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Molecular Identification of *Neurospora* sp. N-1 isolated from Indonesian Red Fermented Cake

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The Indonesian traditional fermented food, red fermented peanut cake (oncom merah) limited to West Java is produced from legume residues by filamentous fungus *Neurospora sp*. Oncom Bandung is prepared from peanut residues after oil extraction. Conidia, usually oval and pink in color, form branched chains at the tips of the aerial hyphae. Since the pigment of the fungus can be utilized in pharmacy and food. We are interested to study the fungus for further utilization in pharmacy. In this study, the molecular identification of *Neurospora sp* N-1 which was isolated from oncom merah was carried out based on the genetic analysis partially on ribosomal DNA included the subunit of 28S rDNA (*D1/D2* region) and Internal Transcribed Spacer (ITS). Results analysis showed that *Neurospora sp* N-1 was homology 100% by amplification on D1/D2 region and 99% by amplification on ITS region to *Neurospora intermedia*. Based on phylogenetic analysis using D1/D2 region and ITS methods, *Neurospora intermedia* N-1 has a close relation to *Neurospora crassa*. We concluded that the fungus *Neurospora sp* N-1 isolated from oncom Bandung is *Neurospora intermedia*. It is a new information since it before that the fungus for oncom merah fermentation was *Neurospora sitophila*.

Key word: Oncom, molecular, homology, *N. intermedia* N-1.

The traditional fermented food, oncom limited to West Java is produced from legume residues by *Neurospora sp*. There are two kinds of oncom, red oncom and black oncom. There are two type of oncom merah, first type is limited to Jakarta-Bogor region and prepared from soybean residues after extraction of protein and curd. The soybean residue is mixed with carbohydrate, inoculated with *Neurospora* conidia and incubated on bamboo trays for two days. The second type, called oncom itself and limited to the Bandung region, is prepared from peanut residues after extraction of oil. The peanut residue is washed, mixed with tapioca flour, boiled, inoculated with *Neurospora* conidia and fermented for two days. The red fermented cake product is covered with a massive coat of living conidia are sold in most market in West Java[1].

The genus *Neurospora* became one of the most important organisms in genetic research when it was used by George Beadle and Edward Tatum in the early 1940s as an experimental model[2]. The two researchers used the fungus to develop their "one gene-one enzyme" hypothesis of gene action. This work initiated the era

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molecular biology. *Neurospora* is particularly useful for genetic because, within each ascus, the four products of meiosis divide once to form eight cells that remain fixed in a row in the order in which they were formed. Each ascocpore in an ascus can be removed in order and its genetic composition can be determined. Morphologically, *Neurospora* produces a loose network of long strands of septate and aerial hyphae. Conidia, usually oval and pink in color, form branched chains at the tips of the aerial hyphae.

The genetic and molecular analyses of the phenomenon of senescence—i.e., irreversible loss of growth and reproductive potential upon subculturing—in *Neurospora intermedia* strain M1991-60A, collected from Maddur in southern India, showed the presence of plasmid pMaddur1, which is homologous to the senescence-inducing circular mitochondrial plasmid[3].

On this study, molecular identification of *Neurospora* sp N-1 strain which was isolated from oncom in Bandung region was carried out. The molecular identification of this fungi based on the genetic analysis partially on ribosomal DNA included the subunit of 28S rDNA (*D1/D2 region*) and Internal Transcribed Spacer (ITS).

MATERIALS AND METHODS

Materials

Indonesian fermented red peanut cake (oncom merah) samples were obtained from some traditional market in Bandung, Indonesia. Extraction of DNA from fungi was carried out by using nucleon PHYTOpure (Amersham LIFE SCIENCE) reagent. The Primer of PCR amplification on D1/D2 region was used NL-1 5'—GCA TAT CAA TAA GCG GAG GAA AAG-3' and NL 4 5'— GGT CCG TGT TTC AAG ACG — 3'. PCR amplification on ITS region was used the Primer ITS 4 5'— TCC TCC GCT TAT TGA TAT GC – 3' and ITS 5 5'—GGA AGT AAA AGT CGT AAC AAG G –3'.

Methods

Isolation of *Neurospora sp* N-1 by TPC method

Neurospora sp was isolated by total plate count (TPC) method. 0.1 gram of spores sample from *oncom merah* was diluted in 10 ml aqua sterile containing 0.1% of tween. The suspension of spores was mixed and diluted to 10^{-7} dilution serial. One ml of a diluted sample was placed in each of sterile petri dish, and then the liquid of potato dextrose agar (PDA) was added and mixed slowly. After the medium was solidified, petri dishes were incubated at 30°C for overnight. The separated colony was removed to PDA slant and incubated at 30°C until the yellow-orange spores was appeared. The isolation process was repeated to obtain the pure isolate. *Neurospora sp* N-1 isolate was selected for further identification.

