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AMPLIFICATION OF *AZOSPIRILLUM* SP. JG3 *GLPD* GENE FRAGMENT USING DEGENERATE PRIMERS GENERATED BY WEB-BASED TOOLS

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ABSTRACT

Primaclade and In Silico web-based tools were used as a strategy to obtain the correct-size PCR amplicon targeting a fragment of gene encoding glycerol-3-phosphate dehydrogenase (*glpD*) of *Azospirillum* sp. JG3. The bacterial strains are soil, Gram-negative PGPR (Plant-Growth Promoting Rhizobacteria) isolated from an agricultural land in Purwokerto, Central Java, Indonesia, which have ability to produce several commercial enzymes. The aim is to obtain a pair of reliable degenerate primers from a limited number of *glpD* sequences from other *Azospirilla* retrieved in GenBank using bioinformatics approach. We demonstrated degenerate primer design that led to successful PCR amplification corresponding to the targeted DNA fragment. Homology analysis showed that the obtained DNA fragment is 61% and 99% similar to sn-glycerol-3-phosphate dehydrogenase genes of *Azospirillum brasilense* and *Stenotrophomonas maltophili* respectively.

Keywords: Degenerate primer, primaclade, *in silico*, colony PCR, *Azospirillum* sp. JG3

INTRODUCTION

To answer many questions of evolutionary developmental biology, it is necessary to identify genes which could be responsible for the studied traits (**Žlůvová, 2007**). PCR amplification of homologous genes using degenerate primers followed by direct sequencing is a standard approach to generate a data set for evolutionary analysis (**Regier and Shi, 2005**).
Degenerate primers are easy and cheap to produce regular unique primers, are useful for amplifying several related genomic sequences, and have been used in various applications (**Linhart and Shamir 2005**). However, degenerate primer design can be still very difficult because of codon degeneracy and the additional degeneracy needed to represent multiple codons at a position in the alignment (**Žlůvová, 2007**). Not only are the calculating tasks heavy, a ranking mechanism for optimization is also very sophisticated. Therefore, the computational aid on primer design is a critical issue in bioinformatics (**Chen, *et al.*, 2003**).
Numerous computer programs are now available allowing users to design oligo-nucleotides nearly without preliminary knowledge. Most of these programs are available for free and straightforward to use from convenient web-interfaces (**Christensen, et al., 2008**). Though not all of these resources are well maintained, some of them, including Primaclade (http://www.umsl.edu/services/kellogg/primaclade.html), are ofhigh quality.
Primaclade is a web-based application that accepts a multiple species nucleotide alignment file as input and identifies a set of polymerase chain reaction (PCR) primers that will bind across the alignment. Primaclade iteratively runs the Primer3 application for each alignment sequence and collates the results (**Gadberry, *et al.*, 2005**). Along with CODEHOP (**Rose, *et al.*, 2003**) Primaclade is one of classical methods for degenerate primer design based on multiple global alignments to identify clear blocks of conserved regions (**Gorrón, et al, 2010**).
In Silico PCR analysis program (http://In Silico.ehu.es/) is a useful and efficient complementary method to ensure primer specificity for an extensive range of PCR applications. This *in-silico* PCR method can assist in the selection of newly designed primers, identify potential mismatches in the primer binding sites and avoid the amplification of unwanted amplicons so that potential problems can be prevented before any "wet bench" experiment (**Yu and Zhang, 2011**).
Recently the application of Primaclade was successful in the evolutionary development study of genus *Begonia* (Neale, et al., 2006) and the genomic island identification study of *E.coli* and *Shigella flexneri* (**Ou, *et al.*, 2006**). In Silico application was also prospering in the multi-copy gene family expression study of *Plasmodium falciparum* (**Bachmann, *et al.*, 2009**) and pseudogene analysis study of human and mouse (**Sun, *et al.*, 2012**). However, a strategy combining of Primaclade and In Silico techniques in *glpD* gene identification study of genus *Azospirillum* has not been implemented.
Azospirillum sp. JG3 strain is a member of *Azospirillum* genus, which members are frequently associated with root and rhizosphere of a large number of agriculturally important crops and cereals (**Saharan and Nehra 2011**). However, lack of media for selective isolation or techniques for specific detection or identification limit the exploration of these rhizobacteria. This has motivated many researchers to design a genus-specific oligo-nucleotide primer pair which could assist in rapid detection of species of the genus *Azospirillum* by means of PCR-specific amplification (**Lin, *et al.*, 2011**).
Despite of its poor genetic and genomic information, the Gram-negative *Azospirillum* sp. JG3 strain was known to have ability to produce industry enzymes, lipase and amylase (**Lestari *et al.*, 2009; Zusfahair and Ningsih, 2012**). This research is an attempt to reveal the genetic properties of *Azospirillum* sp. JG3 bacteria by identifying its *glpD* gene encoding glycerol-3-phosphate dehydrogenase essential for the aerobic growth of bacteria in glycerol or glycerol-3-phosphate (**Choi, *et al.*, 1989**).
The objective of our study was to propose degenerate primers designed using Primaclade which could be used to design primers when only a limited number of sequences with high global similarity to their suspected homologues available. We also demonstrated the use of In Silico web-based tools to simulate a virtual PCR using the obtained primers that can avoid potential problems in our *in-vitro* experiment. We aimed to obtain reliable oligo-nucleotides that can amplify *glpD* gene fragment of strain *Azospirillum* sp. *JG3*.
We show that using a limited number of aligned sequences the amplification of DNA sequences with similarity to glycerol-metabolism related genes in other member of *Azospirillum* genus (possibly homologues) is highly possible using Primaclade. Experimentally, the combination use of Primaclade and In Silico

