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Molecular Identification of Coal Isolate Fungi Based on Internal Transcribed Spacer (ITS) Region Sequence

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Abstract :

Fungi represent a highly diverse group of microorganisms found in various environments, including extreme habitats such as coal deposits. This study aimed to identify fungal species isolated from Indonesian coal based on ITS region sequence analysis and compare the effectiveness of CTAB/NaCl and microwave methods for fungal DNA isolation from coal substrates. Fungal isolates were obtained from coal samples using the dilution method on PDA media. DNA isolation was performed using CTAB/NaCl and microwave methods with power levels 700 W. ITS region amplification used PCR with ITS5-ITS4 primers. Sequence analysis was conducted using BLASTn and phylogenetic reconstruction using the Neighbor-Joining method with 1000x bootstrap replicates. Two fungal isolates (isolate A and B) were obtained, with only isolate A further characterized. Morphological observations showed characteristics similar to the genus Penicillium. The CTAB/NaCl method successfully yielded DNA with purity of 2.01 and concentration of 275.89 ng/µL, producing a single DNA band on electrophoresis. Conversely, the microwave method failed to isolate intact DNA across high power, indicated by absence of DNA bands despite spectrophotometric purity values (2.02-3.28) falling within acceptable range. ITS region amplification of isolate A successfully produced a 592 bp product. BLASTn analysis revealed isolate A shared 94% identity with several Penicillium species. Phylogenetic analysis demonstrated isolate A was closely related to Penicillium sublateritium JX841245.1 (94.66% identity; 68% bootstrap support). The identity value below 97% suggests isolate A has potential as a novel or cryptic species within genus Penicillium. This study represents the first report on molecular identification of fungi from Indonesian coal and comparison of DNA isolation methods on this substrate. The CTAB/NaCl method proved superior to the microwave method for fungal DNA isolation from coal. Further analysis with additional genetic markers is needed to confirm the taxonomic status of isolate A.

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INTRODUCTION

Coal remains one of the world's most abundant fossil energy resources, but its extraction and utilization cause severe environmental impacts, including land degradation, soil contamination, and greenhouse gas emissions. Large quantities of low rank and waste coal, as well as coal mine spoils, accumulate at mining sites and are often considered recalcitrant and biologically inert due to their complex macromolecular structure and low bioavailability of organic carbon. However, accumulating evidence demonstrates that coal and coal-impacted soils host diverse and metabolically active microbial communities, including bacteria, fungi, actinobacteria, and microalgae, which

collectively participate in coal biotransformation and ecosystem processes [1,12].

Fungi are particularly important in these environments because many taxa possess ligninolytic and oxidative enzyme systems (e.g. laccases, peroxidases, hydrolases) capable of attacking lignin like and humic structures present in low rank coals [4,10]. Coal associated fungi such as Penicillium, Aspergillus, Rhizopus, and other filamentous species have been shown to depolymerize coal matrices, release a suite of aromatic and aliphatic compounds, and enhance the production of value added products including humic substances, biomethane, and chemical feedstocks [4,10,12]. Isolated obtained directly from coal or mine environments frequently exhibit enhanced coal-degrading capacity compared with non-indigenous strains, highlighting local adaptation to this carbon-rich but nutritionally habitat [10].

In parallel, soil fungi and arbuscular mycorrhizal fungi in coal mining regions contribute to nutrient cycling, pollutant detoxification, soil aggregation, and vegetation establishment, and are therefore key indicators and drivers of ecological restoration on post mining land [3,13,14]. Despite the recognition of fungal functional importance in coal and coal affected ecosystems, the taxonomic diversity and composition of cultivable fungi directly associated with coal remain insufficiently documented in many coalfields. Accurate identification is essential to link specific taxa to coal degrading traits and to evaluate their potential for biotechnological applications such as coal beneficiation, bioremediation, and bioenergy production [1,12].

