Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.6566

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **6 (3):** 1-10 (2018)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Isolation, Characterization and Gene Sequencing of Partial Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Gene of *Diospyros discolor* Willd.

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Received: 15.05.2018 | Revised: 21.06.2018 | Accepted: 27.06.2018

ABSTRACT

The Diospyros discolor Willd. is an endemic and vulnerable plant species in the Philippines known for its premium kamagong timber and mabolo fruit that possesses nutritional, medicinal and pharmacological properties. However, due to its limited genetic information, it is less likely be used to gene expression studies. This study worked for the initial step – isolation of a specific housekeeping gene, glycerlaldehyde-3-phosphate dehydrogenase. The gDNA from the leaf tissue was extracted using CTAB method, then subjected it to gradient PCR in order to amplify the GAPDH gene in an optimized temperature using the designed primers. Samples K2 and L1 at 57.4°C and 57.8°C were purified and sequenced by the Macrogen, Korea. Results of in silico analyses indicated that the isolated gene is partial GAPDH gene that contains 687 base pairs with an exact reading frame of 300 base pairs which corresponds to 100 amino acids. The conserved domains from the amino acid sequence proved that it belongs to the GAPDH NAD binding domain superfamily and GAPDH C-terminal domain superfamily. The relative plant species was determined using BLASTn and BLASTp where Clematoclethra scandens, Acrostichum aureum, Vittaria graminifolia and Antrophyum latifolium showed the highest relevance with 85% identity. The sequence data were submitted and accepted by GenBank, NCBI with an accession number of MH234383.1. The success of this study enables addition to the genetic background of D. discolor Willd. that can be employed to its gene expression analysis and of other endemic and/or endangered related flora.

Key words: *partial GAPDH gene, Diospyros discolor, genomic DNA, Gradient PCR*

INTRODUCTION

According to the Convention on Biological Diversity, a secretariat under the United Nations' Environment Programme, the Philippines ranks fifth in the number of plant species and upholds 5% of the world's flora[1]. With over 16,000 floral species, about 70% to 80% of these identified angiosperms found within primary forests are endemic species.

Cite this article: Pineda, A.R.R. and Acuña, E.I.F., Paitan, V.P., Isolation, Characterization and Gene Sequencing of Partial Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Gene of *Diospyros discolor* Willd., *Int. J. Pure App. Biosci.* **6(3):** 1-10 (2018). doi: http://dx.doi.org/10.18782/2320-7051.6566

However, 984 of these species are threatened where 179 are critically endangered, 254 are endangered, 406 are vulnerable, and 145 other threatened species that are close to being vulnerable[2]. *Diospyros discolor* Willd. is one of the aforementioned endemic and vulnerable species. It is natively known as Kamagong[3] and is often dubbed as an "iron wood" due to its durable and almost unbreakable timber[4] that is carved into furnitur[5,6]. While its edible fruit is termed as Mabolo because of its hairy texture[7] that possesses several economic importance. It manifests notable nutritional contents which is vital for maintaining good health[8]. It is a good source of vitamin B complex, calcium, zinc, dietary fiber, malic acid and antioxidants. In addition, the mabolo is known as ethnobotanical fruit because of its medicinal and pharmacological properties such as antibacterial, antimicrobial[9] antitumor, anti-inflammatory, anticancer[7,10], thrombolytic and cytotoxic activities[11]. However, one of the most integral pharmacological activities of the crude methanoic extract of the bark and leaves of mabolo is its acetylcholinesterase inhibitory property, reason for its vitality as an effective treatment for Alzheimer's disease[3,12]. Thus, *D. discolor* Willd. is obviously significant in human health not only on its high nutritious value but also to its medicinal properties.

Plants with potential in improving human health should undergone several advanced studies to expose its economic importance especially in the field of molecular biology for these uphold the knowledge of future experiments. One of the most common techniques used in molecular approach in botany is the isolation and sequencing of a specific gene of interest. This provides valuable data that can be utilized in variety of ways – gene expression studies, tracing evolutionary relationship, and usage for broad spectrum of medicine and agriculture. As well, this will add accessible genetic information to the body of knowledge. The present study uses the genomic DNA from the *D. discolor* Willd. for isolation, characterization and sequencing of the partial Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene.