tools saved time and cost by avoiding trial-and-error experiments using unreliable degenerate primers that fail to provide correct-size PCR results.

MATERIAL AND METHODS

Bacterial culture

Azospirillum sp. JG3 strains previously cultured at Microbiology Laboratory, Faculty of Biology, University of Jenderal Soedirman (Purwokerto, Central Java, Indonesia) were collected as generous gift. The culture was then kept temporarily at 4°C before sub-cultured. Bacterial strains were sub-cultured in 2% nutrient agar for 24h at 39°C. The morphology of grown bacteria was observed by transferring a single colony on glass slide with a drop of sterile water and observed under light microscope (Olympus, Japan). The overnight cell culture was directly used as template of PCR amplification.

Degenerate Primer Design

A pair of degenerate primers was designed using EBI sequence set (http://www.ebi.ac.uk) involving three glycerol-phosphate dehydrogenase genes from genus *Azospirillum*: *Azospirillum* sp. B510 (ENA| BAI71044.1), *Azospirillum lipoferum* 4B (ENA| CBS85664.1) and *Azospirillum brasilense* Sp245 (ENA| CCC96273.1). The sequences were aligned using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and the result was converted to FASTA format using an EBI converter tool (http://www.ebi.ac.uk/Tools/sfc/readseq/). A further checking on hairpin possible formation, self-dimerization possibility and 3'-complementarity was carried out using an online oligo calculator (http://www.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx).

In-silico and *In-vitro* PCR

In-silico or virtual PCR amplification using the pair of designed degenerate primers was conducted using In Silico, a web-based tool (Bikandi, et al., 2004). The results were analyzed to determine whether they are accepted to be used for subsequent experiments.
In-vitro colony PCR reaction was conducted separately at annealing temperature of 56.3°C to amplify the targeted DNA sequence. Total DNA of *Azospirillum sp* JG3 was used as PCR templates, each of which was prepared directly from bacterial colony adopted from previous study (**Pedraza and Díaz-Ricci, 2010**). The PCR mixture contained: Tris-HCl 20mM, KCl 50mM, $MgCl_2$ 1,4mM, dNTPs 0,2mM each one, 1 U Taq Polimerase, each primer at 2.5 µM, and final volume 25 µL. PCR cycling condition was 95°C 3 minutes, followed by 35 cycles of 95°C 30 seconds, 56.3°C 1 minute, and 72°C 1 minute, and finally 72°C 5 minutes.

Nucleotide Sequencing and Sequence Analysis

Fragment isolation was done by cutting out targeted PCR band seen on 1.5% agarose gel. The obtained isolate was then purified and sequenced using GPF and GPR primers with ABI PRISM 310 sequencer. Sequence analysis of PCR product was performed using BLAST search software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Bacterial Sample

Azospirillum sp. JG3 bacteria used in this work were well grown with pellicle formation on medium indicating successful growth of *Azospirillum* **(Kanimozhi and Panneerselvam, 2010)**. Members of the genus *Azospirillum* are Gram-negative to Gram-variable, have a curved-rod shape on solid media **(Kim, et al., 2005)**. Similar morphology was shown by our *Azospirillum* sp. JG3 (Figure 1).

