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Research Article

Molecular Characterisation of Bacterial endophytes from the Medicinal Plant *Diplorhynchus condylocarpon*

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Abstract

Endophytes are microbes found within live plant tissues and are known to be reservoirs of bioactive compounds that promote plant growth and antimicrobial activity. This makes them important, but are understudied. In this study endophytes from *Diplorhynchus condylocarpon* plant were characterized at molecular level and their potential to produce bioactive compounds with antimicrobial activity was explored. DNA extraction was carried out on twenty-four endophytes isolated from leaves and twigs of *Diplorhynchus condylocarpon*. To confirm the nature of these endophytes at kingdom level the *16S rRNA* gene was amplified using the Polymerase chain reaction and all indicated to be prokaryotes in nature. Nine *16S rRNA* amplicons out of twenty-four were sequenced and species were identified as *Izhakiella capsodis, Escherichia fergusonii, Shigella dysenteriae, Xernorhabdus szentirmaii, Providencia rettgeri, Dickeya zeae* and *Escherichia albertii*. Agar well diffusion was used to test for antimicrobial activity of twenty-four endophytes. All twenty-four endophytes exhibited antimicrobial activity against E. coli and S. aureus, with wide range of zones ranging from 19 to 65 mm. Using KEGG database it was discovered that sequenced species have potential to produce antibiotics such as fosfomycin and streptomycin. Secondary agar well diffusion was done to compare potency of endophytes extracts with ampicillin, a known antibiotic. Endophytic extracts were observed to be more effective than ampicillin with highest zones of inhibition for extracts and ampicillin as 54 and 36 respectively. In conclusion this study showed that endophytes from *Diplorhynchus condylocarpon* are reservoirs of bioactive compounds with antimicrobial effect. Also, that the *16S rRNA* gene can be used to identify species.

Keywords: *Diplorhynchus condylocarpon*; Endophytes; Molecular Characterization; Bioactive Compounds; Antimicrobial Assay; 16S rRNA

Introduction

Use of traditional plants for medicinal purposes has been one of the most affordable and easy access to treatment of poor communities in Zimbabwe and there is a long history of use of plants for medicinal purposes. About 10% of all species growing in Zimbabwe have medicinal properties. Within 10% shrubs and trees taking 38% each, herbs at 21% and climbers at 3%. *Diplorhynchus condylocarpon* also known as *Mutowa* in Shona is a plant found in Zimbabwe and other tropical, southern African countries. It can grow either as a shrub or a tree and has been used for medicinal purposes.

With an increase in drug resistance which has become a global health issue, new methods to solving this crisis and exploring medicinal properties of endophytes have been adopted by many countries. Endophytes are microbes that reside inside living plant tissues. Endophytes are phylogenetically rich, hyper-diverse species, ecologically important and are under explored species with wide range of genetic and functional diversity. Taxonomic classi-

fication of endophytes begins at the kingdom level (Fungi or bacteria). Almost every plant species across the globe is colonized by endophytes [17]. No visible harm has been noticed to be caused by endophytes in a plant they colonize. Relationship between the plant and endophytes is usually mutualistic [1].

Molecular characterization is a technique used to differentiate and identify organisms by characterizing at the molecular level without depending on factors such as environment, physiological state or development of an organism [4]. Molecular techniques involving the use of small ribosomal RNA (rRNA) sequence variability as a biomarker has been used mostly for the past years to characterize bacterial endophytes. There are several advantages associated with using *16S rRNA* gene sequence as a biomarker in molecular characterization of bacterial endophytes because, these include its presence in all prokaryotes species, high variation across species and presence of many sequences of *16S rRNA* from various organisms are present in the database and this gives reference sequences when identifying species [11].

Despite the identification of endophytes and potential they pose in many biological applications, there is no significant information about endophytes in Africa before 1994. Between 1994 and 2014 Egypt and South Africa were the two leading African countries to have researched on endophytes. In Zimbabwe use of endophytes for medicinal purposes and other biological applications has been approached in recent years [16]. *Diplorhynchus condylocarpon* also known as *mutowa* in Shona is a plant found in Zimbabwe and other tropical, southern African countries. *Diplorhynchus condylocarpon* has been used traditionally to treat several infections such as indigestion, diarrhea, fever, snakebites and infertility. Endophytes from *Diplorhynchus condylocarpon* are distributed in all parts of the plant therefore characterization of these endophytes is less expensive and can be used in biotechnological processes.