MATERIAL AND METHODS

Plant Material

The *D. discolor* Willd. (see figure 1) was protected and grown by the owner at No.31 Lucero St., Brgy. Mabolo, City of Malolos, Bulacan, Philippines. The leaves used in this study were collected between October and November 2017. Moreover, the collected leaves were immediately washed with sterile distilled water (sdH_2O) in order to remove dirt and other filthy parts (see figure 2). Afterwards, the samples were prepared prior to homogenization and extraction of its genomic DNA.

Genomic DNA Extraction

Extraction of the genomic DNA (gDNA) of the *D. discolor* Willd. and the isolation of partial Glycerladehyde-3-phosphate dehydrogenase (GAPDH) gene was performed in the Philippine Genome Center (PGC) located at the 2nd floor of National Institute of Molecular Biology and Biotechnology, UP Diliman, Quezon City, Philippines. The gDNA was extracted from the young leaves of *D. discolor* Willd. using the Cetyl Trimethylammonium Bromide (CTAB) extraction method wherein the protocol provided was by the PGC. This extraction method can provide a sufficient gDNA that can be used for further analysis[13,14].

Primer Design

The primers used in this study were designed by the researchers using the Clustal Omega multiple sequence alignment on different GAPDH gene sequences of the following species: *Navarretia linearifolia*, *N. sinistra*, *Collomia tenella*, *C. wilkenii* and *Allophyllum divaricatum* which are closely related to *D. discolor* in order level. The OligoAnalyzer 3.1 (sg.idtdna.com/analyzer/Applications/OligoAnalyzer/), on the other hand, was the tool used in designing an adequate and acceptable primers. The following parameters of the primers were considered: an optimal length of 18-22 nucleotides, melting temperature ranging from 52°C – 58°C, GC content percentage that range from 40-60%, absence of the hairpin structures, self-dimers or primer-dimers value and lack of self-

complementarities at the 3' end of the primers. Table 1 shows the different parameters of the designed primers for GAPDH gene.

Isolation of partial GAPDH gene

Gradient Polymerase Chain Reaction analysis was done in order to isolate the target gene in an optimize temperature, wherein the PCR was set its annealing stage in different temperatures between 50ºC – 58 ºC. A 10µL reaction of PCR Master Mix was prepared using the KAPA Taq PCR Kit (Kapa Biosystems) following the manufacturer's protocol.

***In silico* Analyses**

The ExPASy tool (https://web.expasy.org/ translate/) was used to translate the nucleotide sequence of the isolated GAPDH gene sequence of *D. discolor* Willd to deduced amino acid sequence. ORF finder (http://www.bioinformatics.org/sms2/orf_find. html) was used to find the exact frame with that will be used in further analysis. Moreover, the physical and chemical parameters of the deduced amino acid sequence were determined using the ProtParam tool (https://web.expasy .org/protparam). Conserved domains were determined using the Conserved Domain Database (CDD) (https://www.ncbi.nlm. nih.gov/Structure/lexington/lexington.cgi).

Also, the homology analysis of the *D. discolor* Willd with other plant species based on its isolated GAPDH gene were determined using BLASTn (https://blast.ncbi.nlm.nih. gov/Blast.cgi?PAGE_TYPE=BlastSearch) and BLASTp (https://blast.ncbi.nlm.nih.gov/ Blast.cgi?PAGE=Proteins). Lastly, ProtScale was used in order to clearly view the raw, exact reading frame and amino acid sequences of the isolated GAPDH gene (https://web.expasy.org/ protscale/).