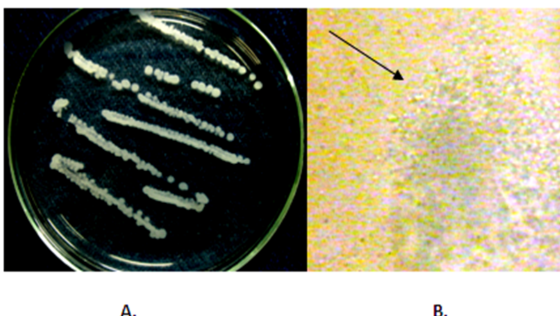

Figure 1 *Azospirillum sp.* JG3 subcultured in 2% nutrient agar medium. A. The cultured bacteria after 24 hour incubation at 37°C. B. A single colony of the culture when observed under the light microscope.

Degenerate primer design

According to literature review, only three sequences associated with *glpD* gene of *Azospirillum* could be found either in GenBank or EBI. A CLUSTALW2 multialignment (http://www.ebi.ac.uk/tools/clustalw2) of the sequences from EBI with score analysis of 79%, 80% and 92% was listed in a section of the program output showing a satisfactory global similarity (data not shown). Such multialignments from limited number of sequence but with highly-conserved regions were a good precedent for primer design and a suitable condition to use Primaclade for degenerate primer design.
The CLUSTALW2 multialignment output file was converted to FASTA format using http://www.ebi.ac.uk/Tools/sfc/readseq/, the result was then saved in notepad as txt file. Primaclade was run under the default condition and it took less than 10 seconds to complete. Compared to manual degenerate primer design procedure it was much a time-saving process.
Totally 26 degenerate primers were successfully generated by Primaclade using the defined sequence set used and the complement sequences of these primers were displayed in the output file. Our further selection to choose a pair of primers to manufacture was relied on maximum theoretical amplicon size and the minimum T_M difference between the primer pairs for easier handling in *in-vitro* experiments.
A pair of forward and reverse primers, GAGAACCGCGAYTAYCTGC (GPF) and GATSGCCAGCACRTTCTTC (GPR) was finally picked to proceed to manufacture for our *in-vitro* PCR experiment. This pair has a relatively close melting point (T_M) making it easier to set the range of its annealing temperature (T_A) for a colony PCR. Based on the position of both theoretical forward and reverse primers (Figure 2), a calculated amplicon size of 450 bp was expected.

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CLUSTAL 2.1 multiple sequence alignment


ENA|BAI71044|BAI71044.1      ATGACAATGATGCCCCCCTCCCCCCTTCACCGCGTCGGCGTCGTCGGCGG 50
ENA|CBS85664|CBS85664.1      ATGAAGACGATGCCCCCCTCTCCCCTCAACCGCATCGGCGTTGTCGGCGG 50
ENA|CCC96273|CCC96273.1      ATGGCGGCAACGGACT---------TCCGGCGCATCGGCGTGATCGGCGG 41
                             ***      * * *          *    *** *******  *******

ENA|BAI71044|BAI71044.1      CGGCGCCTGGGGACGGCGCTGGCGCTGGCGGCCTTGCGGGCCGGGCGCG 100
ENA|CBS85664|CBS85664.1      CGGCGCCTGGGGAACGGCGCTGGCGCTGGCGGCTCTGCGGGCCGGGCGGG 100
ENA|CCC96273|CCC96273.1      CGGGGCCTGGGGCACGGCGCTGGCGCTGGCCGCCCTGCGCGCCGGGCGGG 91
                             *** ******** ***************** **   **** ******** *

ENA|BAI71044|BAI71044.1      AGACGCTGCTGTGGGCACGGGAGCCGGCGGTGGTGGAGGCGATGAGC-CT 149
ENA|CBS85664|CBS85664.1      AGACGCTGCTGTGGGCGCGCGAGCCGGCGGTGGTGGAGGCGATGAGC-CT 149
ENA|CCC96273|CCC96273.1      AGGCCCTGCTGTGGGCGCGCGAGCCGGCGGTGGTCGAGTCGGTCAACACC 141
                             ** * *********** ** ************** *** ** * * * *

