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EVALUATION OF MICROBIAL QUALITY OF UNFERMENTED COCONUT SAP WITH DIFFERENT COLLECTION METHODS

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ABSTRACT

Coconut (*Cocos nucifera*) sap is one of the natural drinks, being traditionally tapped from unopened inflorescences of the coconut palms. In the present study, microbial quality of coconut sap that was collected using three methods; Treatment 1: application of hal bark (*Vateria copallifera)* to the 4L clay pots (TM), Treatment 2: Novel sap collection method (NSM), Treatment 3: application of 5g of hal bark in to 30 cm X 50 cm polythene bag in 4L clay pots (MTM) were evaluated. Sap was collected for 12 h duration and Total Plate Count (TPC) and Yeast and Mold Count (YMC) were determined. Colonies were isolated and preserved. DNA extractions of microbes were done by CTAB (Cetyl trimethylammonium bromide) method with modification. ITS1 forward and ITS4 reverse primers were used to identify yeast species and 27 forward and 1492 reverse primers were used for the bacterial species in Polymerase Chain Reaction (PCR). Amplified products were separated using 1.5 % agarose gel and purified DNA was sent into Macrogene Korea for sequencing. Four types (A, B, C and D) of distinct microbial colonies were isolated from the differently collected coconut sap samples. DNA homology data revealed that, A is *Naumannella halotolerans* only presented in NSM. B and C *Serratia marcescens, are Achromobacter xylosoxidans* contained in TM and MTM. *Saccharomyces cerevisiae* (D) was found in all the collection system. The collection method affects for the microbial quality and quantity of unfermented coconut sap.

1.Introduction

Coconut (*Cocos nucifera*) sap is the nutritive phloem fluid that oozes out from the coconut inflorescence after a special extraction process called tapping. The sap is collected at 12 h or 6 h time intervals. Freshly collected coconut sap (*meera*) is a rich source of sugars, minerals and vitamins (Barh and Mazumdar, 2008). It undergoes natural fermentation by wild microorganisms after contacting with atmosphere and is used for product development. The fermented coconut sap (“*Raa”*) is a popular alcoholic beverage among rural people. Natural fermentation of coconut sap starts with lactic acid fermentation and followed by alcoholic fermentation and finally

acetic acid fermentation. Seventeen (17) species of yeasts and seven (7) genera of bacteria have been identified in the naturally fermented coconut sap (Atputharajah *et al.,* 1986).

In order to reduce the rate of fermentation, lime ($Ca(OH)_2$) and bark of Hal tree (*Vateria copallifera*) are put into clay pots from ancient time (Ratnasooriya *et al.,* 2006). However, these substances has negative impact on odour, taste and color of coconut sap. In addition, the traditionally collected sap is generally contaminated by insects, ants, dust particles and pollen due to the gap between clay pot and coconut inflorescence (Navaratne, 2015). Therefore, novel sap collection method was introduced by Coconut Research Institute (CRI) of Sri Lanka to reduce the fermentation creating sealed equipment with an ice box. This new collection device comprises of a fixing unit, a pipe connector, a male adaptor, a flexible hose for translocation of sap and a cooling compartment. The fixing unit has a soft skin to protect the inflorescence without damaging while providing a sealed condition to avoid contaminations. The cooling compartment with ice box prevents the growth of microorganisms due to its low temperature (<15°C). Therefore, the collection method can affect to the microbial quality and quantity of the coconut sap due to having different mode of contact with the coconut inflorescence and different environment.

Molecular approach and biochemical (traditional) approaches can be utilized to microbial identification. The traditional approaches are time-consuming, labour intensive and often subjective. The applications of molecular methods avoid the weaknesses in the culture-dependent methodologies. DNA extraction is crucial step in molecular DNA analysis. Within the different protocols for DNA extraction, CTAB (cetyl trimethyl ammonium bromide) method is commonly used for plant DNA extraction (Doyle and Doyle 1987). Amplification of universal region of microbial genome is used for identification of bacteria. ITS1 forward (5' TCCG TAG GTGAACCTGCGG 3') and ITS4 reverse primers (5' TCCTC GCTTA TTGATATGC 3') (White *et al.,* 1990) were used for yeast species and 27 forward (5'-AGAGT TTGA TCCTGGCTCAG-3') and 1492 reverse (5'-CGGTTACCTTGTTACGACTT-3') primers were used for bacteria species (Miller *et al.,* 2013). This research was conducted to evaluate the microbial quality and quantity of unfermented coconut sap collected from three types of collection methods by using modified plant DNA extraction protocol for bacterial DNA extraction.