RESULTS AND DISCUSSION

The electrophoretogram of the extracted gDNA of *D. discolor* Willd. showed that the gDNA have a high integrity and was successfully extracted (Figure 3). The total concentrations of the samples were 29.46 ng/µL for sample A and 30.49 ng/µL for sample B. The purity of the gDNA were evaluated using 260/280 nm ratio resulting to 1.79 and 1.84 purity, respectively. Since sample B have shown higher purity ratio, the researchers used this sample as the source for gene isolation. Moreover, the electrophoretogram of the PCR products showed that samples K2 and L1 have a well-defined band with lesser contaminants (Figure 4).

The raw nucleotide sequence resulted to 687 base pairs (see Figure 5) with an open reading frame of 300 base pairs (see Figure 6), which encodes to 100 amino acids (see Figure 7). This was submitted to GenBank, NCBI through BankIt tool. The submitted sequence have been accepted and was given an accession number of MH234383.1 (see Figure 8). In addition, the conserved domains that were examined from the amino acid sequence of the isolated partial GAPDH gene proved that they belong to the Glyceraldehyde-3-phosphate dehydrogenase, NAD binding domain; and Glyceraldehyde-3-phosphate dehydrogenase, C-terminal domain superfamily (see Figure 9). BLASTn (nucleotide BLAST) was performed to determine the relative plant species from the isolated nucleotide sequence. The table 2 shows species with high percent of relevance, where the *Clematoclethra scandens* with 85% identity shows the highest relevance. In contrast, BLASTp (protein BLAST) was also done to determine the relative plant species from the deduced amino acid of the isolated partial GAPDH gene sequence of *D. discolor* Willd. Table 3 illustrates that *Acrostichum aureum*, *Vittaria graminifolia* and *Antrophyum latifolium* shows highest relevance with 85% homology or identity. In addition, different parameters of the amino acid sequence of the partial GAPDH gene of *D. discolor* Willd. were determined using the ProtParam tool. This include the calculation of the physical and chemical properties of the amino acid sequence. The Table 4 and 5 display the molecular weight, isoelectric point and amino acid composition.

LIST OF FIGURES AND TABLES

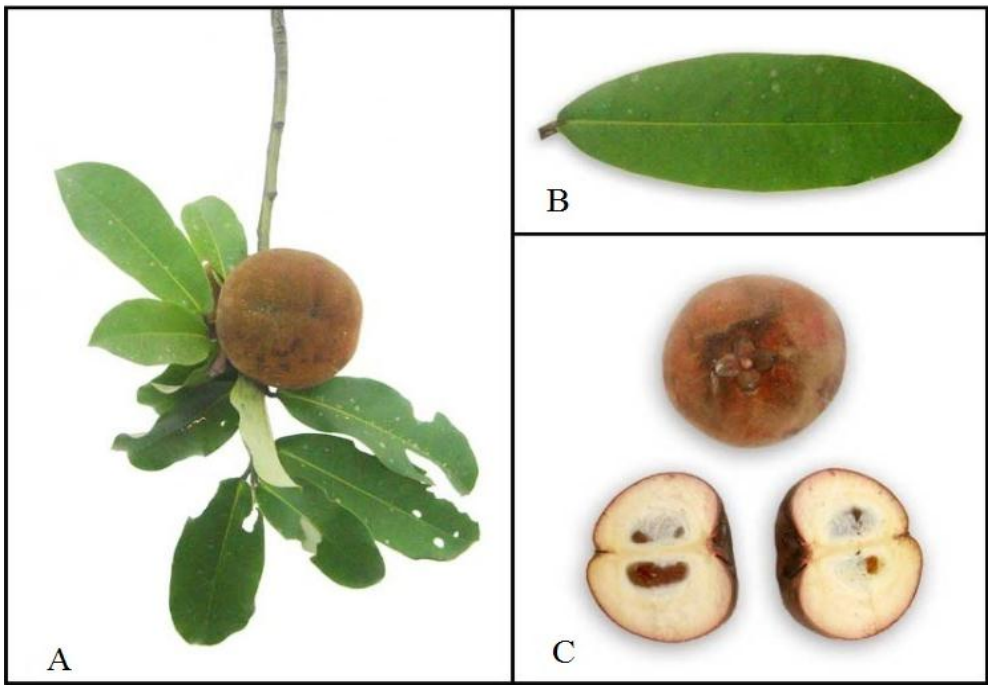


Fig. 1: Velvet Apple, *Diospyros discolo*r Willd. Foliage and its fruit (A) Leaf (B) Cross-section of Fruit (C) (source: http://www.stuartxchange.com/Mabolo.html)