ENA|BAI71044|BAI71044.1      GCGCCGCGAGAACCGCGACTATCTGCCCGGCGTGCCGCTGCCCGACGCGC 199
ENA|CBS85664|CBS85664.1      CCGCCGCGAGAACCGCGATTACCTGCCCGGCGTGCCGCTGCCCGACGCGC 199
ENA|CCC96273|CCC96273.1      GCGC-GGGAGAACCGCGACTATCTGCCGGGCGTGACCTTGCCCGCGGCAT 190
                              *** * *********** ** ***** ****** *  ******   **

ENA|BAI71044|BAI71044.1      TGCGCATCACCGGCGACCTTGCCGATCTCGGCGGGTGCGACGCCGTGCTG 249
ENA|CBS85664|CBS85664.1      TGCGGGTCACCGGCGACCTCGCCGATCTCGGCGGTTGCGACGCGGTGCTG 249
ENA|CCC96273|CCC96273.1      TGCGCGCGACCGGCGATCTGGCCGAGGCCGCCGCCTGCGACGCCATCCTT 240
                             ****     ******** ** *****   ** **   ********  * **

ENA|BAI71044|BAI71044.1      CTGGTGTCGCCGGCCCAGCATGCGCGCGGCGTCACCGCGCGGATGGCGCC 299
ENA|CBS85664|CBS85664.1      CTGGTGTCGCCGGCCCAGCATGCGCGCAGCGTCACCGCGCGGATGGCGCC 299
ENA|CCC96273|CCC96273.1      CTGGTGACCCCGGCCCAGCATCTGCGCAGCGCC--TGCGCCGGCCTCGCC 288
                             ****** * ************  **** *** *   **** *    ****

ENA|BAI71044|BAI71044.1      GCTG--CTGAAGCCGGGTGTGCCCGTCGTCGTCTGCGCCAAGGGGATCGA 347
ENA|CBS85664|CBS85664.1      GCTG--CTGAAGCCGGGCGCGCCGGTGGTCGTCTGCGCCAAGGGGATCGA 347
ENA|CCC96273|CCC96273.1      GCGCATCTGCGGACGGGCACCCCGCTGGTCATCTGCGCCAAGGGGATCGA 338
                             **    ***  * ****    **  * *** ******************

ENA|BAI71044|BAI71044.1      GCTGGACAGCCACGCGCTGATGAGCGAGGCGGTCGCCGCCTCCCTGCCCG 397
ENA|CBS85664|CBS85664.1      GTTGGACAGCCACGCGCTGATGAGCGAGGCGGTTGGCGCCGTGCTGCCGG 397
ENA|CCC96273|CCC96273.1      GCTGGACAGCCACGCCCTGATGAGCGAGGCGGCCGCGGCCGCCCTGCCGG 388
                             * ************** ****************  *  ***   ***** *

ENA|BAI71044|BAI71044.1      CCGGCAACCCGGTGGCGATCCTGTCCGGCCCGACCTTCGCGGCGGAGGTG 447
ENA|CBS85664|CBS85664.1      GCGGCAATCCGGTGGCGATCCTGTCCGGCCCGACCTTCGCGGCGGAGGTG 447
ENA|CCC96273|CCC96273.1      CGGGGACCCCGCTGGCCGTGCTGTCCGGTCCGACCTTCGCGGCGGAAGTG 438
                               ** *  *** ****  * ******** ***************** ***

ENA|BAI71044|BAI71044.1      GCGCGCGGGCTGCCGACGGCGGTGACGCTGGCCTGCGCCGACGGGGCGCT 497
ENA|CBS85664|CBS85664.1      GCGCGCGGGCTGCCGACGGCGGTGACGCTGGCCTGCGCCGACGAGGCGCT 497
ENA|CCC96273|CCC96273.1      GCGCGCGGCCTGCCGACCGCGGTGACGCTCGCCTGCGCCGACGCCGCGCT 488
                             ******** ******** *********** ************   *****

ENA|BAI71044|BAI71044.1      CGGCACGGCGCTGGTCGCGGCGCTGGGCAGCCGCACCTTCCGGCCCTACC 547
ENA|CBS85664|CBS85664.1      GGGCACGGCGCTGGTCGCCGCGCTGGGCAGCCGCACCTTCCGGCCTTACC 547
ENA|CCC96273|CCC96273.1      GGGCGCCCGGCTGGTCGAGGCGCTGGGCAGCCGCACCTTCCGCCCCTACC 538
                              *** *   ********  ************************ ** ****

