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Molecular Diversity of Mold Associated with *Gatotan*

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Gatotan is a raw material to make *gatot*, an Indonesian fermented food, especially in Central Java, made from cassava tubers. Traditionally, the cassava tubers are sun-dried for several days until the black color appears. However, natural fermentation allowed by many types of microorganisms, especially mold in this process could raise concerns about the food safety issues. Previously, the identifications of molds in *gatotan* were based on morphological observation. Here, we reported the diversity of molds associated with *gatotan* using molecular identification method. The molecular identification was based on ribosomal DNA internal transcribed spacer (ITS) amplification sequences using combination of ITS4 and ITS5 primers. A total of ten molds were isolated and phylogenetic trees were constructed based on ribosomal DNA sequences. Our results showed that the molds were classified into *Lasiodiplodia* spp., *Trichoderma* sp., *Aspergillus nomius, Fusarium oxysporum*, and *Cladosporium sphaerospermum.*

Key words: cassava, fungi, *gatot*, internal transcribed spacer

Gatotan adalah bahan baku dalam pembuatan gatot, makanan fermentasi Indonesia, khususnya di Jawa Tengah yang terbuat dari umbi singkong. Pembuatan gatot secara tradisional dilakukan dengan mengeringkan umbi singkong langsung dengan paparan sinar matahari hingga muncul warna kehitaman. Fermentasi alami yang terjadi pada proses ini melibatkan pertumbuhan berbagai mikroorganisme, terutama kapang, yang tidak terkendali dan dapat menimbulkan permasalahan keamanan pangan. Identifikasi kapang yang terlibat dalam pembuatan gatotan telah dilakukan secara morfologi. Pada penelitian ini, identifikasi kapang yang berasosiasi dengan gatotan dilakukan secara molekuler. Identifikasi molekuler dilakukan dengan menggunakan amplifikasi situs *internal transcribed spacer* (ITS) pada DNA ribosom menggunakan pasangan primer ITS4 dan ITS5. Sebanyak sepuluh kapang telah diisolasi dan pohon filogenetik disusun berdasarkan pada sekuen DNA ribosom. Hasil identifikasi yang diperoleh ialah *Lasiodiplodia* spp., *Trichoderma* sp., *Aspergillus nomius, Fusarium oxysporum*, dan *Cladosporium sphaerospermum.*

Kata kunci: cendawan, gatot, *internal transcribed spacer*, singkong

Fermentation of cassava (*Manihot esculenta*) has been considered as one strategy to improve nutrient quality and reduce its natural cyanide content. Recently, *Lactobacillus plantarum*, a lactid acid bacteria commonly found in cassava waste, was reported decrease up to 97% of the cyanide content in cassava after 36 hours of fermentation (Hawashi *et al.* 2018). Furthermore, together with *L. plantarum*, several microorganisms consortium including *Corynebacterium manihot*, *Saccharomyces exiguus*, and *Geotrichum candidum* reduced the cyanide content and improved the protein and fatty acid quality of cassava after 96 hours fermentation (Samson and Akomolafe 2017). In Indonesia, fermentation of cassava is usually used to make *gatot*. Traditionally, cassava tubers are peeled off, cut, and washed until clean, then sun-dried for 3-5 days until become *gaplek*. *Gaplek* is placed outdoor within approximately two weeks until the tubers colour changed to black, indicating natural fermentation to form *gatotan* which can be further processed into *gatot* by steaming (Purwandari *et al.* 2014a).

Gatotan flour had 90.33% antioxidant scavenging activity 18.92 mg × 100 g^{-1} equivalent to vitamin E (Purwandari *et al.* 2014b). Development of *gatot* as noodle would not only increase the blood sugar level quicker, but also reduce it quicker than white bread consumption control, suggesting the low glycemic potential of *gatotan*. Furthermore, the starch resistant of *gatotan* (22.5%) and *gatot* (21.85%) were lower than cassava (24.44%) in glucose oxidase assay

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(Puspaningtyas *et al.* 2018). Additionally, the dietary fiber of *gatotan* (14%) and *gatot* (17.36%) were found to be higher than cassava (8.61%), respectively suggesting a prominent *gatot* as functional food for people with diabetes mellitus (Sari *et al.* 2018).