2. Materials and methods

2.1. Materials

The experiment was conducted during the period from February to August 2017. Coconut sap was collected from twenty four coconut palms (Tall ×Tall variety, 45 years old) in Bandirippuwa Estate of Coconut Research Institute, Lunuwila. The novel sap collection devices were fabricated at Coconut Processing Research Division, Coconut Research Institute, Lunuwila.

2.2. Methods

2.2.1. Collection of coconut sap sample

One type of sap collection method was applied in to one block of coconut palms which is consisted with eight number of trapping trees. Three rotations were done among blocks to collect samples. Three types of treatments were Treatment 1: Traditional method- application of Hal bark to the clay pots of 4L (TM), Treatment 2: Novel sap collection method (NSM), Treatment 3: Modified traditional method - application of 5g of Hal bark in to 30 cm X 50 cm polythene bag in clay pots of 4L (MTM). The environment temperature was fluctuated from 27°C to 31.4 °C during sap collection from 6.00 pm to 6.00 am.

Coconut sap from each treatment was used for microbial studies. Aliquot of coconut sap sample was diluted and inoculated to the sterilized plate with Nutrient Agar (NA) media for Total Plate Count (TPC) and Potato Dextrose Agar (PDA) for Yeast and Mold Count (YMC). TPC and YMC were enumerated using standard

protocols and isolated pure cultures were preserved at -20°C in glycerol.

2.2.2. Microbial DNA extraction

The pure colonies were grown in nutrient broth, in 24 hrs in a shaking incubator (Grant Instruments™ ES-80) at 28°C. The broth was filled into sterilized 1.5ml micro centrifuge tube in Sigma 1-15K and centrifuged at 5000 rpm for 5 min to separate bacteria and yeast. The supernatant was removed and dry pellet was taken to extract DNA using modified CTAB method. The composition of modified CTAB buffer was 10 % of 1M Tris HCl, 4 % of 0.5 M EDTA, 8.19 % of NaCl, 2 % of CTAB at pH 8. Then, 750 µl of buffer solution was added into the tube with pellet and incubated at 65°C for 30 min (Memmert 0810).

Equal volume (750µl) of phenol: chloroform (1:24) was added to the tubes and mixed and centrifuged at 13,000 rpm for 15 min. Supernatant was collected and Phenol: chloroform extraction step was repeated. Two third (2/3) volume of ice cold iso-propenol was added to the supernatant and mixed well. Samples were kept in refrigerator (4±2 °C) for 2hrs and centrifuged at 13,000 rpm for 10 min. Supernatant was removed and the pellet was washed with 20 µl of 70 % alcohol. The mixture was centrifuged at 10,000 rpm for 10 min and pellet was collected removing supernatant. The dried pellet was dissolved in 20µl of TE buffer and stored at -20 °C. The concentration and quality of extracted DNA were measured by Nano drop 2000C.

2.2.3.PCR amplification with universal primers

For bacteria isolates 27 forward (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 reverse (5'-CGGTTACC TTGTTA CGACTT-3') primers was used for amplification using thermo cycler (Takara Bio Inc TP 600). The temperature profile of PCR machine was 95°C in 15 min initial denaturation), followed by 31 cycles of 95°C 1min denaturation, 55.5°C 2min annealing and 72°C 2 min (extension) Samples were kept at 72°C for 10 min for final extension.

For the Yeast amplification universal primers of ITS1 forward (5' TCCG TAG GTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') reverse primers were used with temperature profile of 9 °C in 15 min (initial denaturation), followed by 94°C 1 min denaturation process, 55.5°C 45 s annealing, 72°C 1.5 min extension, 72°C 10 min final extension, 4°C cooling with 35 cycles for yeast ITS1/ITS4 primer amplification.

