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Biochemical and Molecular Characterization of Bacteria Associated With *Cnidoscolus aconitifolius* (Mill.) I. M. Johnston

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Abstract: *Cnidoscolus aconitifolius (Mill.) I. M. Johnston is an economic plant with various medicinal and food values. Little is known about the bacterial organisms associated with this plant. The use of molecular techniques in determining the bacterial composition of biological samples has proved be a more reliable method than the traditional cultural method which is based only on morphological and biochemical characteristics of isolates; in which case, misinterpretation or wrong identification of organisms may occur. In this study, the bacterial organisms associated with Cnidoscolus aconitifolius were isolated and identified using traditional cultural method, biochemical tests and basic molecular techniques. C. aconitifolius leaves and stem showing disease symptoms were collected from the three senatorial zones in Rivers State. Pure cultures of bacteria were obtained using nutrient agar and the bacterial DNA was extracted using Quick-DNA Fungal/Bacterial MiniPrep™ Kit (Zymo Research Group, California, USA). The 16S rRNA gene of the bacterial genome was amplified through polymerase chain reaction using bacterial universal primer pair; I6SF and I6SR. Three bacterial organisms; two strains of Pseudomonas putida and one strain of Pseudomonas sp. were successfully isolated and identified. A phylogenetic tree was constructed which showed the relationship between the isolates and other species on Genebank.*

Keywords: *Cnidoscolus aconitifolius, bacterial organism, polymerase chain reaction, sequencing*

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I. Introduction

Cnidoscolus aconitifolius (Mill.) I. M. Johnston commonly called "Chaya" is an evergreen drought-resistant shrub that originated from Mexico as a vegetable during pre-cambrian period (Ross-Ibarra &Molina-Cruz, 2002; Iwalewa *et al*., 2005). *C. aconitifolius* is a widely distributed plant, ranging from temperate to tropical zones. Chaya has been experimentally planted in research stations in Ghanna and Nigeria (Grubben and Denton, 2004). Today, chaya is found growing in the wild and in cultivation in the Eastern, Northern, Western and Southern parts of Nigeria.

In Nigeria, the plant is called "Efo IyanaIpaja" by the Yorubas; "Akwukwonriohuru" or "Ugu-Oyibo" by the Igbos; "Hospital Too Far" by the Niger Deltans because of its various medicinal values as claimed by the inhabitants and "Catholic vegetable" because it was used by those living in the convents as a vegetable (Donkoh *et al*., 1990; Iwalewa *et al*., 2005).

The peak of growth of *Cnidosolus aconitifolius* is during hot weather as it originated from the tropics. It can withstand harsh conditions such as high temperatures, low light conditions, deep shades and droughts. Chaya requires moderate water for optimum growth. Once the plant is well established, it can withstand severe drought or long periods of excess water supply provided that there is presence of good drainage and roots are not submerged in water-logged soil (Dawn, 2006). Chaya can grow well on a wide range of soils such as heavy clay and sandy soils but the best soil for its growth is well-drained loam (Luis, 2001).

Cnidosolus aconitifolius is widely used in the rural areas in Nigeria because of its medicinal and food values. Hamid *et al*. (2016) reported that the aerial parts of *C. aconitifolius* exhibit antibacterial and antifungal activities. The plant is used as a blood booster in the Southern and Eastern parts of Nigeria where it is usually taken by anemic children and pregnant women. Young shoots and leaves are squeezed with water, drank alone or mixed with tomato paste and milk (Iwalewa *et al*., 2005). Mordi and Akanji (2012) reported that *C. aconitifolius* have antihaemorrhagic, antihypertensive and cardiac depressant properties. The water from the squeezed leaves is also applied as a treatment for scorpion stings. Apart from medicinal and food uses, *C. aconitifolius*is also used as a hedge to barricade farm lands so as to prevent entry of farm animals into cultivated lands. In the rural areas, it is used to demarcate boundaries between farm lands and compounds.

Microorganisms affect the growth and yield of plants. Isolation of these microorganisms is key to finding ways of preventing/ controlling their incidence on plants. In recent times, identification and taxonomy of microorganisms is being geared towards the use of modern molecular techniques which are based on the amplification of specific regions of the organism's genome. This has proved to be a more reliable method than

the traditional cultural method. Therefore, this study was carried out to isolate and identify the bacterial organisms associated with *Cnidoscolus aconitifolius* (Mill.) (Mill.) I. M. Johnston using biochemical and molecular methods.