Molecular tools based on ribosomal DNA markers have become standard for fungal systematics; among these, the nuclear small subunit (18S) rRNA gene is widely used for higher level classification, community surveys, and the identification of environmental isolates because it combines conserved and variable regions suitable for broad fungal coverage [2]. Comprehensive primer toolkits now allow targeted amplification of fungal 18S rRNA across diverse lineages and sequencing platforms, facilitating robust taxonomic assignments when combined with curated databases [2]. In addition, the internal transcribed spacer (ITS) has been recognized as the primary barcode marker for fungal identification due to its high variability at the species level [9,19].

MATERIALS AND METHODS

Coal samples were collected from mining sites in East Java, Indonesia. Samples were stored at 4°C and processed within 24 hours. All glassware and media were sterilized prior to use, and manipulations were performed aseptically in a laminar airflow cabinet.

Fungal isolation was performed using the dilution plate method. Ten grams of coal were added to 100 mL sterile distilled water and shaken at 100 rpm for 30 minutes. Serial dilutions up to 10^{-3} were prepared, and 100 µL aliquots were spread onto Potato Dextrose Agar (PDA) supplemented with 1% carboxymethyl cellulose (CMC) and 1% starch. Chloramphenicol (100 mg/L) was added to inhibit bacterial growth. Plates were incubated at room temperature (25±2°C) for 3-7 days. Emerging fungal colonies were purified by repeated subculturing on fresh PDA and maintained as pure cultures at 4°C.

Macroscopic characteristics (colony color, texture, margin, surface topography) were observed after 7 days of growth. Microscopic observations were performed using the adhesive tape method with lactophenol cotton blue staining. Hyphal structure, conidiophore morphology, and spore characteristics were observed under a compound microscope at 400x and 1000x magnification.

Enzyme activity assay were checked by cellulolytic and amylolytic activities. First, isolates were inoculated on PDA with 1% CMC and incubated for 3 days. Plates were flooded with 0.1% Congo red for 15 minutes, then destained with 1M NaCl. Clear zones indicated cellulase activity. Cellulolytic index was calculated as (clear zone diameter - colony diameter)/colony diameter. Second, isolates were inoculated on PDA with 1% starch and incubated for 3 days. Plates were flooded with 1% iodine solution. Clear zones indicated amylase activity. Amylolytic index was calculated using the same formula.

DNA isolation was performed using two methods for comparison. For the CTAB/NaCl

method, approximately 250 mg fresh mycelium was suspended in 567 µL TE buffer and 33 µL 10% SDS, incubated at 37°C for 1 hour, then added with 100 µL 5M NaCl and 80 µL CTAB-NaCl and incubated at 65°C for 10 minutes. RNase A (10 µL) was added and incubated at 37°C for 10 minutes. Proteins were extracted using phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1). DNA was precipitated with cold isopropanol, washed with 70% ethanol, and resuspended in 50 µL TE buffer. For the microwave method, four tubes containing 250 mg mycelium each were suspended in 500 µL TE buffer and 200 µL 10% SDS, then microwaved at varying power levels (280 W, 420 W, 560 W, and 700 W) for two 1-minute intervals. After centrifugation, pellets were extracted with phenol:chloroform:isoamyl alcohol, and DNA was precipitated with cold ethanol and NaCl, then resuspended in 50 µL TE buffer.

DNA concentration and purity (A260/A280 and A260/A230 ratios) were measured using a NanoDrop 2000 spectrophotometer, while DNA integrity was evaluated by electrophoresis on 1% agarose gels stained with ethidium bromide and visualized under UV transilluminator. The ITS region was amplified using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') in 25 µL PCR reactions containing 2 µL DNA template (50 ng/µL), 1 µL each primer (10 pmol/µL), 12.5 µL 2x PCR master mix, and 8.5 µL nuclease-free water. Amplification conditions included initial denaturation at 95°C for 3 minutes; 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 45 seconds; and final extension at 72°C for 5 minutes. PCR products were visualized on 1.5% agarose gels, purified using GeneJET PCR Purification Kit, and sent to 1st BASE Sequencing Services (Malaysia) for bidirectional sequencing using the same primers. Raw sequences were assembled using DNA Baser software, and consensus sequences were compared against NCBI GenBank using BLASTn, where sequence identity ≥97% indicated same species, 95-97% indicated same genus, and <95% indicated potential novel species. Phylogenetic analysis was performed using MEGA11 software with ClustalW for multiple alignment, and trees were constructed using the Neighbor-Joining method with Kimura 2-parameter model and 1000 bootstrap replicates.