In this study endophytes from *Diplorhynchus condylocarpon* plant were characterized at molecular level and their potential to produce bioactive compounds with antimicrobial activity was explored. The *16S rRNA* sequencing technique is also used for identification of the bacterial species.

Materials and Methods

Collection of samples

The study was carried using endophytes isolated from two *Mutowa* trees obtained from different geographical locations (Kwekwe and Karoi). The endophytic isolates were procured from the glycerol stocks prepared by Ms Jordanca Kugara. To induce growth, the endophytic isolates were cultured on potato dextrose agar (PDA) and incubated at room temperature for 24 hours. Each colony that grew was sub-cultured in Luria Bertani broth (LB) from Kirkhouse Trust England and incubated at room temperature for 4 days.

Media preparation

Agar plates preparation

The media was prepared by dissolving 40 g of PDA powder in 1000 ml distilled water and the mixture was autoclaved for 15 minutes at 121^{o}C. The sterile molten PDA media was poured in sterile petri dishes and left to solidify.

LB broth preparation

The liquid media was prepared by dissolving 11.5 g of LB broth powder in 500 ml of distilled water. The mixture was then autoclaved for 15 minutes and 121^{o}C. The media was allowed to cool before inoculation of endophytic isolates.

Genomic DNA extraction

An aliquot of 500 ul of CTAB buffer was mixed with 500 ul of liquid overnight culture in a 1.5 ml centrifuge tube. The mixture was then incubated at 55 °C for 30 minutes. After the incubation the samples were centrifuged for 5 minutes at 5000 rpm and the supernatants were then transferred to new tubes. Equal volume of chloroform was then added to each tube, centrifuged at 5000 rpm for 3 minutes and the supernatants were transferred to new tubes. Subsequently, an equal volume of ice-cold isopropanol was added to each tube and the mixture was incubated for 20 minutes at -20 °C. Afterwards, the tubes were centrifuged at 10000 rpm for 10 minutes and the supernatants were discarded. The pellets formed were washed by adding 500 ul of ice-cold 70% ethanol and centrifuged at 10000 rpm for 3 minutes. The supernatant was discarded and the pellets were dried in a speed vac for 10 minutes. An aliquot of 50 ul of TE buffer was added into each tube to dissolve the nucleic acids. The quality of DNA in each sample was evaluated by electrophoresis in 0.8% agarose gel at 100 V for 30 minutes. Afterwards, the agarose gel was visualized under UV light and a picture of the DNA bands was taken.

16S PCR assay

The bacterial *16S rRNA* was amplified using the genomic DNA template and 27F (5'- AGAGTTTGATCCTGGCTCAG -3') forward primers and 1492R and (5'-CGGTACCTTGTTGTTACGACTT-3')

reverse primer. The PCR reactions were carried out with 25 ul master mix containing (5 ul 5 x PCR buffer,

2.5 ul 2 mM dNTPs, 1.5 ul 25 mM MgCl2, 3 ul Taq DNA polymerase, 1 ul 27F primer, 1 ul 1492R primer and 1 ul endophytic genomic DNA). The PCR was carried out in a thermo cycler (Applied Biosystems, model 2720) under these conditions, 95°C for 5 minutes for denaturation, 95°C for 45 seconds for further denaturation, 60°C for 40 seconds for annealing of DNA with primers, primer elongation at 72°C for 3 minutes and a final extension for 5 minutes still at 72°C to complete the full cycle. The PCR machine was set to stop after thirty-five cycles. The amplified DNA was then evaluated by electrophoresis in 1% gel at 100 V for 30 minutes. The gel was visualized using UV light and the picture of bands was taken.

16S rRNA gene sequencing

The *16S rRNA* gene amplicons were sent to Stellenbosch University in South Africa for sequencing.