2.2.4. Identification of PCR products

Amplified products were separated using 1.5 % (w/v) agarose gel with 100 bp ladder and amplified bands were observed under a transilluminator (Cell Bioscience). The bands were excised and purified with wizard PCR clean-up system. The purified products were sent to Macrogen Inc. Korea for sequencing.

2.2.5. Data analysis

Parametric data was analyzed using Mini Tab 16 using ANOVA. Sequences were blasted and compared with data in NCBI data bank.

3. Results and discussions

3.1. Quantity of Microbes in different collection systems

3.1.1. Total plate count and Yeast count

Microbial counts obtained by colony forming units (CFU) were different in three methods. TPC of sap collected from traditional method (TM) ($1.19*10^7$) and poly bag collection method ($5.2*10^6$) did not show a significant difference ($p<0.05$). The seal environment of NSM with its cooler compartment has retarded the microbial contamination. Therefore, the bacteria and yeast count of NSM showed a significantly ($p<0.05$) lower number of TPC (3.64×10^6) and YMC (7.67×10^6).

3.2. Colony characteristics of isolates

Four types of distinct microbial colonies were isolated from the sap of three different methods (Figure 1; A, B, C and D). A, B and C colonies were bacteria while D colony was a yeast strain. Different types of color patterns and culture characteristics were observed in these

four different isolates (Figure 1). Type "A" isolate was observed in samples collected from NSM while B and C isolates were observed in sap samples of TM and MTM. D colony was observed in samples of all collection methods. The A isolate was light yellow in colour while B isolate had an attractive bright purple colour. the C isolate produced gummy or jelly like colonies. White colour D isolate showed a budding growth and it could be identified as S*accharomyces* strain.

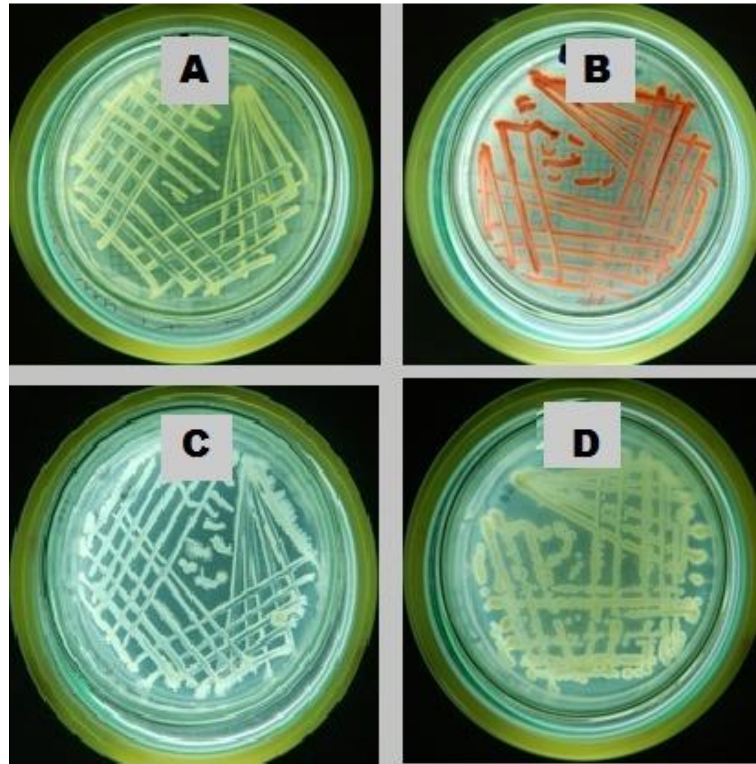

Figure 1. Microbial cultures Isolated from unfermented coconut sap grown in NA (A, B and C) and PDA(D) media

3.3. Quality of extracted DNA by modified CTAB method

The concentrations of DNA of three different isolates revealed that the modified CTAB method for microbial DNA extraction is a feasible method to extract bacterial and yeast DNA with only a few steps while using the same chemicals used for plant DNA extraction. DNA concentrations of three isolates were 3155.1ng/µl, 1567.5ng/µl, 1013.0ng/µl and 3800.3ng/µl for A, B, C and D respectively.