II. Materials And Methods

2.1 Study Area and Sampling

The study was conducted at the Regional Centre for Biotechnology and Bioresources Research Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria. Sequencing of the PCR products were done at the International Institute for Tropical Agriculture (IITA) Ibadan.

Cnidoscolus aconitifolius leaves and stem showing disease symptoms were obtained from the three senatorial zones in Rivers State. Obio-Apor, Ikwere, Port Harcourt and Emuoha LGAs for River East; Ahoada-East, Ahoada-West, Ogba/Egbema/Ndonni and Degema LGAs for Rivers West; and Tai, Eleme and Oyigbo LGAs for Rivers South East senatorial zones.

2.2 Isolation of bacteria from *Cnidoscolus aconitifolius*

Isolation of bacterial organisms was carried out using traditional media culturing. The microbial population of the sample was determined using serial dilution and spread plate method as described by Aneja (2003). One gram each of *Cnidoscolus aconitifolius* leaves and stem showing disease symptoms were separately surface sterilized in 70% ethanol for 3minutes. The leaves and stem were rinsed three consecutive times with sterile distilled water and transferred into separate sterile mortar. The samples were grounded and then transferred into 100ml conical flasks. 10mls of sterile normal saline was added to each conical flask and the contents shaken for 1 minute. From the stock solution, 1ml was pipette out and transferred into 9ml of sterile normal saline in a 20ml test tube forming 10^{-1} dilution, 1ml was aseptically collected from the 10^{-1} and transferred into another 9ml of sterile normal saline to form 10^{-2} dilution and this process was repeated in that sequence up to 10^{-10} that is, ten-fold serial dilution of the supernatant were made up to 10^{-10} dilution.Aliquot (0.1ml) of 10^{-3}, 10^{-5} and 10^{-7} diluents were plated into nutrient agar and spread evenly with a sterile bent glass rod. The plates were incubated at 37^{o}C for 48hours after which bacteria colony count was taken.

After enumeration, the different morphological types that appeared on the plates were carefully picked with sterile wire loop and sub-cultured by streaking each colony into prepared nutrient agar and incubated at 37^{o}C for 24hours to get pure cultures of bacteria.Pure cultures based on the results obtained were inoculated into sterile nutrient agar in Bijou bottles and incubated at 27^{o}C by streaking the culture on the sterile and solidified nutrient agar. The stock culture was then preserved in the refrigerator for further identification or use.

Gram staining and motility tests were performed using the isolates to check whether they are gram positive or gram negative bacteria and to check for the presence of locomotive organelles respectively.

2.3 Biochemical Tests for Bacteria isolates

Biochemical tests were carried out with the isolates as described by Collins *et al*. (1989), and Pacarynuk and Danyk (2004). The bacteria isolates were examined for their ability to produce catalase and oxidase. Production of indole from tryptophan, citrate utilization, MRVP test, production of Hydrogren sulphide using Triple Sugar Iron Agar (TSIA), ability of the isolates to ferment some sugars and to hydrolyse starch were also examined.

2.4 Bacterial DNA Extraction, DNA Concentration and Purity Check

Genomic DNA was extracted following the protocol of Quick-DNA^{TM} Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA) as described by the manufacturer, with modifications. Each bacterial culture was scrapped off from the surface of the culture medium using sterilized surgical blade. This was transferred into a sterilized mortar. 750µl of Bashing bead buffer was added to each bacterium in a mortar and the sample was homogenized using Liquid Nitrogen. Samples were transferred to Eppendorf Tubes (1.5ml). 200µl of distilled water was added to each sample and centrifuged in a refrigerated centrifuge at 10,000 x *g* for 1 minute. 400µl of the supernatant was transferred to a Zymo-Spin IIIF Spin Filter in a collection tube and was centrifuged at 7,000 x *g* for 1 minute. 1,200µl of Genomic Lysis Buffer was added to the filtrate in the collection tube and mixed properly. 800µl of the mixture was transferred into a Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000x *g* for 1 minute. The flow through from the collection tube was discarded and the above step was repeated. 200µl of DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000 x *g* for 1minute. 500µl of g-DNA Wash Buffer was added to the Zymo-Spin IIC Column in a new collection tube and centrifuged at 10,000 x *g* for 1minute. The Zymo-Spin IIC Column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of DNA Elution Buffer was added directly to the column matrix and then centrifuged at 10,000 x *g* for 30 seconds to elute the DNA.