RESULTS AND DISCUSSION

Fungal Isolation from Coal Samples

Fungal isolation from coal samples using the dilution plate method yielded two distinct fungal colonies, designated as isolate A BTBR and isolate K BTBR (Figure 1). The isolates exhibited contrasting macroscopic characteristics: isolate A BTBR displayed grayish-green coloration with a velvety texture, while isolate K BTBR showed cream-colored colonies with a cottony appearance. Both isolates grew well on PDA media supplemented with either CMC or starch, indicating their ability to utilize these carbon sources.

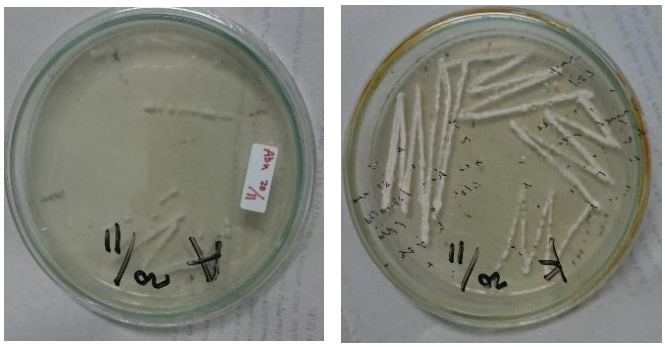

Figure 1. Fungal isolates from coal samples: (a) isolate A BTBR, and (b) isolate K BTBR

The successful isolation of fungi from coal samples aligns with previous studies demonstrating the presence of diverse fungal communities in coal environments [5,14]. Coal represents an oligotrophic environment where fungi have evolved mechanisms to utilize complex organic compounds, making them potentially valuable for biotechnological applications [1,12]. The isolation of only two distinct morphotypes from coal samples may reflect the selective nature of the isolation conditions, including the use of specific media and incubation temperatures. Previous studies have demonstrated that comprehensive fungal diversity assessment requires multiple isolation approaches, including different media compositions, incubation temperatures, and pre-treatment methods [5, 15]. The relatively low fungal diversity recovered in this

study may also reflect the extreme nature of coal environments, which select for specialized fungal taxa adapted to oligotrophic conditions [7, 10].

Enzymatic Activity Screening

Both isolates were screened for cellulolytic and amylolytic activities to assess their potential for industrial enzyme production (Figure 2). Both isolates demonstrated cellulolytic activity, as evidenced by clear zones surrounding colonies on CMC-containing media following Congo red staining. The relatively narrow clear zones suggest that these coal-derived fungi may possess limited cellulolytic capacity compared to specialized cellulolytic fungi, possibly reflecting adaptation to the complex carbon sources present in coal was calculated as 1.14 [4, 12].

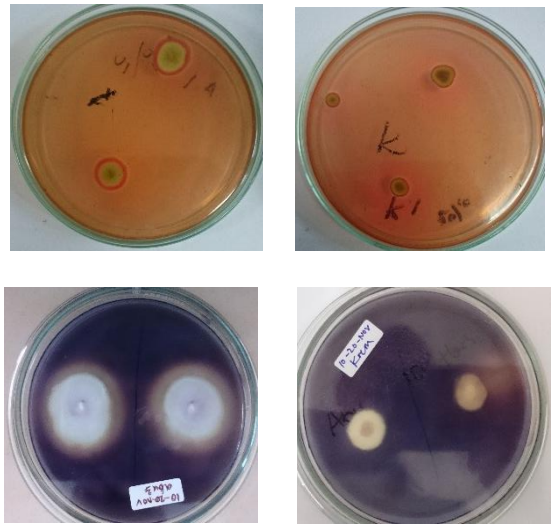

Figure 2. Enzymatic activity assays: cellulase activity in A (isolate A BTBR) and B (isolate K BTBR); amylase activity in C (isolate A BTBR) and D (isolate K BTBR)