Considering the benefits of *gatot* as fermented food product, identification of microorganisms involving in this process were reported. Mold, such as *Aspergillus flavus*, *Rhizopus oryzae*, and *Lasiodiplodia theobromae* were showed to be associated with *gatotan* from various regions in Indonesia (Purwandari 2000). In addition, phenotypic identification showed predominant type of fungi, *Botryodiplodia theobromae*, *R. oligosporus*, together with indigenous lactic acid bacteria *L. fermentum* and *L. manihotivorans* were potential as starter culture for controlled *gatotan* fermentation (Astriani *et al.* 2018). Moreover, spontaneous fermentation of cassava to make *gatot* resulted in growth of *R. oryzae* and *Acremonium charticola*, proposed as indigenous fungi which had salt-tolerant properties and potential antioxidant activity (Yudiarti and Sugiharto 2016; Sugiharto *et al.* 2016). As fungal consortium in *gatotan* fermentation was broadly diverse, its clustering needs to be re-confirmed using molecular approaches. However, there was limited report about molecular identification regarding fungal consortium in *gatot*.

Among many DNA barcode for fungal identification, nuclear ribosomal internal transcribed spacer (ITS) is considered as the most accurate region to differentiate the gap between inter- and intraspecific variations (Schoch *et al.* 2012). ITS region, comprised of ITS1, ITS2, and small 5.8S ribosomal RNA (rRNA), is located between the large 18S and 28S ribosomal RNA in fungal nuclear cistron. Splitting process in posttranslational modification would remove ITS1 and ITS2 since they act as non-coding region (Schoch *et al.* 2012). Distinguish properties of this region influenced efficacy for systemic identification, including in diagnostic mycology area (Ciardo *et al.* 2010). The universal primer combination of ITS4 and ITS5 anneals to tip part of large rRNA subunits, consequences in amplification of whole ITS region for phylogeny construction (White *et al.* 1990). Furthermore, improved ITS primer combination has been evaluated to be capable for food fungal community profiling (Walters *et al.* 2016). Thus, ITS would be a promising tool as a key of mold identification in *gatotan*. This research aimed to identify the diversity of mold associated with *gatotan* using molecular approach of ITS region.

MATERIALS AND METHODS

Gatotan. *Gatotan* were made from cassava (*M. esculenta*) tubers in Bogor. All the water used was distilled water using GFL Water Distillation Unit 2008 (GFL, Burgwedel, Germany). Potato dextrose base (Oxoid, Hampshire, United Kingdom) was mixed with 15 g L^{-1} agar base for potato dextrose agar (PDA), while *A. flavus* and *A. parasiticus* agar (AFPA), and dichloran 18% glycerol (DG18) agar were made according to the protocols (Hocking and Pitt 1980; Pitt *et al.* 1983). The DNA extraction reagent DNA Phytopure™ Kit Extraction was provided by GE Healthcare Life Sciences (Amersham, United Kingdom). Primer pairs of ITS4 (5'-TCCTCCGCTTATTGATATGC-3') dan ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') were synthesized by Integrated DNA Technologies (Singapore).

Mold Isolation. The methods of mold isolation were carried out using direct and dilution methods. In direct isolation method, aseptically, inside part of *gatotan* were placed on PDA and incubated at 30 °C. In dilution method, AFPA and DG18 medium were used. First, 25 g of *gatotan* were suspended with 250 mL aquadest. The suspension was diluted 10^{-2}, 10^{-4}, and 10^{-6} using physiological salt solution. A total of 1 mL diluted suspension was mixed with warm (45 °C) AFPA and DG18 medium prior to solidification. Solid mixtures were incubated at 30 °C. Mold growth was monitored everyday for total four days. Different growing colonies were placed in new PDA for further examination.