ENA|BAI71044|BAI71044.1      GCTCGGACGACGTCATCGGCTCCCAGGTCGGCGGGGCGGTGAAGAATGTG 597
ENA|CBS85664|CBS85664.1      GCTCGGACGACGTCGTCGGCTCGCAGATCGGTGGGGCGGTGAAGAATGTG 597
ENA|CCC96273|CCC96273.1      TGTCCGACGACGTGGTGGGCTCGCAGATCGGCGGGGCGGTGAAGAACGTG 588
                              ** ********  * ***** *** ***** ************** ***

ENA|BAI71044|BAI71044.1      CTGGCGATCGCCTGCGGCGTCGTCGAGGGGCG------------------ 629
ENA|CBS85664|CBS85664.1      CTGGCCATCGCCTGCGGCGTGGTCGAGGGGCGGCGGCTGGGCGACAATGC 647
ENA|CCC96273|CCC96273.1      CTG----------------------------------------------- 591
                             ***

ENA|BAI71044|BAI71044.1      ---
ENA|CBS85664|CBS85664.1      CCG 650
ENA|CCC96273|CCC96273.1      ---

Results Summary
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Figure 2 A CLUSTALW2 multialignment output before converted into FASTA format. Stars showed the highly conserved regions of *glpD* genes from 3 members of genus *Azospirillum*. Black arrows indicated the theoretical positions of our forward and reverse primers.

In-silico and *In-vitro* PCR

In-silico PCR amplification was carried out three times using each of three genomic sequences of genus *Azospirillum* available in In Silico database as theoretical PCR templates. The output of the *in-silico* PCR was shown in Figure 3. Based on results of this *in-silico* simulation, our designed primers 100% could amplify correct PCR product from three genomic DNA sequences of

Azospirillum species used in this study with an expected size between 400 and 500 bp, which equally matched to 450-bp value corresponding to the calculated amplicon size based on primer positions.
The information in a primer pair is combined by an *in silico* PCR identifies potential amplicons by both identity and size. The *in silico* PCR report allows the user to accept or reject potential primer pairs for experimental use (**Boutros & Okey, 2004**). According to this, it can be inferred that our primers were theoretically accepted and were potential to proceed for *in-vitro* PCR use to amplify targeted part of genomic DNA.

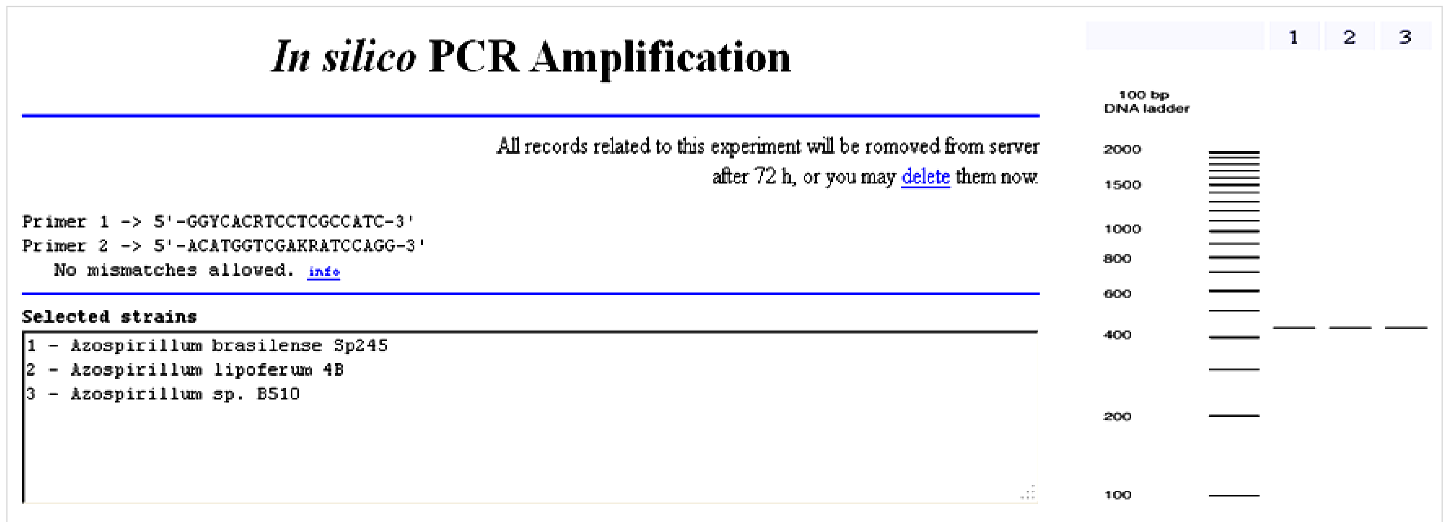

Figure 3 The result of *in-silico* PCR amplification using In Silico tool (http://In Silico.ehu.es/) with the designed degenerate primers obtained from Primaclade and the genome sequences of *Azospirillum* sp. B510, *Azospirillum brasilense* Sp245, and *Azospirillum lipoferum* 4B used as templates.