Fig. 2: Preserved leaf samples

Table 1: Parameters of GAPDH both forward and reverse primers

PRIMER NAME	SEQUENCE (5'- 3')	%GC	T_m (^oC)	Amplicon (bp)
GAPDH Forward	CAACATTATTCCCAGCAGCAC	47.6%	54.5	21
GAPDH Reverse	GGAGACCACATCATCTTCAGTG	50%	55.1	22

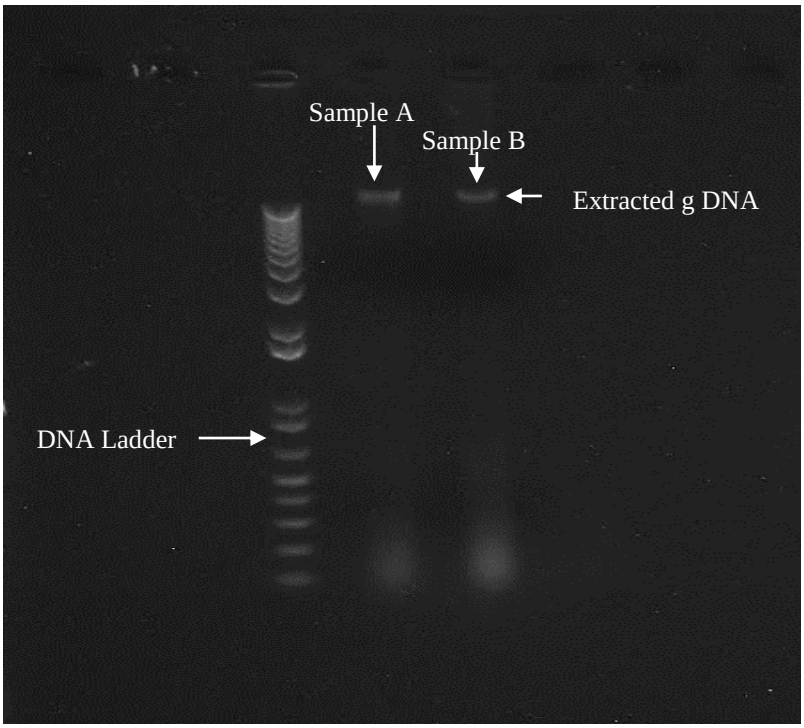


Fig. 3: Electrophoretogram of the extracted gDNA from *D. discolor* Willd. leaf tissue