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA and a ratio of ~2.0 is generally accepted as "pure" for RNA (Glasel, J.A, 1995). DNA concentration of 260/280 showed that, all values were over the limit of 1.8. The values of 260/280 were 1.84, 1.96, 2.14 and 2.13 for A, B, C and D respectively showing a high quality without contaminants**.** Expected 260/230 values are commonly in the range of 2.0 - 2.2. If the ratio is appreciably lower than expected, it indicates the presence of contaminants which absorb at 230 nm. The ratio of 260/230 of A, B, C and D were 2.52, 1.58, 2.11 and 2.03 respectively. Gel electrophoresis of PCR products clearly showed that, A B and C isolates extracted from the coconut sap amplified with the 27 forward and 1492 reversed primers and observed as thick bands. The yeast species were amplified with ITS1 forward and ITS4 reversed primers. Homology search results of the sequenced data are represented with accession

number, identity, query cover and isolated collection system (Table: 1).

Table 1. Homology results of the sequenced data

Isolate	Accession Number	Homologous genome / Organism	Identity %	Query Cover %	Isolated collection system
(A)	KC429590.1	*Naumannella halotolerans*	95	94	NSM
(B)	FJ360759.1	*Serratia marcescens*	100	98	TM and MTM
(C)	KP236255.1	*Achromobacter xylosoxidans*	99	98	TM and MTM
(D)	Z75578	*Saccharomyces cerevisiae*	96	69	NSM, TM and MTM

TM: Traditional method- application of Hal bark to the clay pots of 4L, NSM: Novel sap collection method (NSM), MTM: Modified traditional method - application of 5g of Hal bark in to 30 cm X 50 cm polythene bag in clay pots of 4L

3.4. Identification of microorganisms in unfermented coconut sap

Four different isolates A,B, C and D were *Naumannella halotolerans, Serratia marcescens, Achromobacter xylosoxidans and Saccharomyces cerevisiae* respectively. Seventeen strains of yeast of *Saccharomyces* (10 strains), *Pchia* (2 strains), *Torulopsis* (2 strains), *Candida* (2 strains), *Wingae* (1 strains) have been isolated from the coconut toddy (fermented sap) in Sri Lanka. Perera *et al,* (1978) found four strains of *Saccharomyces cerevisiae* and four strains of *Saccharomyces exguus* as the most predominant species. It has been revealed that the fresh sap of *Cocos nucifera* in India contains microorganisms such as *Kloeckera apiculata*, *Schizosaccharomyces pombe*, *Candida glabrata* and in fermented *toddy Kloeckeraa piculata, Schizosacc haromyces pombe, Pichiaangophorae* and *Bacillus firmus* (Kalaiyarasi *et al.,* 2013). The current study reveals that, the changes in collection methods and climatic condition affect microbial community in coconut sap.

Naumannella halotolerans, is a Gram-positive, non-motile, non-spore forming aerobic coccus of the family Propioni bacteriaceae. The organism was previously isolated from a pharmaceutical room and food (Gernot *et al*, 2012). It is one of the endophytic bacteria (plant beneficial) that colonizing on the shoot-tips of banana and it has shown antagonistic activity against the Panama wilt pathogen (*Fusarium oxysporum)* (Sivamani and Gnanamanickam, 1998).

Serratia marcescens is a gram-negative, rod shaped, bright purple color bacterium in the family Enterobacteriaceae. This bacterium is found abundantly in the environment and is a phloem-colonizing, Squash bug -transmitted bacterium and is the causal agent of cucurbit yellow vine disease. *Serratia marcescens* has become an important nosocomial pathogen and it has an antibacterial characteristic (Lapenda, 2015) due to its Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene)-methyl]-2-methyl-3-pentyl-1Hpyrrole) (Moraes, 2009). When, the isolated culture of *S. marcescens* was tested for diffusion method with 2% phenolic extracts of Hal bark, it (B) didn't show antimicrobial property for phenolic compounds of hal bark compared with isolation A (Figure: 2).

Results emphasized that, antimicrobial property of hal bark does not affect the growth of *S. marcescens* due to its prodigiosin.

Unhygienic practices of clay pot and hal bark collection can be the main routes of contamination of *S. marcescens* in to coconut sap.