Amylolytic activity screening revealed differential capabilities between the two isolates. Isolate A BTBR exhibited pronounced amylolytic activity with an amylolytic index of 1.3, characterized by distinct clear zones surrounding colonies on starch-containing media following iodine staining. In contrast, isolate K BTBR showed minimal amylolytic activity with an index of 1.0. The higher amylolytic activity of isolate A BTBR suggests greater capacity for starch degradation, which may reflect ecological adaptations or genetic differences between the isolates. Amylolytic enzymes from coal-derived fungi may possess unique properties, including stability under extreme conditions, given the challenging environment of coal deposits [17]

Morphological Characterization

Macroscopic and microscopic observations were performed to preliminarily identify the isolated fungi based on morphological characteristics. Isolate A BTBR exhibited colonies with grayish-white coloration, entire margins, undulate surface topography, and circular colony form. Microscopic examination revealed septate hyphae with characteristic branching patterns, including swollen vesicle-like structures at hyphal tips (Figure 3). These morphological features, particularly the presence of brush-like conidiophores with phialides producing chains of conidia, are characteristic of the genus *Penicillium* [16, 19].

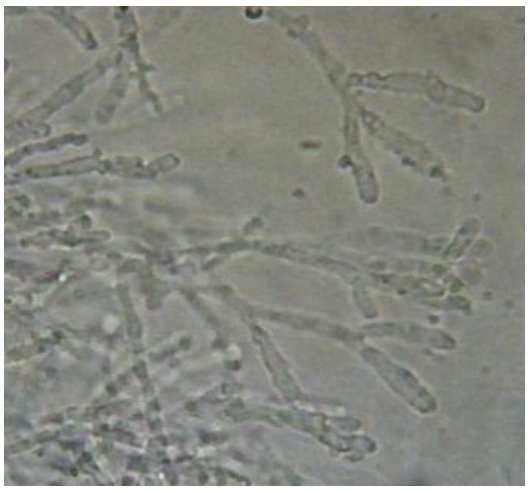

Figure 3. Microscopic morphology of isolate A BTBR showing septate hyphae and characteristic *Penicillium*-like structures (400x magnification)

The morphological similarity between isolate A BTBR and *Penicillium* species is consistent with the frequent isolation of this genus from diverse environments, including soil, plant material, and extreme habitats such as coal deposits. *Penicillium* species are known for their metabolic versatility and ability to colonize various substrates, making them common inhabitants of carbon-rich

environments [16]. However, morphological identification alone is insufficient for definitive species determination, as many *Penicillium* species exhibit overlapping morphological characteristics and phenotypic plasticity in response to cultural conditions [19]. This limitation underscores the need for molecular identification approaches to complement morphological observations [9].

DNA Isolation Using CTAB/NaCl Method

DNA isolation using the CTAB/NaCl method yielded high-quality genomic DNA suitable for downstream molecular applications. Spectrophotometric analysis revealed DNA purity of 1.97 (A260/A280 ratio) with a concentration of 269.53 ng/µL. The A260/A280 ratio falling within the optimal range of 1.8-2.0 indicates minimal protein and polyphenolic contamination. The relatively high DNA concentration obtained from 250 mg of mycelium demonstrates the efficiency of the CTAB/NaCl method for filamentous fungi.

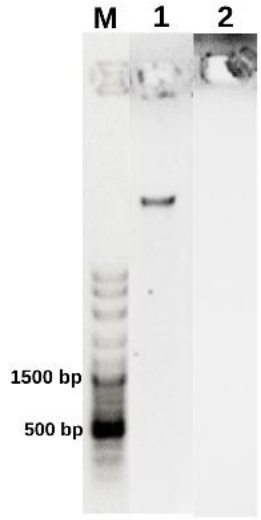

Figure 4. Agarose gel electrophoresis of DNA isolated using CTAB/NaCl method (Lane 1) and microwave method at varying power levels: Lane 2 (700 W).