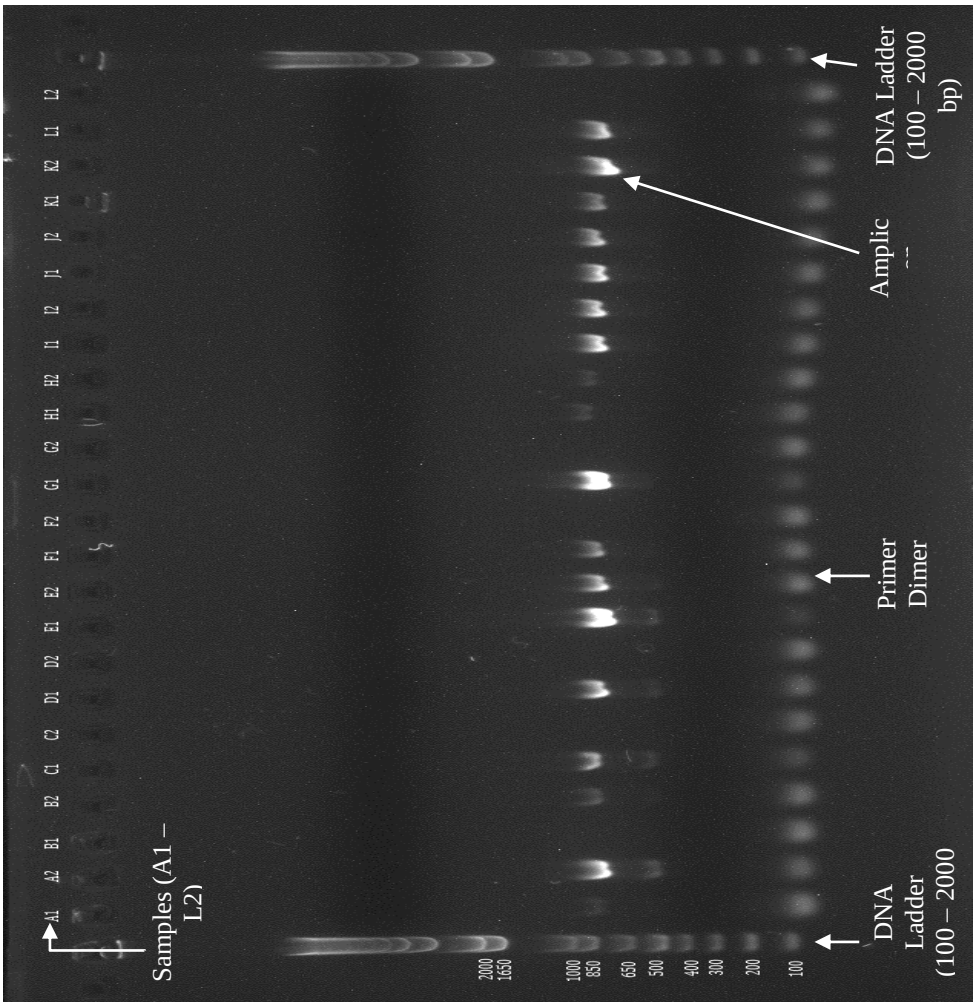


Fig. 4: Gradient PCR products in agarose gel visualized and examined using *AlphaImager® Mini* (ProteinSimple).

```
        10          20          30          40          50          60
CGTTTCTCTT CTCGGTTCGA GACTTATGCA TCTCACTGTC ATTACGTGTG CCAGTTCTTG

        70          80          90         100         110         120
AATGTGTTAT GATTTTTTAG CAATTACCGT CAACCTTGTT CCTCAGCTGA ATTTCTTGAC

       130         140         150         160         170         180
AAAGCTATGA TTATTAGAGT AGCCACTATT TAGACTTCAC CAACTCTTAA AAAGGGTGAG

       190         200         210         220         230         240
CCTGGGTGGC AGTGGTAGGG TTAGTTGCTC AACCATGAAT GGAACGTCAC GACTCACTTC

       250         260         270         280         290         300
TCTTGACTCG TGCAGAGCAT GGAGCCTTTT GCATTGGGAT ATCTTTTTTT ATTTACCCTC

       310         320         330         340         350         360
TACGTTTACA TGTTTGTTTT GTAATGCTGT TCATCTTCTT TTTTGATTTG TATTTGTTGC

       370         380         390         400         410         420
TCCTCACACA GGCGGTTGGA AAGGTTCTGC CTTCACTGAA TGGGAAGCTG ACCGGAATGT

       430         440         450         460         470         480
CCTTCCGCGT TCCAGTTGCT GATGTTTCGG TTGTGGACCT CACTGTGAGG CTTGAGAAGC

       490         500         510         520         530         540
CGGCTACTTA CCAGGAAATC AAAAATGTTG TCAAGTGACA CAATTCTTCC CCTCTAAAAC

       550         560         570         580         590         600
TTGAATTTGA AGATATGCTG ACCGGATCGT CAATGACTAG TCGCATAACT ACTCTTCTAT

       610         620         630         640         650         660
GGCTGTTTCT TTGTTAATAT CAAGGAGGAG TCAGAGGGCA AACTCAAGGG GATACTGGGG

       670         680
TACACTGAAT ATGATGTGAG TCTCCAA

SEQUENCE LENGTH: 687
```