Phylogenetic analysis

Multiple sequence alignment was done on *16S rRNA* gene sequences using Clustal omega program. This program makes use of seeded guide trees and Human Markov Models profile-profile technique in aligning sequences. After aligning sequences this program builds a phylogenetic tree.

Antibacterial assay

Extraction of bioactive compounds from endophytes

An aliquot of 200 ul 10% SDS was mixed with 600 ul of liquid 4 days culture in a 2 ml centrifuge tube. The mixture was then mixed and incubated at room temperature for 4 minutes. After incubation samples were centrifuged for 5 minutes at 10000 rpm. Supernatants were transferred to new tubes. Equal volume of acetone was added to each tube to, centrifuged for 3minutes at 5000 rpm. After centrifugation top aqueous layer which contained extracted bioactive compounds was then transferred to different boats of known mass and samples were dried overnight in the laminar hood. Dried extracts were then resuscitated using 120 ul 2% DMSO. The same process was repeated using two other solvents methanol and ethyl acetate. However, for ethyl acetate samples were dried and resuscitated within the same day because ethyl acetate extracts dry fast.

Preparation of tester plates

Test microorganisms, *E. coli* (ATCC 35218) and *S. aureus* (ATCC 25923) were obtained from University of Zimbabwe department of biochemistry. Overnight cultures of bacterial tester strains were prepared by mixing 1 ml of tester strain and 10 ml of LB broth and incubated at 37^{O}C. The following day OD of the cultures was measured using spectrophotometer and standardized to 1 OD by diluting with LB broth. In the hood a volume of 100 ul of 1 OD bacterial tester strain culture was then spread on top of PDA plates. Plates were then left for 1 hour and then 3 holes were created on the plates using the back of 200 ul pippete tips. In the first hole 10 ul of endophytic extract was added, on the other holes 25 ul and 50 ul volumes of extract were added. Plates were placed in the 37^{O}C incubator and observed after 24, 48 and 96 hours.

Preparation of plates with ampicillin as a control

Tester plates were prepared almost the same way as mention above. The only difference is that acetone alone was used as a solvent and in place of 25 ul extract, 10 ul of ampicillin was added. Plates were then incubated and observed after 24 and 48 hours.

Results and Discussion

Antibacterial activity of endophytes

Twenty-four bacterial endophytes were isolated from two sets of mutowa tree (Kwekwe and Karoi respectively). 60% of endophytes were isolated from the leaves and 40% from twigs. All endophytes isolates were able to inhibit *E. coli* and *S. aureus* in agar well diffusion assay and zones of inhibitions ranged from 22 mm to 65 mm for 50 ul extract (Table 3). Extracts were effective on *S. aureus* to a greater extent compared to *E. coli*. This study showed broad antimicrobial activity spectrum against both Gram negative and Gram-positive bacteria. Significant difference in antimicrobial effect was noted between two strains used (*S. aureus* and *E. coli*). *S. aureus* was more susceptible compared to effect on *E. coli*. This correlates to study carried out by [10] that observed that Gram positive bacteria and more susceptible to antibiotics compared to Gram negative, this is because Gram negative have largely impermeable cell wall. Also [3] who found out that endophytes from *Azadirachta indica* produced bioactive compound that had antibacterial effect against both gram negative and gram positive bacteria.

From this study it was observed that most acetone extracts had highest zones of inhibition followed by ethyl acetate and low antimicrobial potency was observed in methanol extracts. According to [2] acetone and ethyl acetate are polar aprotic solvents meaning they are capable of extracting both polar and non- polar compounds while methanol is a polar protic solvent that can extracts polar compounds. Difference in yield between acetone and Ethylacetate indicate that acetone is a better solvent. Different volumes of extracts from endophytes were used for the agar well diffusion assay, with the 50 ul showing a greater inhibition compared to the 10ul and 25 ul volumes (Table 1 and 2). Highest zones of inhibition were

observed from sample 5, 8, 15, 18, 20, 22. All samples were observed to have broad spectrum against both tester strains with all the three solvents. Nonetheless sample 6, 7, 9, 13, 14 and 21 showed less potency.