DNA concentration was determined using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, Delaware, USA). DNA Purity check was done using agarose gel electrophoresis in a 1X TBE buffer. The genomic DNA was visualized by adding ethidium bromide (0.5 µg /ml) to the gel and viewing under a UV trans-illuminator (Cleaver Scientific Ltd, UK).

2.5 PCR Amplification of 16S rDNA and Sequencing

16S universal primers 16SF, forward (5´- GTGCCAGCAGCCGCGCTAA-3´) and 16SR, reverse (5´-AGACCCGGGAACGTATTCAC-3´) were used to amplify fragments of the 16S ribosomal DNA (rDNA). Polymerase Chain Reaction was carried out in a final volume of 10µL containing 2µL of genomic DNA (10ng/µL), 0.1µL of Taq polymerase, 1.0µL of 10X PCR buffer, 1.0 µL of DMSO, 0.8µL of 2.5mM DNTPs, 1.0µL of 25mM $MgCl_2$ (Promega), 0.5µL of each primer (5µM) and 3.1µL of Nuclease-free water. Amplifications were performed in a thermal cycler (GeneAmp® 9700 PCR System, Applied Biosystems, California, USA) using an initial denaturation step of 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and elongation at 72°C for 45 seconds with a final extension at 72°C for 7 minutes. The amplicon was held at 10°C. The amplicon from the above reaction was subjected to gel electrophoresis in 1.5% agarose gel using TBE 1X and the gel stained with Ethidium bromide. Hyper ladder (1kb) from Bioline (London, United Kingdom) was used. The set up was allowed to run for 40 minutes at 100volts and the gel photographed under UV using an Enduro Gel Documentation System (Aplegen, California, USA).

After amplification, PCR products were sequenced. The sequences obtained were blasted against sequences on the National Centre for Biotechnology Information (NCBI) database.

2.6 Phylogeny

A phylogenetic tree was constructed using maximum composite likelihood method (Tamura *et al*., 2004) to show the relationship between the isolates and other species on GeneBank. The phylogenetic tree was constructed using the best BLAST hits which aligned by Clustal X. The evolutionary analysis was conducted in MEGA X (Kumar *et al*., 2018).

III. Results

Total Population Count of bacteria from *Cnidoscolus aconitifolius*

The number of bacterial colonies on Petri dishes containing grounded *Cnidoscolus aconitifolius* leaves and stem plated separately on nutrient agar was calculated (Table 1). Rivers South East Senatorial zone had the highest Bacterial colonies from the leaves, 6.5×10^5 $cfug^{-1}$; followed by Rivers East (5.1×10^5 $cfug^{1}$) and lastly Rivers West (3.8×10^5 $cfug^{-1}$). Rivers South East Senatorial zone also had the highest Bacterial colonies from the stem (4.7×10^5 $cfug^{-1}$), followed by Rivers East (4.3×10^5 $cfug^{-1}$) and lastly Rivers West (3.3×10^5 $cfug^{-1}$). Pure cultures of isolated bacteria are presented in Plate 1.

Table 1: Total Population Count of bacteria from *Cnidoscolus aconitifolius* from three Senatorial zones in Rivers State

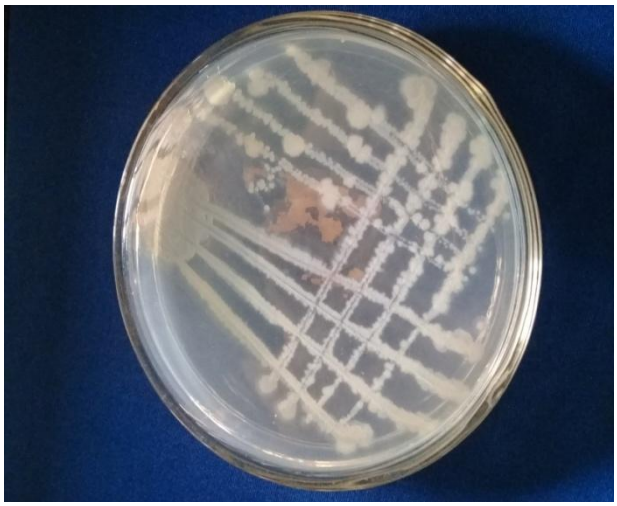
Senatorial Zone	**Colony Count ($cfug^{-1}$)**	
	Leaves	**Stem**
Rivers East	5.1×10^5	4.3×10^5
Rivers West	3.8×10^5	3.3×10^5
Rivers South-east	6.5×10^5	4.7×10^5