Fig. 5: Raw nucleotide sequence of the GAPDH gene of *D. discolor* Willd. (https://web.expasy.org/protscale/)

```
        10          20          30          40          50          60
ATGGAACGTC ACGACTCACT TCTCTTGACT CGTGCAGAGC ATGGAGCCTT TTGCATTGGG

        70          80          90         100         110         120
ATATCTTTTT TTATTTACCC TCTACGTTTA CATGTTTGTT TTGTAATGCT GTTCATCTTC

       130         140         150         160         170         180
TTTTTTGATT TGTATTTGTT GCTCCTCACA CAGGCGGTTG GAAAGGTTCT GCCTTCACTG

       190         200         210         220         230         240
AATGGGAAGC TGACCGGAAT GTCCTTCCGC GTTCCAGTTG CTGATGTTTC GGTTGTGGAC

       250         260         270         280         290         300
CTCACTGTGA GGCTTGAGAA GCCGGCTACT TACCAGGAAA TCAAAAATGT TGTCAAGTGA

SEQUENCE LENGTH: 300
```

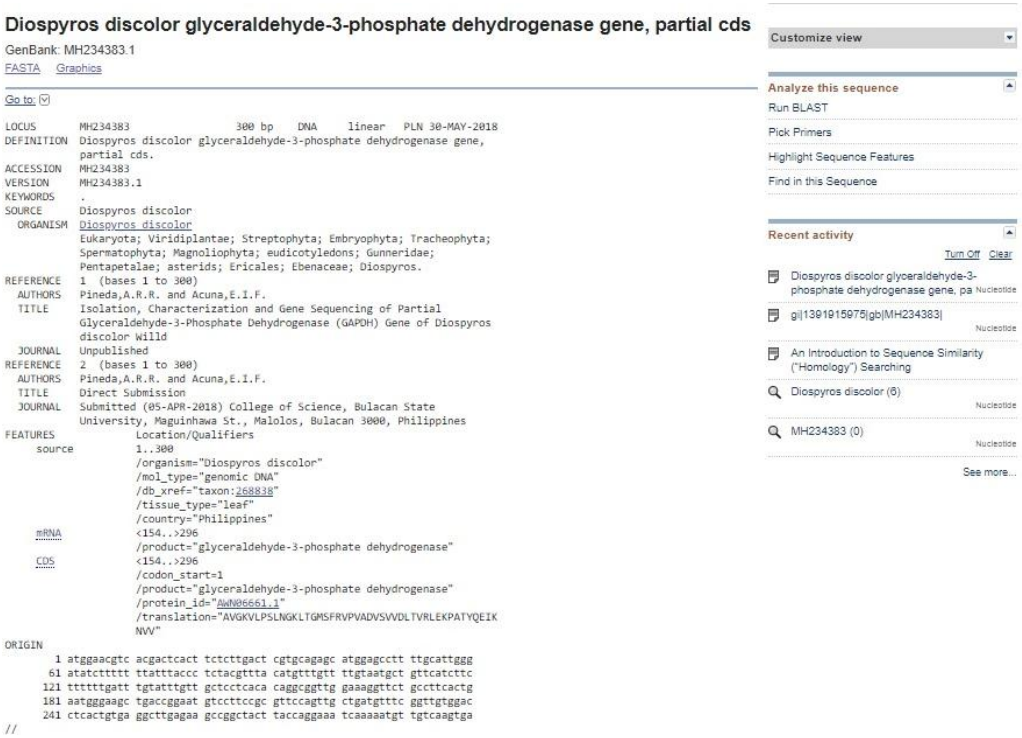
Fig. 6: Cleaned up nucleotide sequence of the GAPDH gene of *D. discolor* Willd. (https://web.expasy.org/protscale/)

```
         10         20         30         40         50         60
MERHDSLLLT RAEHGAFCIG ISFFIYPLRL HVCFVMLFIF FFDLYLLLLT QAVGKVLPSL

         70         80         90        100
NGKLTGMSFR VPVADVSVVD LTVRLEKPAT YQEIKNVVKX
```