DNA Isolation, PCR Amplification, and Sequencing. Four days old mycelia grown on PDA was mixed with distilled water, then centrifuged at 10,000 rpm for 10 min. Pellet were mixed with glass beads (Oxoid, Hampshire, United Kingdom) and vortexed to disrupt the cell wall. DNA were extracted using DNA Phytopure™ Kit Extraction (GE Healthcare Life Sciences, Amersham, United Kingdom) according to manufacturer's protocol. A total of 200 ng DNA was amplified using ITS4 and ITS5 primer pairs to obtain ITS rDNA sites (White *et al.* 1990). The PCR procedures were carried out using Go Taq Green® Master Mix PCR 2× (Promega) with reaction as follows: pre-denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min with final elongation at 72 °C for 5 min (Abe *et al.* 2007). The visualization was done on 1% agarose gel

electrophoresis (Mini-Sub® Cell GT Cell BioRad) at 60 V for 90 min followed by ethidium bromide staining and imaging using Gel Doc™ XR System (BIO-RAD, Germany). PCR products were sent to First BASE (Malaysia) for sequencing.

Phylogenetic Analysis. The nucleotide sequence were analyzed using basic local alignment search tool (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.* 1990) to acquire the mold species. The sequences were aligned using SeqTrace software version 0.9.0 (Stucky 2012). The phylogenetic trees were obtained using maximum likelihood (ML) parameter on molecular evolutionary genetics analysis 6 (MEGA6). Kimura 2 model was chosen to represent the phylogenetic tree. Strength of the internal branch of phylogenetic tree was analyzed with 1,000 bootstraps (BS) (Felsenstein 1985). The reference and outgroup sequence were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank) (Benson *et al.* 2005).

RESULTS

A total of ten mold strains were isolated and identified, including *Lasiodiplodia* (4 strains), *Trichoderma*, *Aspergillus*, *Fusarium* (3 strains), and *Cladosporium* (Table 1). Identification based on ITS sequence on four strains of *Lasiodiplodia* spp. showed that these particular strains were found on the same clade with *Botryosphaeria rhodina* with low BS value (0.47) (Fig 1). The ITS region sequence resolution on phylogenetic tree could not describe *Lasiodiplodia* spp. to species level. There were two species types in one same cluster, namely *B. rhodina* with its anamorphic name *L. theobromae* and *L. parva*.

Strain ST4 of *Trichoderma* sp. was found on the clade consisting of *Trichoderma reesei, T. longibrachiatum, T. orientale,* and *Hypocrea orientalis* with BS value 0.80 (Fig 2). ITS region sequence resolution on phylogenetic tree based on ML analysis could not differentiate the intraspecies variations. Two specimens, *T. reesei* and *T. longibrachiatum*, were in the same cluster. However, the comparison result from the NCBI database showed 95% similarity of strain ST4 with *T. longibrachiatum,* respectively (data not shown).

One *Aspergillus* strain was identified as *A. nomius* (Fig 3). According to the ITS tree produced from ML analysis, STP5 is included in the clade *A. nomius* with high BS value 0.99. In addition, three of *Fusarium* strains were identified as *F. oxysporum* (Fig 4). Strains STA2, STA4, and STA5 were found in the *F. oxysporum* clade with BS value 0.63. Meanwhile, strain STD1 was identified as *Cladosporium sphaerospermum* with BS value 0.86 (Fig 5). The phylogenetic tree formed specific clade of *C. sphaerospermum* with STD1 isolate included.

DISCUSSION

Cassava fermentation into *gatotan* involves several molds to produce unique and distinct taste. Several previous studies showed the involvement of this particular mold genera in *gatotan* fermentation including *A. flavus*, *R. oryzae*, *R. oligosporus* and *L. theobromae* (Astriani *et al.* 2018; Purwandari 2000). Our results showed similar mold genera were identified using molecular identification (Fig 1 and 3). These include *Lasiodiplodia* spp. and *A. nomius*. Importantly, *Lasiodiplodia* spp. was isolated and characterized in every *gatotan* investigation. However, the morphological characterization showed inconsistent identification, while Purwandari (2000) identified this mold as *L. theobromae* and Astriani (2018) as *B. theobromae*. In addition, based on the reference Index Fungorum and Mycobank,

Table 1 Molecular identity of mold strains associated with *gatotan* from Bogor

Isolation medium	Strain code	Molecular identity
Potato dextrose agar	ST2	*Lasiodiplodia* sp.
	ST3	*Lasiodiplodia* sp.
	ST4	*Trichoderma* sp.
	STP5	*Aspergillus nomius*
	STP6	*Lasiodiplodia* sp.
Aspergillus flavus and *parasiticus* agar	STA1	*Lasiodiplodia* sp.
	STA2	*Fusarium oxysporum*
	STA4	*Fusarium oxysporum*
	STA5	*Fusarium oxysporum*
Dichloran glycerol 18% agar base	STD1	*Cladosporium sphaerospermum*