In-vitro PCR amplification using manufactured degenerate primers designed by Primaclade and genomic DNA templates from cultured *Azospirillum* sp. J3 was successfully carried out and the result is shown in Figure 4 where a band with a size around 450 bp was obtained as the main product. This *in-vitro* PCR result is in accordance with the predicted one previously done by *in-silico* PCR.

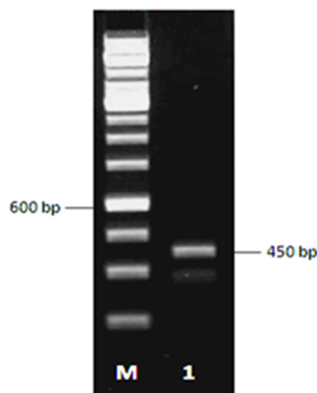

Figure 4 Visualization of *in-vitro* PCR products by agarose gel electrophoresis using degenerate primers from Primaclade: M, 100-bp ladder (Vivantis); Lane 1, PCR products.

Accession number and sequence analysis

The nucleotide sequence of isolated DNA from gel was obtained and shown in Figure 5 and then submitted in GENBANK database with an accession number of AB826685. Results from BLAST search indicated that the DNA sequence shared high identity with *glpD* sequences of other rhizobacteria (*Azospirillum brasilense* and *Stenotrophomonas maltophilia* K279a retrieved in GeneBank databases) at about 61% and 99% identity respectively implying that it was likely a part of a *glycerol-3-phosphate dehydrogenase* gene.

```
BASE COUNT     57 a     137 c     162 g     71 t
ORIGIN
      1 gggattatct gagctgcgtg ccaccaccga cctggcgtcg gcggtggagg gcgcggcctg
     61 gatcctggtg gtgaccccct cgcatgcctt cggcgaaacc gtgcgtgcgc tggcgccgct
    121 gcgtcctgcc ggtgccggcg tggcctgggc caccaagggc ttcgaacccg gttcgggccg
    181 cttcctgcat gaagtggcgc gcgaagtgct gggtgaggac gtgccgctgg ccgttgtcac
    241 cgggccgtcg ttcgccaagg aagtgaccca gggcctgccg accgcgatca ccgtgcacgg
    301 cgacgtgccc gagttcgcgc agatggtggc cgaggcgatg catggcccgg cgttccgcgc
    361 ctacaccggc gacgacatgg ttggtgccga gttgggcggc gcgatgaaga acgtgctggc
    421 catcgaa
//
```

Figure 5 Nucleotide sequence obtained from *in-vitro* PCR product using our degenerate primers.

CONCLUSION

Using degenerate primers generated by Primaclade and In-Silico, PCR amplicon with the correct size could be obtained from both *in-silico* and *in-vitro* PCR amplifications. The primers were able to amplify partially a fragment of (candidate) *glycerol-3-phosphate dehydrogenase* gene of *Azospirillum* sp. JG3. Based on the sequence comparison either at the nucleic acid or deduced amino acid level, we concluded that the fragment we obtained indeed code for Glycerol-3-phosphate dehydrogenase as evidenced by their high identity to other rhizobacterial *glpD* sequences. Our research therefore confirmed that Primaclade provides a quick, easy, powerful and freely available solution for researchers who want to design PCR primers across multiple species as previously declared (**Gadberry, *et al*., 2005**) although secondary structure checking was still required to verify the best possible primers from the program output.

Additional Comments: The result from *in-silico* or digital PCR showing only single amplified band was an indication that ideally such similar specific result could be achieved using *in-vitro* PCR. Therefore, in order to maximize specificity and yield of amplified DNA band as targeted, it is suggested to optimize *in-vitro* PCR condition including the composition of polymerase mix and the amount of other PCR reaction components.

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