Molecular identification of *Neurospora sp* N-1

Molecular identification of *Neurospora sp* N-1 was carried out based on genetic analysis partially to that of ribosome DNA of fungi, included the 28S rDNA (D1/D2 region) subunit and Internal Transcribed Spacer (ITS). Isolation of DNA was carried out by inoculated the isolate in liquid medium Potato Dextrose Broth (PDB*)* and incubated for 72 hours. Biomass of mycelia was then harvested for DNA extraction process. PCR product purification was carried out by PEG precipitation method[4] and continued by sequencing cycle. The purification of this product was repeated by ethanol purification method. The analysis of nitrogen base sequence was read using the automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems). Raw data's from the sequencing was then trimmed by MEGA 4 program and assembling by BioEdit program, then converted in FASTA format. DNA sequencing data's in FASTA format was continued using BLAST program to identified the homology by on line method in data base center on DDBJ (http://www.ddbj.nig.ac.jp) or NCBI (http://www.ncbi.nlm.nlh.gov/). The last step of identification was analysis the phylogenetic tree using Clustal X and NJ plot program.

RESULTS AND DISCUSSION

Neurospora sp N-1 isolate was selected for molecular identification because of its good capability in fermentation using waste tofu substrate. DNA sequencing data's in FASTA format was continued using BLAST program that shown on Fig. 1. 28S rDNA is a large sub unit of eukaryotic cell and ITS (internal transcribed spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript.

Results of homology analysis to *Neurospora sp* N-1 to that of ribosome DNA of fungi using 28S rDNA (D1/D2 region) subunit and Internal Transcribed Spacer (ITS) are shown on Table 1.

Family and phylogenetic trees of *Neurospora intermedia* N-1 isolate were analyzed using Clustal X and NJ plot program. To construct the phylogenetic trees, the homology sequence from BLAST and FASTA format results was

Table 1. Results of molecular identification to *Neurospora sp* N-1

Region	*Neurospora sp*	Homology (%)
D1/D2 of 28S rDNA	*Neurospora intermedia*	100
ITS1, 5.8S, ITS2 of rDNA	*Neurospora intermedia*	99

AY681149.1 Neurospora intermedia 28S large subunit
ribosomal RNA gene, partial sequence.
Length = 831 Score = 1009 bits (509), Expect = 0.0
Identities = 509/509 (100%) Strand = Plus / Minus

Query: 1 tgcagatgcgcgaacctcggtcccggcgagggcattacgcccggggctataacactcccg 60
Sbjct: 519 tgcagatgcgcgaacctcggtcccggcgagggcattacgcccggggctataacactcccg 460

Query: 61 gaggagctacgttccccgaacctttatccccccgccaaaaccgatgctggcctgagccgt 120
Sbjct: 459 gaggagctacgttccccgaacctttatccccccgccaaaaccgatgctggcctgagccgt 400

Query: 121 cccgagtgcaccggtgagaacaccggatgatcggaacggcgcaagtctggtcacaaacgc 180
Sbjct: 399 cccgagtgcaccggtgagaacaccggatgatcggaacggcgcaagtctggtcacaaacgc 340

Query: 181 ttcccctttcaacaatttcacgtgctattaaccctcttttcaaagtgcttttcatctttc 240
Sbjct: 339 ttcccctttcaacaatttcacgtgctatttaaccctcttttcaaagtgcttttcatctttc 280

Query: 241 gatcactctacttgtgcgctatcggtctctggccggtatttagctttagaagaaatttac 300
Sbjct: 279 gatcactctacttgtgcgctatcggtctctggccggtatttagctttagaagaaatttac 220

Query: 301 ctcccattttgagcagcattcccaaactactcgactcgtcgaaggagctttacattggat 360
Sbjct: 219 ctcccattttgagcagcattcccaaactactcgactcgtcgaaggagctttacattggat 160

Query: 361 cggcatccgactatacggggctctcaccctctatggcgccccgttccaggggactcagaa 420
Sbjct: 159 cggcatccgactatacggggctctcaccctctatggcgccccgttccaggggactcagaa 100

Query: 421 ggtgcctcaccaaaagcttcctctacaaattacaactcgggccgaagccagatttcaaat 480
Sbjct: 99 ggtgcctcaccaaaagcttcctctacaaattacaactcgggccgaagccagatttcaaat 40

Query: 481 ttgagctgttgccgcttcactcgccgtta 509
Sbjct: 39 ttgagctgttgccgcttcactcgccgtta 11

Fig. 1. BLAST result of 28S rDNA (D1/D2 region) PCR product

calculated the data for tree construction by Clustal X, and then conversion of calculated data into trees by NJ plot. Neighbors-joining (NJ) method is the simple program to construct the phylogenetic trees from evaluationary distance data by finding the pairs of operational taxonomic units (neighbors) that minimize the total branch length at each stage of clustering of neighbors starting with a star-like tree[5]. The phylogenetic trees of D1/D2 and ITS region were shown on Fig. 2 & 3.