Sample		Acetone			Methanol		Ethyl acetate		
	10 ul	25 ul	50 ul	10 ul	25 ul	50 ul	10 ul	25 ul	50 ul
	mm	mm	mm	mm	mm	mm	mm	Mm	mm
1-Leaf	20	26	29	23	31	36	25	33	50
2-Leaf	28	34	43	25	29	33	23	30	38
3-Twig	26	39	33	29	32	43	27	35	45
4-Leaf	30	41	42	10	21	26	22	35	37
5-Leaf	29	34	53	25	26	32	15	19	27
6-Twig	33	35	35	22	28	34	--	--	--
7-Twig	37	37	38	24	29	41	--	--	--
8-Twig	39	32	37	30	32	40	25	25	30
9-Leaf	25	36	55	23	25	39	--	--	--
10-Leaf	36	34	35	19	24	38	27	27	40
11-Twig	19	24	35	24	25	45	22	22	30
12-Leaf	37	37	40	23	27	35	21	28	35
13-Twig	27	27	38	30	28	32	--	--	--
14-Leaf	39	30	40	27	23	39	21	34	35
15-Twig	17	35	33	18	22	31	25	40	51
16-Leaf	35	35	47	21	30	34	35	35	46
17-Twig	26	33	39	22	23	35	19	25	37
18-Leaf	24	29	55	21	32	34	28	30	42
19-Leaf	16	23	41	27	32	35	27	33	50
20-Leaf	22	29	48	24	26	30	39	36	52
21-Leaf	19	22	34	30	24	32	--	--	--
22-Leaf	20	32	59	22	23	40	33	44	58
23-Leaf	23	36	37	23	25	40	27	31	31
24-Twig	23	43	41	20	19	30	26	30	35

Table 1: Zones of inhibition observed at day 4 in well diffusion assays using solvent extracts (acetone, methanol an ethyl acetate) from 24 bacterial endophytes at different concentrations. *S. aureus* was used as tester strain. Zones were measured in millimeters (mm).

Sample		Acetone			Methanol		Ethyl acetate		
	10 ul	25 ul	50 ul	10 ul	25 ul	50 ul	10 ul	25 ul	50 ul
	mm	mm	mm	mm	mm	mm	mm	Mm	mm
1-Leaf	29	35	33	18	22	26	20	24	35
2-Leaf	19	19	36	15	20	23	31	31	48
3-Twig	34	40	45	13	18	26	32	32	45
4-Leaf	15	22	24	15	18	32	20	21	28
5-Leaf	17	21	29	18	21	26	29	38	40
6-Twig	16	20	30	20	25	31	21	27	27
7-Twig	20	20	38	16	19	31	--	--	--
8-Twig	27	28	45	15	20	22	23	37	55
9-Leaf	30	30	36	19	24	44	32	33	46
10-Leaf	25	31	31	--	17	25	33	40	40
11-Twig	22	22	24	15	19	27	25	36	44
12-Leaf	17	18	22	20	26	30	25	34	34
13-Twig	16	20	22	17	20	25	25	38	41
14-Leaf	18	19	30	17	25	26	20	20	20
15-Twig	14	24	30	14	19	26	27	34	40
16-Leaf	19	20	24	19	20	29	23	30	30
17-Twig	19	35	35	20	20	31	20	30	38
18-Leaf	18	19	22	21	25	30	21	35	39
19-Leaf	13	22	29	33	33	58	31	31	50
20-Leaf	14	25	26	15	17	22	29	42	65
21-Leaf	29	30	30	22	25	27	--	21	29
22-Leaf	13	18	24	32	32	38	--	17	19
23-Leaf	11	18	23	21	26	30	26	26	46
24-Twig	17	21	35	16	19	25	26	27	34

Table 2: Zones of inhibition observed at day 4 in well diffusion assays using solvent extracts (acetone, methanol an ethyl acetate) from 24 bacterial endophytes at different concentrations. *E. coli* was used as tester strain. Zones were measured in millimeters (mm).