SEQUENCE LENGTH: 100

Fig. 7: The translated amino acid sequence of the partial GAPDH gene of *D. discolor* Willd. from ExPASy Translate Tool (https://web.expasy.org/cgi-bin/translate/dna2aa.cgi)



Diospyros discolor glyceraldehyde-3-phosphate dehydrogenase gene, partial cds

GenBank: MH234383.1

FASTA Graphics

Go to:

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DEFINITION  Diospyros discolor glyceraldehyde-3-phosphate dehydrogenase gene,
            partial cds.
ACCESSION   MH234383
VERSION     MH234383.1
KEYWORDS    .
SOURCE      Diospyros discolor
  ORGANISM  Diospyros discolor
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; Gunneridae;
            Pentapetalae; asterids; Ericales; Ebenaceae; Diospyros.
REFERENCE   1  (bases 1 to 300)
  AUTHORS   Pineda,A.R.R. and Acuna,E.I.F.
  TITLE     Isolation, Characterization and Gene Sequencing of Partial
            Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Gene of Diospyros
            discolor Willd
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 300)
  AUTHORS   Pineda,A.R.R. and Acuna,E.I.F.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-APR-2018) College of Science, Bulacan State
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                     NVV"
ORIGIN
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       61 atatcttttt ttatttaccc tctacgttta catgtttgtt ttgtaatgct gttcatcttc
      121 ttttttgatt tgtatttgtt gctcctcaca caggcggttg gaaaggttct gccttcactg
      181 aatgggaagc tgaccggaat gtccttccgc gttccagttg ctgatgtttc ggttgtggac
      241 ctcactgtga ggcttgagaa gccggctact taccaggaaa tcaaaaatgt tgtcaagtga
//
```

Customize view

Analyze this sequence

Run BLAST

Pick Primers

Highlight Sequence Features

Find in this Sequence

Recent activity

Turn Off Clear

Diospyros discolor glyceraldehyde-3-phosphate dehydrogenase gene, pa Nucleotide

gi|1391915975|gb|MH234383| Nucleotide

An Introduction to Sequence Similarity ("Homology") Searching

Diospyros discolor (6) Nucleotide

MH234383 (0) Nucleotide

See more...

Fig. 8: GenBank database of *D. discolor* Willd. partial GAPDH gene sequence.

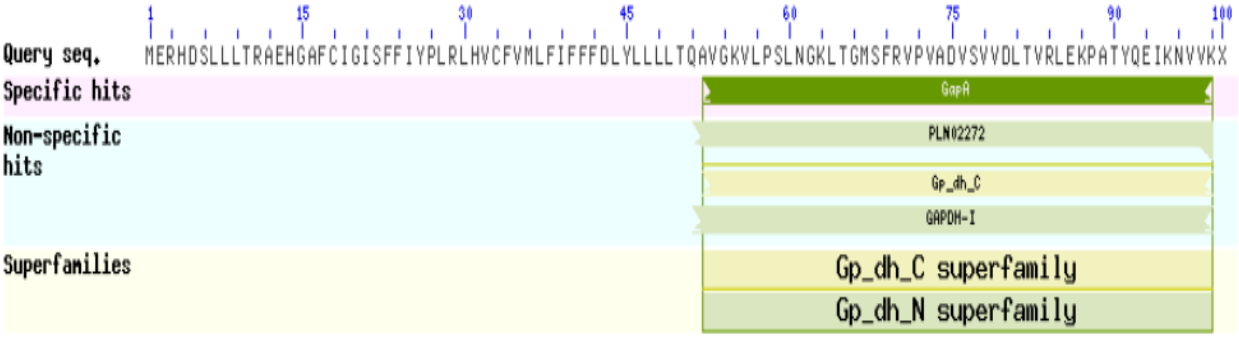

Fig. 9: Standard results for the identified conserved domains of the GAPDH gene of *D. discolor* Willd. using Conserved Domain Database (CDD), NCBI

Table 2: Plant species that shares identity from the partial GAPDH gene of *D. discolor* Willd. using BLASTn (Nucleotide BLAST)

ACCESSION NUMBER	ORTHOLOGUES	IDENTITY (%)	E-VALUE
EU281591.1	*Clematoclethra scandens* subsp. tomentella clone G105 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	85%	9e-36
EU281576.1	*Actinidia arguta* var. purpurea clone G004 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	85%	4e-34
EU281577.1	*Actinidia eriantha* clone G032 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	83%	1e-34
EU281578.1	*Actinidia kolomikta* clone G112 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	83%	4e-34
EU281580.1	*Actinidia melliana* clone G019 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	82%	4e-34
EU281583.1	*Actinidia chinensis* var. rufopulpa clone G159 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	82%	2e-33