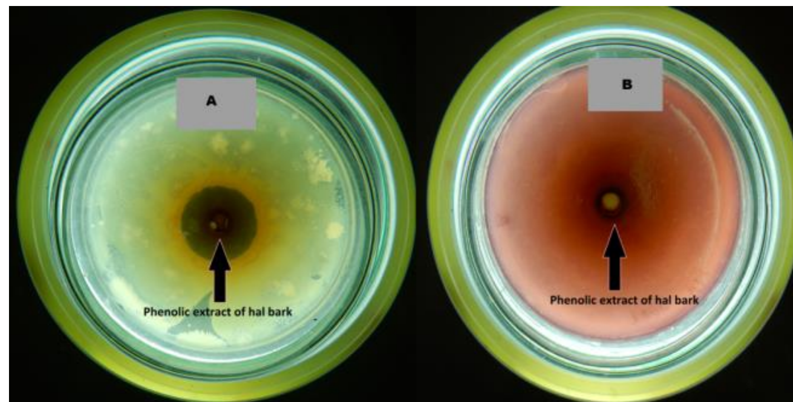

Figure 2. Growth of sap containing microbes A (*Naumannella halotolerans*) and B (*Serratia marcescens*) with phenolic extracts of hal bark

The organism C was identified as *Achromobacter xylosoxidans*. It is a Gram-negative, aerobic, rod shaped motile bacterium. It is generally found in wet environments. *A. xylosoxidans* is considered as an opportunistic pathogen and cause various diseases like bacteremia, meningitis, urinary tract infections, abscesses, osteomyelitis, corneal ulcers, prosthetic valve endocarditis, peritonitis, and pneumonia in both immunocompetent and immunocompromised hosts (Holmes *et al*, 1977). *Saccharomyces cerevisiae* is a unicellular eukaryote that can reproduce both asexually and sexually (Kumar and Srivastava, 2016) and it also known as 'budding yeast' or 'baker's yeast', belongs to kingdom Fungi. The species is widespread and commonly found in fermented fruits, delicious trees and high sugar environment such as nectar and sap. *Saccharomyces cerevisiae* is the main microorganism that has been identified in alcoholic fermentation and odorants production in coconut toddy. It has higher potential to production of alcohol at fairly high temperature (Chandrani, 1998) by converting the sugary sap to alcohol (Amoa-Awua *et al*, 2007).

Three types of sap collection methods did not produce a fully sterile condition. Thus, it facilitates contamination with natural microorganisms. But NSM restricted the colonization of nosocomial pathogen or opportunistic pathogen in unfermented coconut sap compared to other two collection methods. Further, hygienic practices are needed to be applied to increase the quality of coconut sap. The pathogenic nature of isolates restricts the direct consumption of unfermented coconut sap and it needs to go through thermal treatment during the production process to eliminate opportunistic pathogens. However, microbial analysis should be an essential step in processing of sap based products.

4. Conclusions

Microbial DNA can be extracted through modified CTAB method. Three types of unfermented sap samples were contaminated with *Saccharomyces cerevisiae. Naumannella halotolerans* is present in novel sap collection system. *Serratia marcescens* and *Achromobacter xylosoxidans* are identified in sap samples collected from clay pot and poly-bags including *hal* bark. Unfermented sap can't be directly consumed as in the case of fermented sap and it needs to be undergo production processes such as thermal treatment.

5. References

Amoa-Awua, W.K., Sampson, E., Tano-Debrah, K.(2007). Growth of yeasts, lactic and acetic acid bacteria in palm wine during

tapping and fermentation from felled oil palm (Elaeis guineensis) in Ghana. *Journal of Applied Microbiol0gy,* 102, 599–606. DOI: 10.1111/j.1365-2672.2006.03074.x.

Atputharajah, J.D., Widanapathirana, S., Samarajeewa, U. (1986). Microbiology and biochemistry of natural fermentation of coconut palm sap. *Journal of Food Microbiology,* 3 (4), 273-280. DOI:org/10.1016/0740-0020(86)90009-2.

Barh, D., Mazumdar, B.C. (2008). Comparative Nutritive Values of Palm Saps Before and after Their Partial Fermentation and Effective Use of Wild Date (Phoenix sylvestrisRoxb) Sap in Treatment of Anemia. *Research Journal of Medicine and Medical Sciences*, 3(2), 173-76.