Sample	Acetone		Methanol		Ethyl acetate	
	S. aureus Inhibition zones/mm	*E. coli* Inhibition zones/mm	*S. aureus* Inhibition zones/mm	*E. coli* Inhibition zones/mm	*S. aureus* Inhibition zones/mm	*E. coli* Inhibition zones/mm
1-Leaf	29	33	36	26	50	35
2-Leaf	40	36	33	23	38	48
3-Twig	39	45	43	26	45	45
4-Leaf	42	24	26	32	37	28
5-Leaf	53	29	32	26	27	40
6-Twig	35	30	34	31	--	27
7-Twig	38	38	41	31	--	--
8-Twig	39	45	40	22	30	55
9-Leaf	55	36	39	44	--	46
10-Leaf	35	31	38	25	40	40
11-Twig	35	24	45	27	30	44
12-Leaf	40	22	35	30	35	34
13-Twig	38	22	32	25	--	41
14-Leaf	40	30	39	26	35	--
15-Twig	35	30	31	26	51	40
16-Leaf	47	24	34	29	46	30
17-Twig	39	35	35	31	37	38
18-Leaf	55	22	34	30	42	39
19-Leaf	41	29	35	58	50	50
20-Leaf	48	26	30	22	52	65
21-Leaf	34	30	32	27	--	29
22-Leaf	59	24	40	38	58	19
23-Leaf	37	23	40	30	31	46
24-Twig	43	35	30	25	35	34

Table 3: Highest zones of inhibition observed at day 4 for well diffusion anti-microbial assays of extracts from 24 bacterial endophytes extracted using different solvents. Zones were measured at 50 ul of extracts with *S. aureus* and *E. coli* being used as tester strain.

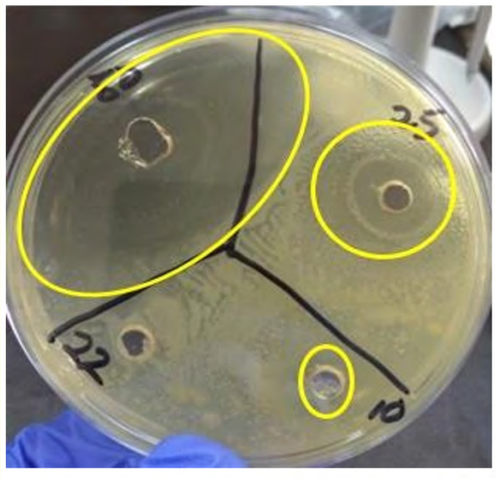

Figure 1: Zone of inhibition of the most potent bioactive endophytic extract 22 viewed after 72 hours of incubation at 37^{O}C against *S. aureus*.

Antibacterial assay with ampicillin as a control

After testing the effect of extracts from 24 endophytes against *E. coli* and *S. aureus* antimicrobial activity was noted and secondary assay was done. For the secondary assay, acetone extracts were plated on the same plate together with ampicillin in order to compare potency against *S. aureus.* The secondary disc diffusion assay was used where effect of 10 and 50 ul of extracts and 10 ul of ampicillin were measured. Zones of inhibition of ampicillin were ranging from 23 to 36 mm. For the 24 samples volumes of 10 ul gave zones of inhibition ranging from 26 mm to 38 mm whilst 50 ul gave 35 mm to 58 mm. zones of inhibition were noted after 48 hours (Table 4).

16S ribosomal RNA

Amplification of *16S rRNA* gene was successful in all twenty-four samples as shown in figure 3. This indicated that all endoph-

Sample	Ampicillin	Extract	
	10 ul Inhibition zones/mm	10 ul Inhibition zones/mm	50ul Inhibition zones/mm
1-Leaf	26	27	54
2-Leaf	26	30	50
3-Twig	36	38	45
4-Leaf	27	26	35
5-Leaf	31	31	38
6-Twig	27	33	44
7-Twig	29	34	49
8-Twig	32	30	48
9-Leaf	31	35	45
10-Leaf	31	30	51
11-Twig	25	31	51
12-Leaf	30	31	46
13-Twig	25	30	38
14-Leaf	27	34	46
15-Twig	25	29	49
16-Leaf	36	30	44
17-Twig	32	27	43
18-Leaf	30	29	49
19-Leaf	26	32	58
20-Leaf	28	30	47
21-Leaf	33	32	40
22-Leaf	29	32	45
23-Leaf	26	32	40
24-Twig	29	32	40

Table 4: Relative sizes of zones of inhibition obtained after incubating tester strain (*S. aureus)* with 24 different endophytes obtained from twigs and leaves of *mutowa* tree and ampicillin (control) for 48 hours.