Agarose gel electrophoresis revealed a single, thick DNA band without smearing for the CTAB/NaCl-isolated sample (Figure 4, Lane 1). The presence of a single band indicates high molecular weight DNA with minimal degradation, while the band intensity reflects the relatively high DNA concentration. The absence of smearing suggests successful removal of contaminants that could interfere with PCR amplification. Several studies have evaluated multiple DNA isolation methods for fungi and found that the CTAB/NaCl method consistently produced DNA with purity values within the acceptable range for wide variety of fungal species [18]. The success of the CTAB/NaCl method can be attributed to the detergent properties of CTAB, which effectively disrupts fungal cell walls and membranes while simultaneously complexing with polysaccharides and removing them from the aqueous phase during chloroform extraction. While, microwave method has not shown any band, which mean the DNA not successful isolated.

ITS Region Amplification

PCR amplification of the ITS region using ITS5-ITS4 primer pairs successfully generated a single product of approximately 600 bp from isolate A BTBR DNA isolated by the CTAB/NaCl method (Figure 5). The amplified product exhibited a concentration of 159.28 ng/µL, sufficient for downstream sequencing applications. The observed fragment size falls within the expected range of 500-800 bp reported for fungal ITS regions in numerous studies [9, 19].

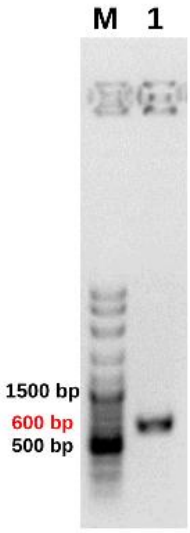

Figure 5. Agarose gel electrophoresis of ITS region PCR products. M is a marker and 1 is amplified DNA

The successful amplification of the ITS region from CTAB/NaCl-isolated DNA confirms the

suitability of this extraction method for providing template DNA free from PCR inhibitors commonly encountered in fungal samples. The presence of a single, clean band without non-specific amplification products indicates optimal PCR conditions and primer specificity. The ITS5-ITS4 primer pair is widely recognized for its broad applicability across diverse fungal taxa, targeting conserved regions flanking the variable ITS1 and ITS2 spacers [9].

Sequence Analysis and Molecular Identification

Figure 4. ITS region sequence of isolate A BTBR (592 bp)

The ITS region sequence of isolate A BTBR was determined to be 592 bp in length (Figure 6.), consistent with the size estimated from gel electrophoresis and within the typical range for fungal ITS region. BLASTn analysis against the NCBI GenBank database revealed that isolate A BTBR shared the highest sequence identity (94%) with several species of the genus Penicillium. This identity value falls below the commonly accepted threshold of 97% for species-level identification [9]], suggesting that isolate A BTBR may represent a distinct species or potentially a novel taxon within the genus *Penicillium* [9, 19].

The highest identity value (95.61%) was observed with *Penicillium simplicissimum* HQ607882.1, followed closely by *Penicillium sublateritium* JX841245.1 (94.66%). However, sequence alignment revealed that the similarity with *P. simplicissimum* involved longer gaps compared to the alignment with *P. sublateritium*, potentially affecting the phylogenetic placement. Gap placement and length are important considerations in sequence alignment and phylogenetic reconstruction, as they may reflect genuine evolutionary events or alignment artifacts [9].

Table 1. Sequence identity values between isolate A BTBR and closely related *Penicillium* species based on BLASTn analysis.

Species	GenBank Accession	Identity (%)	Query Coverage (%)
Penicillium sublateritium	JX841245.1	94.66	98
Penicillium simplicissimum	HQ607882.1	95.61	95
Penicillium ochrochloron	JX841228.1	94.12	97
Penicillium janthinellum	KP016783.1	93.88	98

Phylogenetic Analysis

Phylogenetic reconstruction using the Neighbor-Joining method placed isolate A BTBR in a clade with *Penicillium sublateritium* JX841245.1 with moderate bootstrap support (68%) (Figure 7). Despite the slightly higher raw identity value with *P. simplicissimum*, the phylogenetic analysis suggested closer evolutionary relationship with *P. sublateritium* based on alignment characteristics and tree topology. This apparent discrepancy highlights the importance of comprehensive phylogenetic analysis beyond simple pairwise identity comparisons for accurate taxonomic placement.