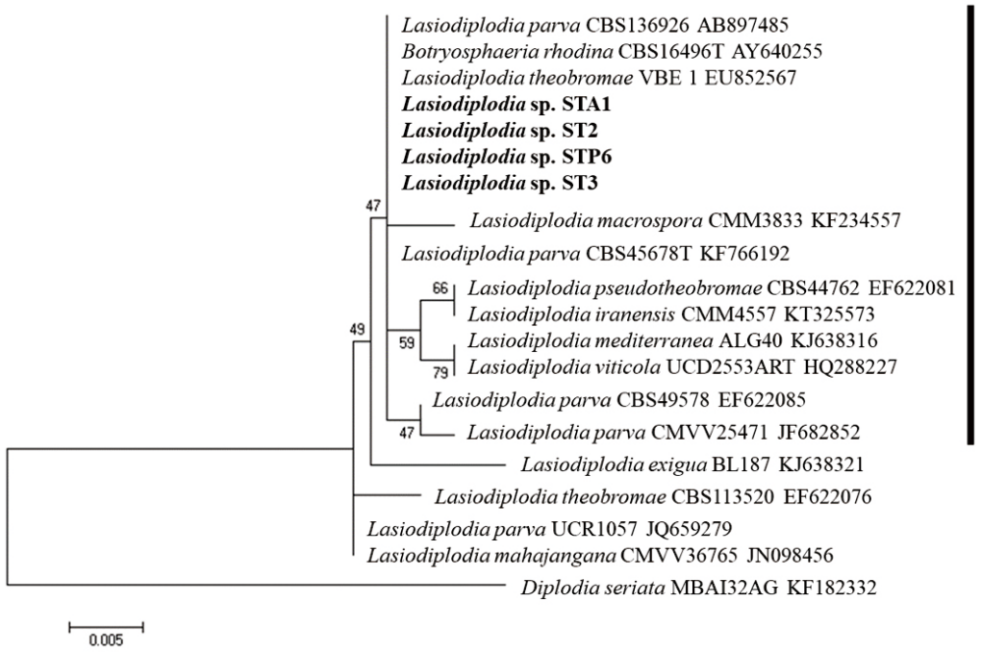

Fig 1 Phylogenetic tree and morphological characteristics of *Lasiodiplodia* spp. *Diplodia seriata* was used as an outgroup. Phylogenetic tree was made based on maximum likelihood analysis with Kimura2 model (1,000X bootstraps).

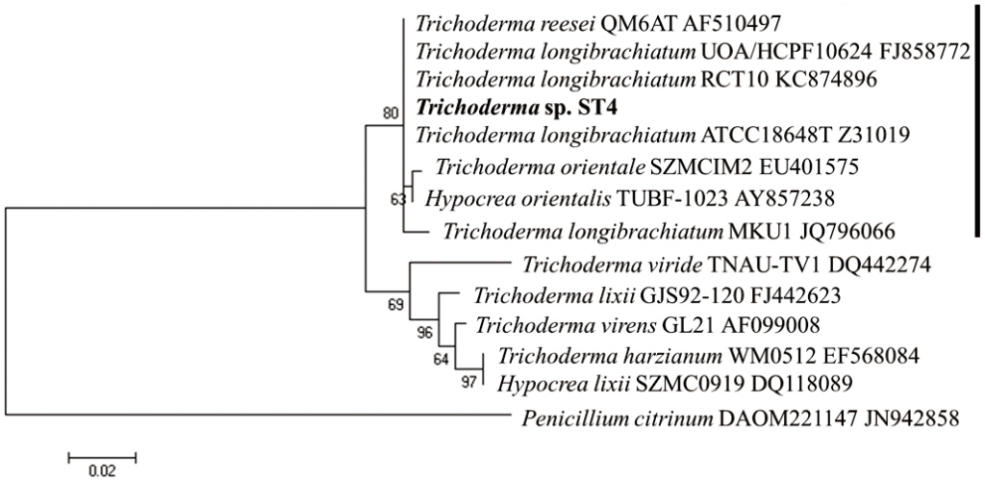

Fig 2 Phylogenetic tree of *Trichoderma* sp. *Penicillium citrinum* was used as an outgroup. Phylogenetic tree was made based on maximum likelihood analysis with Kimura2 model (1,000X bootstraps).