Bacterial organisms associated with *Cnidoscolus aconitifolius*

Three bacterial organisms were isolated from *Cnidoscolus aconitifolius* leaves and stem (Table 2). Samples B14 and B15 had a round entire and smooth surface, creamy in colour while sample B16 had a creamy circular colony with a smooth and shiny surface. Pure cultures of isolated bacteria are presented in Plate 1.

Sample B14

Sample B15



Sample B16

Plate 1: Pure cultures of bacterial organisms isolated from *Cnidoscolus aconitifolius* leaves and stem at room temperature (27± 2°C)

Table 2: Morphological and biochemical characteristics of bacteria isolates of *Cnidoscolus aconitifolius* leaves and stem

Characteristics & morphology	**Sample ID**	**GS**	**O**	**C**	**M**	**MR**	**VP**	**IND**	**CT**	**H_2S**	**Ga**
SH	**G**	**L**	**Suspected Organism**								
Round entire and smooth surface	B14	-rods	+	+	+	+	-	-	+	-
-	-	+	+	*Pseudomonas* sp.							
Round entire and smooth surface	B15	-rods	+	+	+	+	-	-	+	-
-	-	+	+	*Pseudomonas* sp.							
Circular creamy colony with	B16	-rods	+	+	+	+	-	-	+	-
-	-	-	+	*Pseudomonas* sp.							
smooth surface											

Key:
+ = positive
- = negative
GS = Gram Staining
O = Oxidase
C = Catalase
M = Motility

MR = Methyl Red test-
VP = VogesProskauer's test
IND = Indole test
CT = Citrate
H_2S = Hydrogen Sulphide test
Ga = Gas production
SH = Starch Hydrolysis
G = Glucose
L = Lactose

DNA Extraction, Quantification and Gel Electrophoresis

Bacterial genomic DNA was successfully extracted from the pure cultures isolated from *Cnidoscolus aconitifolius* leaves and stem using Quick-DNA^{TM} Fungal/Bacterial MiniPrep Kit (Zymo Research Group, California, USA). Nanodrop spectrophotometer revealed the concentration and purity of the DNA as presented in Table 3.

Table 3: Concentration and Purity of DNA extracted from Bacterial Pure Cultures Isolated from *Cnidoscolus aconitifolius* Leaves

Sample ID	DNA concentration (ng/µl)	DNA Purity (A260/280nm)
B14	247.60[b]	1.89[a]
B15	266.60[b]	1.91[a]
B16	216.83[a]	1.90[a]
Total	243.68	1.90
ANOVA (F-Test)	17.599	0.913
p-value	0.003	0.451

Each value is a mean of three test replicates at 95% confidence limit
Row mean with different alphabet is significant at 5%.
The ANOVA result showed that for concentration, samples are significant at p-value (0.003) <5% significant level.
ANOVA result showed that for purity, sample are not significant at p-value (0.451) > 5% significant level.

Gel Electrophoresis

The quality of the genomic DNA was determined on 1% agarose gel. The extracted DNA of all isolates was of good quality as all isolates showed bands on gel as presented in Figure 1.

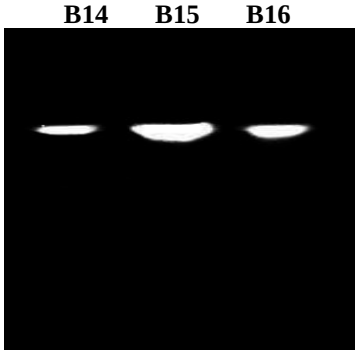


Figure 1: Gel electrophoresis of genomic DNA extracted from bacterial pure cultures isolated from *Cnidoscolus aconitifolius*

Polymerase Chain Reaction

All extracted DNA amplified using the 16S primer pair; 16SF, forward (5´- GTGCCAGCAGCCGCGCTAA-3´) and 16SR, reverse (5´- AGACCCGGGAACGTATTCAC-3´). The PCR products obtained from the isolates are represented with bands on gel as presented in Figure 2.