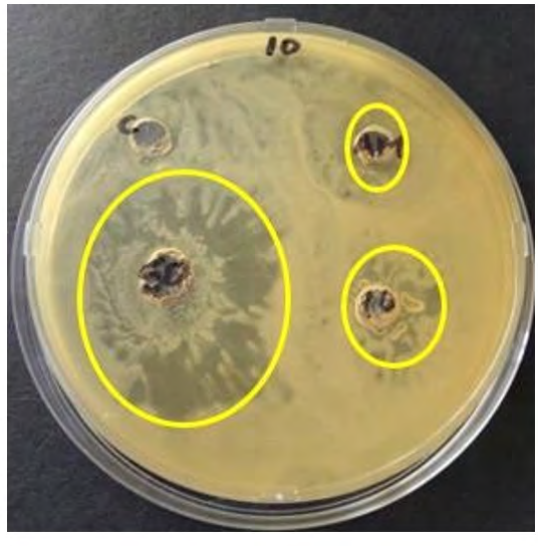

Figure 2: Zones of inhibition of endophytic extracts in comparison to ampicillin (AM) of concentration 16mg/ml observed after 48 hours of incubation at 37^{O}C against *S. aureus*.

ytes were prokaryotic in nature. 27F and 1492R primers were used because they can bind to highly conserved regions within the *16S rRNA* gene, present in all prokaryotic cell [15].

DNA sequence analysis

Sequencing of *16S rRNA* gene amplicons was delayed due to the Covid 19 pandemic inconveniences therefore only isolates 5, 7, 8, 9, 15, 16, 22, 23 and 24 were sequenced. These isolates were selected basing on those that gave higher zones of inhibition on antimicrobial assays. BLAST was used to reveal sequence identities which are summarized in table 5. Sequencing of the *16S rRNA* gene identified the isolates to strain level.

Izhakiella capsodis species is still a novel species as it was first described in 2016 and it has not been fully characterized. *Providencia rettgeri* was first described by Rettger in 1904 from a cholera like epidemic in chicken. This organism is a member of *enterobacteriaceae* family. Also known of its properties to ferment mannitol, Gram negative motile rod shaped and being a potential opportu-

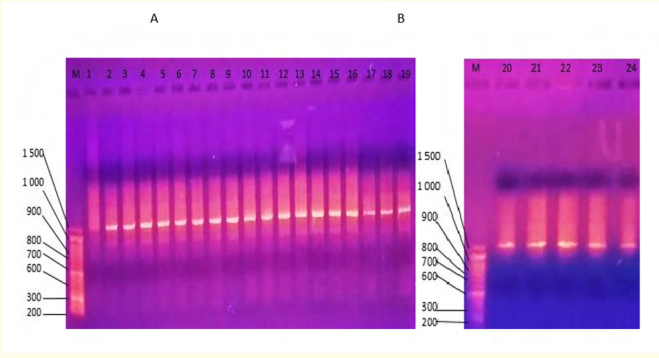

Figure 3: A and B showing ethidium bromide stained gel electrophoresis of 16S rRNA gene polymerase chain reaction from extracted genomic DNA.