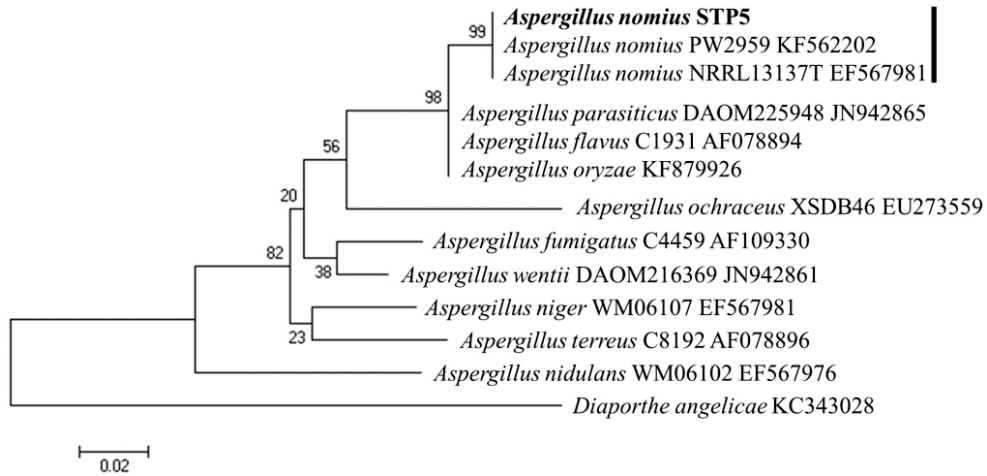

Fig 3 Phylogenetic tree of *Aspergillus nomius*. *Diaporthe angelicae* was used as an outgroup. Phylogenetic tree was made based on maximum likelihood analysis with Kimura2 model (1,000X bootstraps).

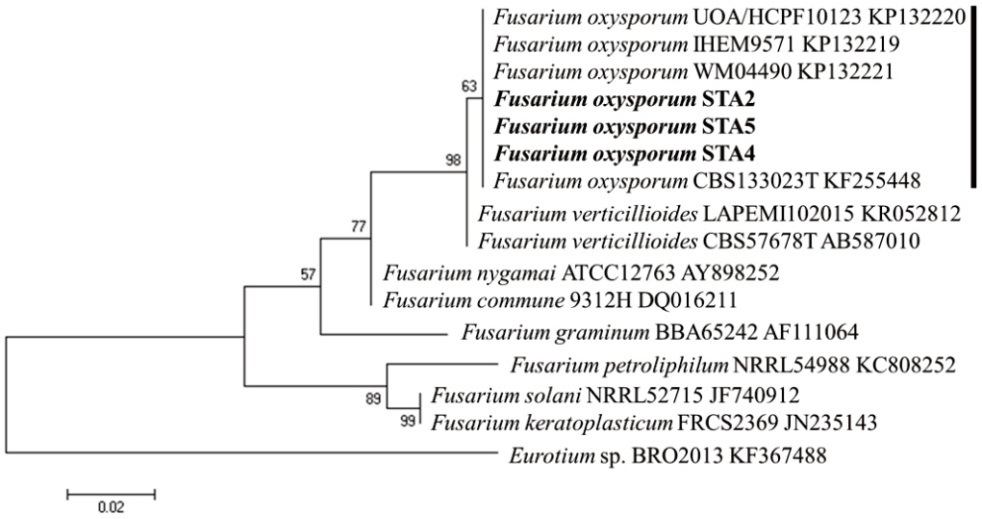

Fig 4 Phylogenetic tree of *Fusarium oxysporum*. *Eurotium* sp. was used as an outgroup. Phylogenetic tree was made based on maximum likelihood analysis with Kimura2 model (1,000X bootstraps).