Based on phylogenetic analysis using D1/D2 region and ITS methods (Fig. 2 and Fig. 3), *Neurospora intermedia* N-1 has a close family with *Neurospora crassa*. *N. crassa* and *N. intermedia* are similar enough in crossing behavior to be confused with each other, these species produce large normal-looking perithecia when crossed with each other. *N.intermedia* strains are orange and are indistinguishable in appearance from typical *N. crassa* [6]. Detmann

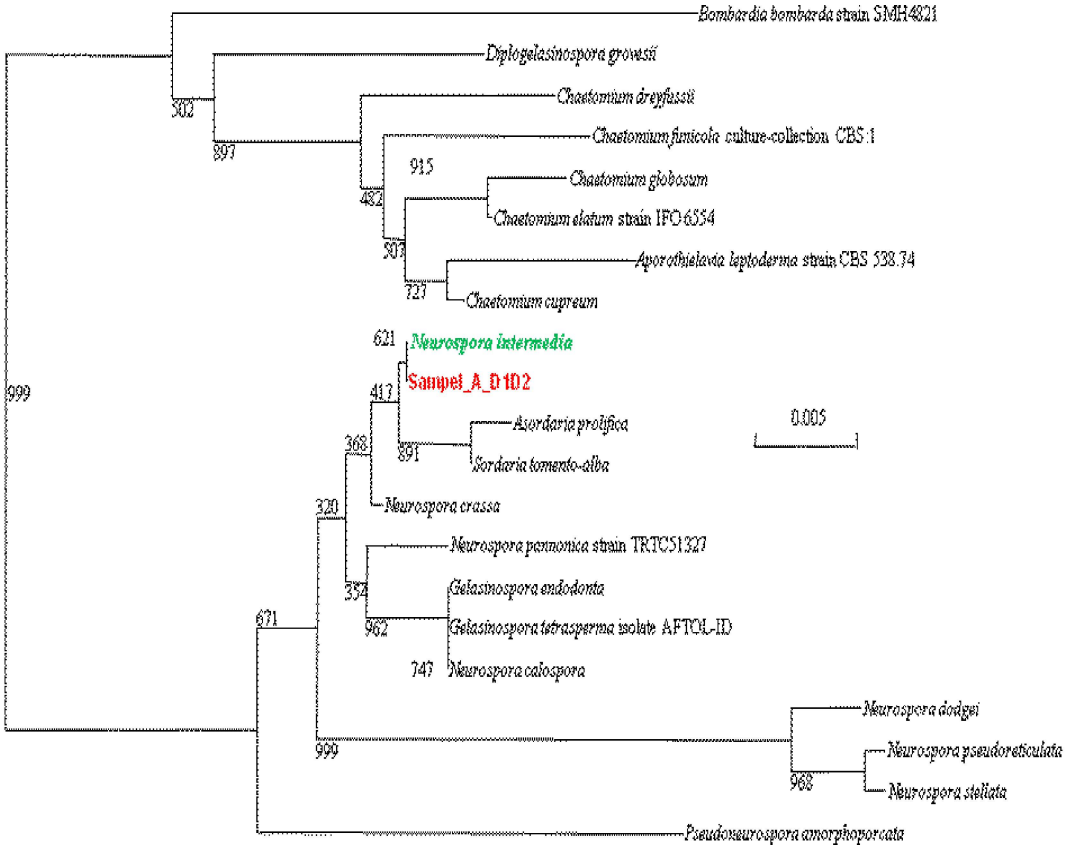

Fig. 2. Phylogenetic tree of D1/D2 region of *Neurospora sp* N-1

et al., [7] reported the examined methods for recognizing species in the model filamentous fungal genus *Neurospora* by comparing traditional biological species recognition (BSR) with more comprehensive applications of both BSR and phylogenetic species recognition (PSR). Each of the putative *N. crassa* x *N. intermedia* hybrids included in this study was confidently assigned to a single species, using both PSR and BSR. A short branch indicates that little reproductive isolation was observed between the two groups of individuals. The range of *N. crassa* overlaps with that of *N. intermedia*