Chandrani, W.S. (1998). Temperature tolerance and other properties of two ethanol producing *Saccharomyces* strains isolated from coconut toddy Journal of National Science council Sri Lanka 26(2),77-91. DOI: 10.4038/jnsfsr.v26i2.3556.

Doyle, J.J., Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull, 19, 11–15.

Gernot, R., Siegfried, S., Mareike, W. (2012). Isolation and Identification of Shoot-Tip Associated Endophytic Bacteria from Banana cv. Grand Naine and Testing for Antagonistic Activity against Fusarium oxysporum f. sp. Cubense, *International Journal of Systematic and Evolutionary Microbiology*, 62, 3042–3048.

Glasel, J.A. (1995). Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios, Biotechniques 18, 62-63.

Holmes, B., Snell, J. J. S., LaPage, S. P. (1977). Strains of Achromobacter xylosoxidans from clinical material. *Journal of Clinical Pathology.* 30, 595-601. DOI: 10.1136/jcp.30.7.595.

Kalaiyarasi, K., Rajarajan, S., Sangeetha, K. (2013). Comparative study on the microbial flora of the fresh sap from cut inflorescence and fermented sap (toddy) of *Borrassus flabellifer Linn* (Palmyrah tree) and of *Cocos nucifera Linn* (Coconut tree) to identify the microbial fermenters. *International Journal of Research in Pure and Applied Microbiology*, 3(3), 43-37.

Kumar, R., Srivastava, S.(2016). Quantitative proteomic comparison of stationary/G0phase cells and tetrads in budding yeast. Science Reports.; 6: 32031. DOI:https://doi.org/10.1038/srep32031.

Lapenda, J.C., Silva, P.A, Vicalvi, M.C, Sena, K.X, Nascimento, S.C. (2015). Antimicrobial activity of prodigiosin isolated from *Serratia marcescens* UFPEDA 398. World Journal of Microbiol Biotechnology, 31: pp 399-406. DOI: 10.1007/s11274-014-1793-y.

Miller, C.S., Handley, K.M., Wrighton, K.C., Frischkorn, K.R., Thomas, B.C., Banfield, J.F. (2013). Short-read assembly of full-length 16S amplicons reveals bacterial diversity in subsurface sediments 8(2), 56018.DOI:org/10.1371/journal.pone.0056018.

Moraes, C.S., Seabra, S.H., Albuquerque, J.M., Castro, D.P., Genta, F.A., De Souza, W., Brazil, R.P., Garcia, E.S., Azambuja, P. (2009). Prodigiosin is not a determinant factor in lysis of Leishmania (Viannia) braziliensis after interaction with Serratia marcescens d-mannose sensitive fimbriae. Exp. Parasitol. 122, 84-90.DOI: 10.1016/j.exppara.2009.03.004.

Navaratne, S.B. (2015). Determination of the Effectiveness of Hal Bark (*Vateria copallifera*) as a /Natural Preservative for Food Security of Confectionery Industry,.*Journal of Tropical Forestry and Environment* ,5(2), 60-66. DOI: 10.31357/jtfe.v5i2.2661

Perera, M., Ruwan, A., Pathirana, Kandasamy, S., Edwin E., Jeyaraj. (1978).Yeasts of the coconut palm wine of Sri Lanka. *Journal of science of food and agriculture*, 29 (11), 975–978. DOI: 10.1002/jsfa.2740291112

Ratnasooriya, W.D., Lelwala, L.H.D.S., Kannangara, K.N.K., Sandanayake, S.D.D. (2006). Sedative activity of steam bark of Sri

Lankan Endemic Plant, Fitoterapia, 77,331-332.

Sivamani, E., Gnanamanickam, S.S. (1988). Biological Control of *Fusarium oxysporum* f. sp. *cubense* in Banana by Inoculation with *Pseudomonas fluorescens*. *Plant and Soil*, 107, 3-9.
DOI: https://doi.org/10.1007/BF02371537

White, T., Burns, T., Lee, S., Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds). PCR Protocols. San Diego: Academic Press, 315-322.