Isolate	Identity	Percentage identity	Accession Number
5- Leaf	*Izhakiella capsodis*	96%	NR_148767.1
7- Twig	*Escherichia fergusonii*	96%	NR_074902.1
8- Twig	*Izhakiella capsodis*	95%	NR_148767.1
9- Twig	*Escherichia fergusonii*	96%	NR_114079.1
15- leaf	*Shigella dysenteriae*	93%	NR_026332.1
16- Twig	*Xenorhabdus szentirmaii*	93%	NR_042328.1
22- Leaf	*Providencia rettgeri*	87%	NR_042415.1
23- Leaf	*Dickeya zeae*	96%	NR_041923.1
24- Leaf	*Escherichia albertii*	96%	NR_025569.1

Table 5: Species identities.

nistic pathogen anaerobe that is rarely associated with human infections [14]. *Escherichia fergusonii* was first described as a new species of *enterobacteriaceae* family in 1985. It is a Gram-negative rod-shaped species, closely related to *Escherichia coli*. This bacterium was previously known as *Enteric* group 10. This was due to its biochemical nature compared to other *enterobacteriaceae* genus species. *Escherichia fergusonii* has been isolated from blood, urine and faeces of clinical human samples. As of 2017 there has been one reported case of this organism in Africa and was isolated in fish in Egypt [8].

Escherichia albertii was classified as Hafnia alvei like strain in early 1990s after being isolated from human stool [9]. It was then recognized as a unique enteropathogen in 2003 being associated with diarrhea and abdominal pain in Bangladeshi children. It is a Gram negative and rod shaped bacteria belonging to the *enterobacteriaceae* family [7]. *Shigella dysenteriae* was first described in 1897 by. It is Gram negative, facultative anaerobe of genus *Shigella* and is the principal agent of bacillary dysentery. This organism was not expected to be found in a plant where traditionally people used its decoction for medicinal purposes without having bacillary dysentery. In a study carried out by [13], they discovered that type III secretion system used by pathogenic strains to inject effector proteins to modulate host response were not highly conserved. And through genome analysis they discovered that some known pathogens do not have the type III SS when associated with plants as endophytes. This might explain the reason why Shigella *dysenteriae* is found *mutowa* tree endophyte and did not express the pathogenic system that could cause the disease.

Xenorhabdus szentirmaii. This organism belongs to *Xenorhabdus* genus of Gram negative, motile bacteria from *morganellaceae* family. *Xenorhabdus* species are usually found living in close symbiosis with nematodes from *Steinernema* genus [6]. According to study carried out by [6], who discovered that *Xenorhabdus szentirmaii* produces szentiamide class of depsipeptide and Fabclavine that have activity against gram positive, gram negative bacteria, *Plasmodium falciparum, Saccharomyces cerevisiae Trypanosoma brucei,* and *Trypanosoma cruzi.* [11] also stated that *Xenorhabdus szentirmaii* produced ngrA- dependent antibiotics which are active against several microbes and *Xenorhabdus nematophila.* The ability of *Xenorhabdus szentirmaii* to produce antibiotics as explained above can be used to produce effective antibiotics.

Phylogenetic analysis

The nine sequences were used to generate a phylogenetic tree (Figure 4). Three notable branches were observed from the tree. *Escherichia albertii* had no recent relative to any of the sequences other than the ancestral root. This indicates that *Escherichia albertii* diverged early and has a few changes from the most ancestral stain [10]. *Shigella dysenteriae, Xernorhabdus szentirmaii, Providencia rettgeri* and *Dickeya zeae* emerged from a common ancestor indicating that they are closely related. However, *Providencia*

rettgeri and *Dickeya zeae* share most recent ancestor making them more closely related species and recently diverged species in that cluster. Location of two strains of *Escherichia fergusonii* indicated that they diverged at the same time which is expected as they are same strains. Phylogenetic analysis indicated that two species of *Izhakiella capsodis* diverged at different times. Full genome sequencing would have given more insight on genetic differences between the two *Izhakiella capsodis* strains.

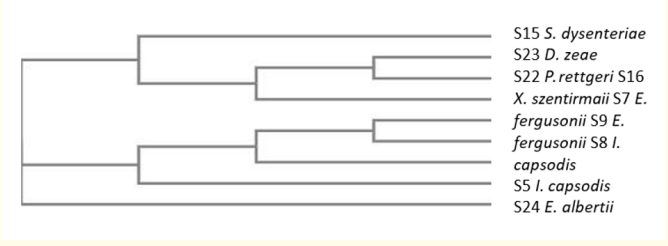

Figure 4: Phylogenetic tree constructed with sequences of the 16S rRNA regions of endophytic bacteria isolated from *mutowa* tree.