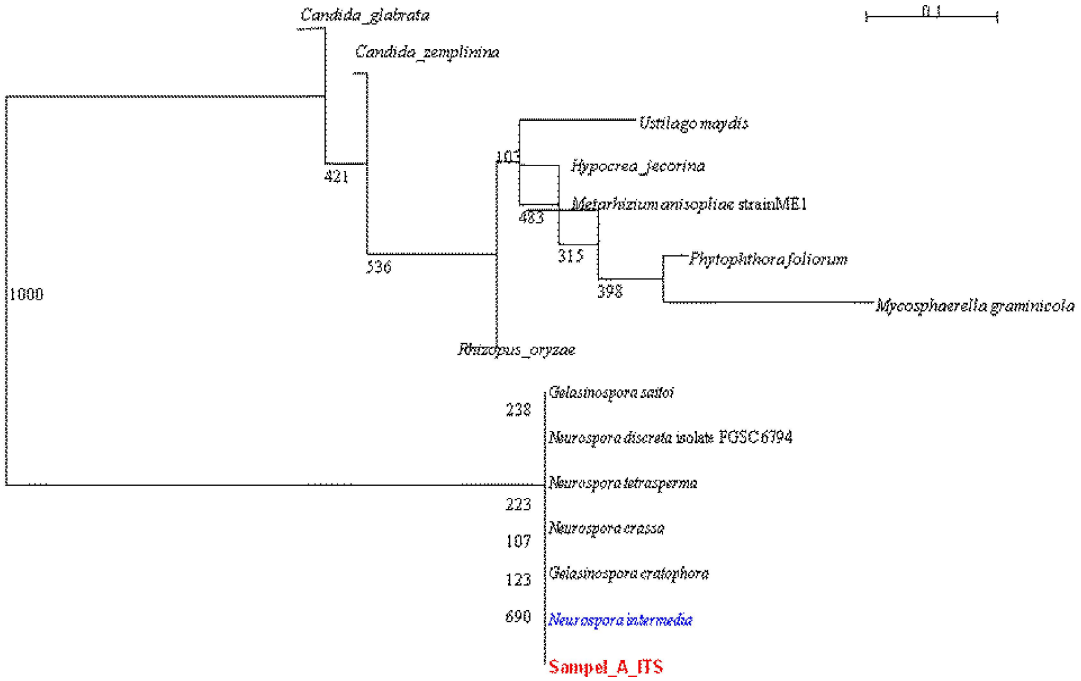

Fig. 3. Phylogenetic tree of ITS region of *Neurospora sp* N-1

CONCLUSION

Homology analysis to *Neurospora sp* N-1 to that of ribosome DNA of fungi using 28S rDNA (D1/D2 region) subunit and Internal Transcribed Spacer (ITS), the isolate is homology to *N. intermedia*. Based on phylogenetic analysis using D1/D2 region and ITS methods, *N. intermedia* N-1 has a close family with *N. crassa*.

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REFFERENCES

1. C.C. Ho, Microbiology of Oncom, an Indonesian Fermented Food. Proceedings of The First ASEAN Workshop On Solid Substrate Fermentation, ASEAN Sub-Committee On Protein, Bandung, Indonesia. 1978; 145-147
2. Pelczar M.J., E.C.S. Chan, Noel R. Krieg. Microbiology: concepts and applications. Mc. Graw Hill. International Edition 1993; 282
3. Anthony D. D'Souza *et al.,* Characterization and prevalence of a circular mitochondrial plasmid in senescence-prone isolates of *Neurospora intermedia*, *Curr Genet.* 2005; **47**: 182–193
4. Hiraishi,A., Kamagata, Y., & Nakamura, N. Polymerase chain reaction amplification and restriction fragment length polymorphism analysis of 16S rRNA genes from methanogens. *Journals of Fermentation Bioengineering*. 1995; **79**: 523-529.
5. Nakagawa Y. 16S rRNA sequencing analysis. Training course Bioindustries II. NBRC-NITE. Japan 2006.
6. Turner B.C., David D. Perkins,1 and Ann Fairfield. *Neurospora* from Natural Populations: A Global Study. *Fungal Genetics and Biology.* 2001; **32**: 67-92
7. Dettman J. R.,1,2 David J. Jacobson. Reproductive Isolation and Phylogenetic Divergence in *Neurospora*: Comparing Methods of Species Recognition in a Model Eukaryote. *Evolution,* 2003; **57**(12): 2721–2741.