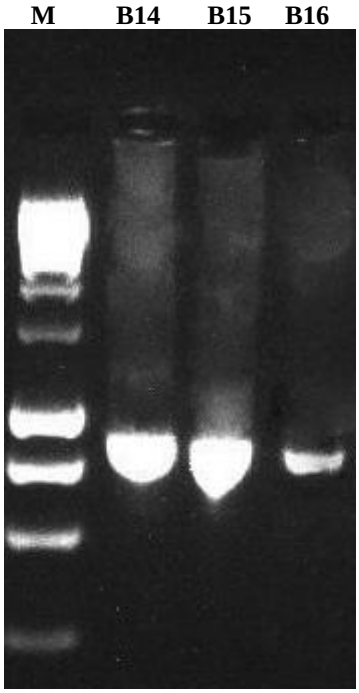


Figure 2: Gel electrophoresis showing PCR amplification generated from bacterial gDNA using bacterial Universal Primer pair (16SF and 16SR).
M- DNA Marker (1kb Ladder from Bioline)

Identification of Bacterial isolates

After sequencing, the blast results revealed the species identity of the bacterial isolates to be: *Pseudomonas putida* Trevisan for samples B14 and B15 and *Pseudomonas* sp. Migula for sample B16 (Table 4).

Table 4: Putative Taxonomic Affinities of Sequence Types Inferred from Blast Searches of 16S Sequences obtained from bacterial samples isolated from *Cnidoscolus aconitifolius*

Sample ID	Taxonomic affinity (Gene bank no)	Percentage Similarity (%)
B14	*Pseudomonas putida* isolate 191031(LN610443.1)	89
B15	*Pseudomonas putida* strain EPAn40-2(JF911379.1)	91
B16	*Pseudomonas* sp. strain 52(2015) (KT025907.1)	89

The nucleotide length of each sequence obtained from the bacterial isolates of *Cnidoscolus aconitifolius* was determined to be: 560 base pairs for the two strains of *Pseudomonas putida* (samples B14 and B15) and 480bp for *Pseudomonas* sp. The sequences of the bacterial isolates were submitted to GeneBank and accession numbers were assigned to each sequence as follows:

- *Pseudomonas putida* (MK937566) strain RCBBR_AEANKB14
- *Pseudomonas putida* (MK937572) strain RCBBR_AEANKB15
- *Pseudomonas sp.* (MK937583) strain RCBBR_AEANKB16

Phylogenetic Analysis

The relationship between the bacterial isolates of *Cnidoscolus aconitifolius* and other closely-related isolates on GeneBank was analysed using a phylogenetic tree. Samples 14 and 15 are more closely related as they belong to the same species of the genus, *Pseudomonas*. The phylogenetic analysis is presented in Figure 3.