EU281581.1	*Actinidia hemsleyana* clone G028 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	82%	2e-32
EU281617.1	*Actinidia callosa* var. discolor clone G107 glyceraldehyde 3-phosphate dehydrogenase (g3pdh) gene, partial cds	81%	9e-31
JN571725.1	*Brassica oleracea* glyceraldehyde 3-phosphate dehydrogenase gene, partial cds	78%	1e-14

Table 3: Plant species that shows highest identity to the deduced amino acid sequence of GAPDH gene of *D. discolor* Willd. using Protein BLAST.

ACCESSION NUMBER	ORTHOLOGUES	IDENTITY (%)	E-VALUE
AFX63162.1	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase [*Acrostichum aureum*]	85%	2E-20
AGV22168.1	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase [*Vittaria graminifolia*]	85%	4E-20
AGV22171.1	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase [*Antrophyum latifolium*]	85%	5E-20

Table 4: Amino Acid Composition of the GAPDH gene of *D.*

Amino Acids	Composition
Alanine	5%
Arginine	5%
Asparagine	2%
Aspartic Acid	4%
Cysteine	2%
Glutamine	2%
Glutamic Acid	4%
Glycine	5%
Histidine	3%
Isoleucine	5%
Leucine	16%
Lysine	5%
Methionine	3%
Phenylalanine	9%
Proline	4%
Serine	5%
Threoline	5%
Tyrosine	3%
Valine	12%

Table 5: Physical and Chemical Properties of the GAPDH gene of *D. discolor* Willd.

Properties	Values
Molecular Weight (g/mol)	11,373.81
Isoelectric point (pI)	8.69
Half-life in mammalian reticulocytes	30 hours
Half-life in yeast	>20 hours
Half-life in *E. coli*	>10 hours
Instability Index (II)	20.84

CONCLUSION

The partial GAPDH gene was successfully isolated, characterized and sequenced from the genomic DNA of the *D. discolor* Willd using the primers designed by the researchers. The raw nucleotide sequence resulted to 687 base pairs with an open reading frame of 300 base pairs encoding to100 amino acids. The conserved domains from the amino acid sequence proved that they belong to the Glyceraldehyde-3-phosphate dehydrogenase, NAD binding domain superfamily; and Glyceraldehyde-3-phosphate dehydrogenase, C-terminal domain. Also, the relative plant species was determined from the isolated nucleotide sequence using BLASTn and BLASTp, where the *Clematoclethra scandens* also known as Teng shan lui plant, *Acrostichum aureum* known as golden leather fern, *Vittaria graminifolia* also called as shoestring fern and *Antrophyum latifolium* shows 85% identity and have the highest relevance. The success of this study enables the *D. discolor* Willd from the family of Ebenaceae to be a representative species for having isolated GAPDH gene from its gDNA. In addition, the results of this study can be employed and used for further gene expression analysis and studies, particularly, the *D. discolor* Willd and other endemic and/or endangered related flora.

Acknowledgement

The authors would like to extend their utmost gratitude to Almighty God for without His endless grace, this study would be non-existent. Boundless appreciation and genuine gratefulness are encompassed to the succeeding persons who supported the authors, in any way possible, in the accomplishment of this study.

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