Bootstrap values (≥50%) from 1000 replicates are shown at nodes. The bootstrap support value of 68% for the *P. sublateritium*-isolate A clade indicates moderate statistical support, suggesting that while a relationship is indicated, additional data would strengthen the inference. The relatively low bootstrap values in this region of the tree may reflect the limited phylogenetic signal in ITS sequences for resolving relationships among closely related *Penicillium* species, or may indicate that

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isolate A occupies an intermediate phylogenetic position between described taxa.

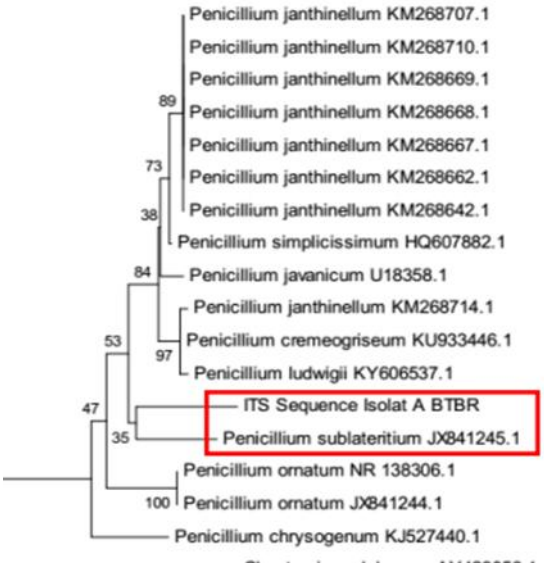

Figure 7. Neighbor-Joining phylogenetic tree showing the relationship of isolate A BTBR with closely related *Penicillium* species.

The placement of isolate A BTBR within the *Penicillium* clade is consistent with morphological observations and BLAST results, confirming the genus-level identification. However, the sequence divergence from described species (>3%) and the phylogenetic position suggest that isolate A may represent an undescribed species or a cryptic species within the *Penicillium* subgenus. Cryptic species morphologically similar but genetically distinct taxa are increasingly recognized in fungi through molecular phylogenetic studies [9]. The coal environment may harbor unique *Penicillium* lineages adapted to this extreme habitat that have not been previously characterized [7, 10].

The identity value below 97% and the phylogenetic placement provide strong evidence that isolate A BTBR is not conspecific with any previously described *Penicillium* species represented in GenBank. However, definitive taxonomic conclusions require consideration of several factors. First, the ITS region alone, while sufficient for genus-level identification and preliminary species discrimination, may not provide complete resolution for closely related *Penicillium* species [9, 19]. Second, the public databases may not contain sequences of all described *Penicillium* species, and the closest match in GenBank may not represent the true phylogenetic neighbor. Third, intraspecific variation in ITS sequences can occasionally exceed 3% in some fungal genera, though this is uncommon in *Penicillium* [9].

CONCLUSIONS

This study successfully isolated two fungal isolates from Indonesian coal samples, with isolate A BTBR characterized in detail. Morphological observations suggested affinity with the genus *Penicillium*, which was confirmed by molecular analysis. Comparative evaluation of DNA isolation methods demonstrated that the CTAB/NaCl method effectively yielded high-quality DNA (purity 2.01, concentration 275.89 ng/µL) suitable for PCR amplification, while the microwave method failed to isolate intact DNA across all power variations tested despite acceptable spectrophotometric purity readings. ITS region amplification produced a 592 bp product, and sequence analysis revealed 94-95% identity with various *Penicillium* species. Phylogenetic analysis placed isolate A BTBR closest to *Penicillium sublateritium* JX841245.1 with 94.66% sequence identity and 68% bootstrap support. The identity value below 97% suggests that isolate A BTBR may represent a novel or cryptic species within the genus *Penicillium*, warranting further taxonomic investigation with additional genetic markers. This study provides the first report of molecular characterization of fungi from Indonesian coal and contributes to our understanding of fungal diversity in extreme environments.

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Author name1: Shinta Wulansari conceptualized and designed the study, performed phylogenetic analysis, supervised the research, and wrote the manuscript; Author name2: Muhammad Shobihul Khoir conducted fungal isolation, DNA extraction (CTAB/NaCl and microwave methods), PCR amplification, sequencing analysis, morphological characterization, and enzymatic activity assays. Both authors reviewed and approved the final manuscript.

AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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