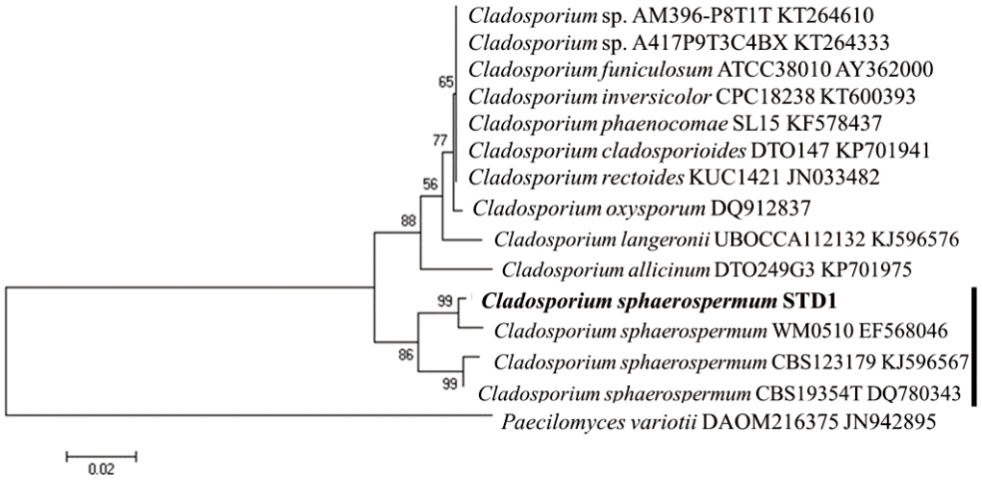

Fig 5 Phylogenetic tree of *Cladosporium sphaerospermum*. *Paecilomyces variotii* was used as an outgroup. Phylogenetic tree was made based on maximum likelihood analysis with Kimura2 model (1,000X bootstraps).

Lasiodiplodia is an anamorphic form from *Botryosphaeria* and the usage of the name *Lasiodiplodia* refers to the determination of one fungus having one name (Hawksworth *et al.* 2011; Robert *et al.* 2013). Importantly, since *L. theobromae* had been isolated in every *gatotan* studies, the role of this mold would be crucial in fermentation of *gatotan* (Purwandari 2000; Astriani *et al.* 2018).

Other mold genera also reported to be associated with *gatotan* despite of their controversial contribution and safety for food fermentation. *Trichoderma* sp. isolated from *gatotan* had been classified as unwanted mold since its potency to produce toxin "(Astriani *et al.* 2018). In addition, *Fusarium* and *Cladosporium* were isolated and identified as the major contaminants in *gaplek*, dried cassava tubers before fermented to *gatotan* (Susanti 2010). However, *T. longibrachiatum*, in particular, was reported to inhibit the growth of *F. oxysporum*, *Penicillium oxalicum*, *Rhizoctonia solani*, and *Sclerotium rolfsii* that caused post-harvest disease in yam (Dania *et al.* 2016). Nonetheless, *Trichoderma* spp. also has an important role in solid substrate fermentation, particularly in cassava to reduce the cyanide content and improve the nutritional value (Hawashi *et al.* 2019).

Latterly, *A. niger*, one of generally recognized as safe microorganism, isolated from *gatot* was observed to produce extracellular starch degrading enzyme, α-amylase (Angelia *et al.* 2019). Both crude and partially purified α-amylase enzyme showed similar degrading activity of starch to glucose and maltose with commercial enzyme. Importantly, black colonies of *A. niger* were also isolated in *gatotan* samples from various region in Indonesia, suggesting their presence

as unique characteristic of *gatotan* fermentation (Astriani *et al.* 2018; Purwandari 2000). Beside *L. theobromae*, *Rhizopus* spp. was reported to have an important role in *gatotan* fermentation. *R. oligosporus*, together with *B. theobromae*, *A. niger*, and *Trichoderma* sp., was isolated as one of indigenous fungi in *gatot* fermentation (Astriani *et al.* 2018). However, in this study, we didn't identify *Rhizopus* spp. using molecular technique, despite of their presence during isolation.

In conclusion, this study identified *Lasiodiplodia* spp., *Trichoderma* sp., *F. oxysporum*, *C. sphaerospermum,* and *A. nomius* as molds associated with *gatotan* using ITS identification. *Lasiodiplodia* spp. and *Aspergillus* spp., were always found in *gatotan*, thus they may provide essential contribution in *gatotan* fermentation.

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