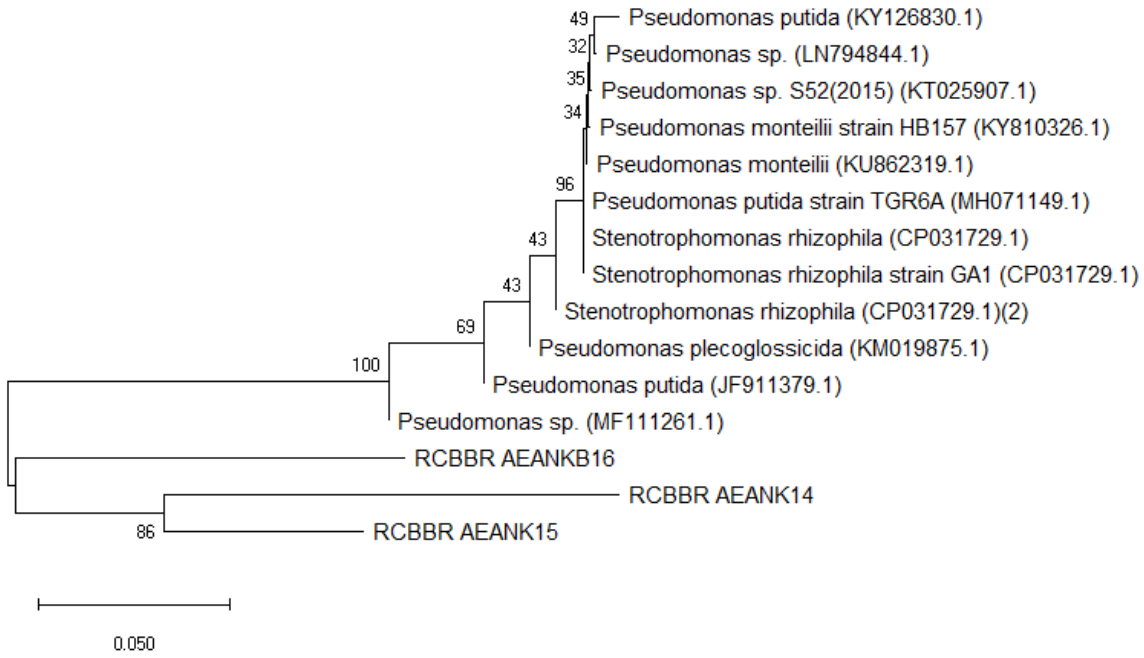


Figure 3: Phylogenetic tree generated by maximum composite likelihood analysis based on the 16S rDNA gene sequences.

IV. Discussion

Three bacterial organisms were isolated and identified from *Cnidoscolus aconitifolius* using traditional cultural method, biochemical and basic molecular techniques. The bacteria isolated and identified include: two strains of *Pseudomonas putida* and one strain of *Pseudomonas* sp. The DNA of the three samples amplified with the16S primer pair. The nucleotide length of each sequence obtained from the bacterial isolates was determined to be: 560 base pairs for the two strains of *Pseudomonas putida* and 480bp for *Pseudomonas* sp.

Pseudomonas species survive as saprophytes and/or parasites in plants (Moore, *et al*., 2006). They are pathogenic to many plants causing several diseases on crops. Twenty-one plant pathogenic *Pseudomonas* species exist. *P. syringae* is said to be the most important of these species as it has more than 50 pathovars (Höfte *et al*., 2007). Beiki *et al.* (2016) reported *P. monteilii*, *P. lurida*, *P. orientalis, P. moraviensis, P. syringae, P. viridiflava* and *P. simiae* as being pathogenic to citrus.

Pseudomonas putida strains have been reported to be present in water and also colonize the rhizosphere of many plants; creating a symbiotic relationship (Molina *et al*., 2000; Matilla *et al*., 2010; Fernandez *et al*., 2015). Matilla *et al*. (2010) reported that *P. putida* is a bio-control agent. As it was able to protect *Arabidopsis thaliana* against disease caused by *Pseudomonas syringae* pv. tomato.

There is limited information on microorganisms associated with *Cnidoscolus aconitifolius*. Few reports have been recorded on the viral diseases of chaya. Elliot and Zettler (1987) reported the occurrence of cassava common mosaic virus (CCMV) of the genus Potexvirus on *Cnidoscolus aconitifolius* detected by serology in 23 out of 33 samples (69.7%) collected in Yucatan State (Mexico). Iyanyi and Ataga (2019) isolated six fungal organisms associated with *Cnidoscolus aconitifolius* in Nigeria using basic molecular techniques. To our knowledge, this is the first record on the bacterial organisms associated with *C. aconitifolius* in Nigeria.

The bacterial organisms were successfully isolated using traditional cultural techniques and identified using biochemical tests and basic molecular methods. The sequences obtained were aligned and the phylogenetic analysis showed the relationship that exists between the isolates and other closely related species.

V. Conclusion

Three bacterial organisms were isolated from *Cnidoscolus aconitifolius* using traditional cultural method and identified using biochemical tests and basic molecular techniques. The identification of bacteria by biochemical and morphological characteristics is helpful, but is not enough to differentiate bacterial species from each other. From the results, the accurate identification or characterization of bacterial organisms can only be attained by combining cultural, biochemical and molecular methods. The preparation of pure cultures and

biochemical tests only aided in the processes that led to the correct identification of the isolates. Molecular characterization by amplifying the 16S ribosomonal DNA (rDNA) gene sequence of the isolates was effective in the identification of the isolates. To our knowledge, this is the first report of bacterial organisms associated with *Cnidoscolus aconitifolius* in Nigeria. This study will help enlighten scientist on the bacterial organisms associated with chaya in order to help reduce the incidence of these organisms on the plant.

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