomonas* sp. exhibited

an increase in the biomass as well as tolerance towards Ni toxicity [36]. Similarly, IAA-producing strains of *Bacillus, Pseudomonas* and *Staphylococcus* were shown to increase the IAA content and growth of *Triticum aestivum* var. Inqalab-91 [37]. Additionally, the level of the IAA production is also an important factor in plant growth promotion. A study showed that, when *Medicago truncatula* were treated with an IAA-overproducing strain of *Sinorhizobium meliloti*, the IAA contents in roots and nodules as well as the salt tolerance of the plant host were increased [38]. However, the plant-growth-promoting efficiency of endophytic bacteria is not always in a direct proportion with their IAA production levels. In a previous study, inoculation of canola seedlings with an IAA-overproducing mutant strain of *Pseudomonas putida* resulted in inhibition of root elongation compared to those inoculated with the wild-type strain of *P. putida* [39]. This was explained by the interaction between IAA and the phytohormone ethylene. The excessive amount of IAA that was incorporated into plant roots may have increased the biosynthesis of the 1-aminocyclopropane-1-carboxylate (ACC) synthase enzyme. This potentially led to the accumulation of ACC that was subsequently converted to ethylene which inhibited root growth [40]. Based on these previous reports, the consideration for the utilization of endophytic bacteria as biofertilizers may be primarily based on their IAA production levels. However, more study on the effects of these endophytic bacteria on economically important crops will also be needed.

Table 2. IAA production by endophytic bacteria.

Isolate	Genus	IAA content (µg/mL)
L2-88	*Deinococcus*	713.2 ± 11.6^{a}
L2-71	*Janibacter*	37.1 ± 10.7^{j}
L2-35	*Starkeya*	18.7 ± 0.9^{j}
L2-40	*Bacillus*	15.3 ± 3.4^{j}
L2-75	*Bacillus*	20.2 ± 11.1^{j}
L2-48	*Bacillus*	$215.0 \pm 12.7^{c,d,e}$
L1-14	*Bacillus*	14.9 ± 1.1^{j}
L1-3	*Bacillus*	$126.5 \pm 15.8^{f,g,h,i}$
L1-4	*Bacillus*	$225.7 \pm 28.7^{b,c,d}$
L1-6	*Bacillus*	$221.0 \pm 13.1^{b,c,d}$
L1-11	*Bacillus*	296.5 ± 25.6^{b}
L1-12	*Bacillus*	235.7 ± 42.9^{c}
L1-17	*Bacillus*	$149.8 \pm 1.3^{d,e,f,g}$
L1-27	*Bacillus*	$209.8 \pm 21.9^{c,d,e}$
L1-28	*Bacillus*	$66.4 \pm 13.8^{h,i,j}$
L1-S4	*Bacillus*	$184.5 \pm 7.4^{c,d,e,f}$
L1-S16	*Bacillus*	$138.2 \pm 11.0^{e,f,g,h}$
L1-S17	*Bacillus*	$151.6 \pm 0.7^{d,e,f,g}$
L1-S19	*Bacillus*	$203.4 \pm 15.9^{c,d,e,f}$
L1-S20	*Bacillus*	22.7 ± 3.2^{j}
L1-S29	*Bacillus*	$189.0 \pm 17.7^{c,d,e,f}$
L1-S35	*Bacillus*	$174.5 \pm 16.8^{c,d,e,f}$
L1-S10	*Bacillus*	$93.7 \pm 6.4^{g,h,i,j}$
L2-80	*Bacillus*	28.4 ± 8.7^{j}
L2-32	*Paenibacillus*	$124.1 \pm 13.9^{g,h,i,j}$
L2-70	*Acinetobacter*	29.2 ± 8.7^{j}
L2-72	*Brevundimonas*	46.2 ± 3.4^{j}

Means ± SD with different letters are significantly different at the 0.05 level by the Tukey's test (n=3).

4. Conclusion

In this study, the combination of morphological traits and the DNA-based marker is effective for a characterization of *L. punctata* isolates A1 and A2. We also examined the biodiversity of endophytic bacteria isolated from the two duckweed isolates. Our results indicated that members of the phyla *Actinobacteria*, *Deinococcus-Thermus*, *Firmicutes* and *Proteobacteria* colonized the internal tissues of the two duckweed isolates. Additionally, the diversity of these endophytic bacteria was likely influenced by environmental factors. The IAA production levels were variable among endophytic bacteria. These IAA-producing endophytic bacteria are potential candidates for further study and utilization as biofertilizers in economically important crop plants.

5. Acknowledgement

This work is financially supported by the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang.

6. References

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