Restriction mapping

The link http://www.takara-bio.co.jp/enzyme/enzyme_search.php was used to develop restriction maps for two sequences of *Izhakiella capsodis.* This was done to compare similarity and differences of the *16S rRNA* gene of the two sequences. From the results obtained using restriction enzyme *EcoRI* and *Hind III* it was observed that the sequences belonged to two different species of *Izhakiella capsodis* as shown in figure 5.

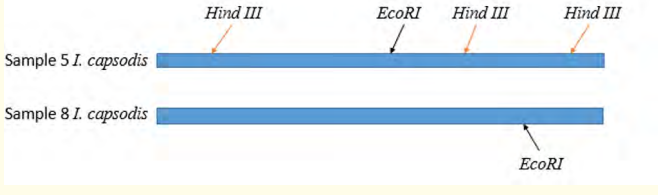

Figure 5: Restriction sites of restriction enzymes (*Hind III* and *EcoRI*) on the 16S rRNA gene sequence of two *Izhakiella capsodis* species (sample 5 and 8).

Pathway mapping

Pathway maps of sequenced isolates were obtained using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. From the database only pathway maps for *Shigella dysenteriae, Providencia rettgeri, Dickeya zeae*, *Escherichia albertii* and *Escherichia fergusonii* were found. Pathway maps for *Izhakiella capsodis* and *Xenorhabdus szentirmaii* were not found on the database. Pathway maps showed that isolates have potential to biosynthesize antibiotic compounds shown in table 6. KEGG pathway mapping results were in agreement with the antimicrobial assays findings.

Species	Bioactive compounds	Function
(*Escherichia fergusonii, Escherichia albertii* and *Shigella dysenteriae*)	Streptomycin, Monobactam, Novobiocin Carbapenem	Highly effective antibiotics, used to treat serious bacterial infections *Beta* lactam highly effective antibiotic used to treat high risk bacterial infections. Therapeutic drug monitoring important
	Acarbose	Slows digestion of carbohydrates, helps low blood sugar levels. It is used treat type 2 diabetes
	Validamycin	Aminoglycoside antibiotic and fungicide
Dickeya zeae	Streptomycin, Monobactam Novobiocin, Roseoflavin and Carbapenem Acarbose Validamycin	Highly effective antibiotics Highly effective antibiotics Used to treat type 2 diabetes Fungicide and antibiotic
	Cycloserine	Used to treat active drug resistant tuberculosis
Providencia rettgeri	Streptomycin, Monobactam, Bacilysin, Aurachin, Novobiocin, Kanosamine, Fosfomycin Acarbose Validamycin, Capsaicin Podopyllotoxin Puromycin Fumiquinazoline D	Highly effective antibiotics Highly effective antibiotics Used to treat type 2 diabetes Aminoglycoside antibiotic and fungicide Used to treat arthritis, backache and as an analgesic Used to treat genital warts and molluscum cotagiosum Aminonucleoside antibiotic, inhibits protein synthesis Antibiotic activity against gram positive and gram negative

Table 6: Predicted bioactive compounds synthesized by sequenced strains from *Diplorhynchus condylocarpon using KEGG.*

Conclusion

The *16S rRNA* gene is a useful molecular marker that can be used to identify species. Endophytes from *Diplorhynchus condylocarpon* are capable of synthesizing and are reservoirs of bioactive compounds. Some of these bioactive compounds are the ones that made it possible to use extracts from *Diplorhynchus condylocarpon* in antimicrobial assays and should be explored further. These endophytes produce highly effective antibiotics that uses numerous mechanisms to attack both Gram-negative and Gram-positive bacteria. Bioinformatics tools such as KEGG are useful in analyzing genes present in a species. Through KEGG not only antibiotic biosynthesis genes were identified but also compounds such as Acarbose which can be used in the treatment of diabetes. Ability of endophytes to produce antibiotics and other bioactive compounds can be exploited for pharmaceutical use.

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