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# Geographic and genetic diversity of edible *Tylopilus griseipurpureus* in Vietnam's *Acacia* hybrid plantations

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**Abstract** An edible *Tylopilus* species has long been recorded in the *Acacia* plantation forests in Vietnam and is widely traded in local markets. In this study, mushroom specimens associated with *Acacia* plantations were collected from different geographical regions across Vietnam for analysis. Morphological examinations and phylogenetic analyses based on internal transcribed spacer (ITS) and nuclear large ribosomal subunit (nrLSU) sequence data confirmed that these specimens belong to *Tylopilus griseipurpureus*. The genetic diversity among 19 *T. griseipurpureus* specimens from across Vietnam was further investigated using inter-simple sequence repeat (ISSR) markers.

**Keywords:** ITS, Inter simple sequence repeat, *Tylopilus griseipurpureus*

## Introduction

The planted forest area in Vietnam covers approximately 4.65 million hectares, of which *Acacia* plantations account for 2.2 million hectares (Hai, 2022; Ministry of Agriculture and Rural Development, 2023). These forests are mainly distributed in the midland and northern mountainous regions, the central coastal region, and the Central Highlands. A key species widely planted throughout Vietnam is the natural hybrid between *Acacia mangium* × *Acacia auriculiformis*,

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commonly referred to as *Acacia* hybrid (Harwood and Nambiar, 2014; Kha, 2001). Over the years, this hybrid has become an important reforestation species, with its cultivation area continuing to expand.

Information on the diversity of wild edible mushrooms associated with *Acacia* plantations is limited. In India, *Lycoperdon utriforme* and *Scleroderma citrinum* has been reported in association with *Acacia* species (Sridhar, 2018). In Thailand, *Boletus griseipurpureus* has been commonly observed during the rainy season in plantations of *A. auriculiformis* and *A. mangium* (Aungaudchariya *et al*., 2010; Seehanan and Petcharat, 2008). In Vietnam, during the early rainy season, a bitter-tasting edible mushroom resembling *Tylopilus* commonly appears throughout *Acacia* plantations (Figure 1). In regions where *Acacia* plantations are extensive, this mushroom occurs in relatively high abundance and has become a prominent local commercial product, particularly in Quảng Trị province, Hue City, Kien Giang Province, and Dong Nai Province. The taxon was previously identified as *Tylopilus felleus* (Kiet, 2013; Pham, 1972); however, its taxonomic position remains unresolved, and no voucher specimens have been preserved to confirm the earlier identifications.

The genus *Tylopilus* (Boletaceae, Boletales) is closely related to genera such as *Boletus* and *Xerocomus*. It was first established as cited by Singer (1986), with *Tylopilus felleus* (≡ *Boletus felleus* Bull.) designated as the type species (in Singer, 1986). This species is distributed across East Asia, northern Europe, and eastern North America. Many species of *Tylopilus* are well known for their distinctly bitter taste. Species of *Tylopilus* are typically ectomycorrhizal with both broadleaf and coniferous hosts from families such as Dipterocarpaceae, Myrtaceae, Fagaceae, and Pinaceae (Li and Yang, 2023). Morphologically, the genus is characterized by pileate-stipitate basidiomes; pilei that range in color from pink, brown, gray to purplish; a hymenophore composed of tubes with pinkish to grayish tones; stipes that may or may not exhibit reticulation; and context that usually remains unchanged when cut or exposed. The spore print is pale pink to pinkish brown and the basidiospores are ellipsoid to fusiform and non-amyloid. Basidia are typically four-spored, and cystidia may be present (Singer, 1986; Wu *et al*., 2014, 2016).

According to Index Fungorum (http://www.indexfungorum.org), *Tylopilus* currently comprises 246 accepted species (accessed on 2 September, 2025). Recent phylogenetic studies based on multilocus analyses indicate that *Tylopilus sensu lato* can be separated into several well-supported clades. Advances in molecular systematics have greatly improved the classification of *Tylopilus*, leading to the reassignment of several former *Tylopilus* to newly erected genera such as *Abtylopilus* Yan C. Li & Zhu L. Yang, *Anthracoporus* Yan C. Li & Zhu L. Yang, *Austroboletus* (Corner) Wolfe, *Chiua* Yan C. Li & Zhu L. Yang, *Fistulinella* Henn., *Harrya* Halling, Nuhn & Osmundson, *Hymenoboletus* Yan C.

Li & Zhu L. Yang, *Indoporus* A. Parihar, K. Das, Hembrom & Vizzini, *Leccinellum* Bresinsky & Manfr. Binder, *Mucilopilus* Wolfe, *Porphyrellus* E.-J. Gilbert, *Pseudoaustroboletus* Yan C. Li & Zhu L. Yang, *Retiboletus* Manfr. Binder & Bresinsky, *Royoungia* Castellano, Trappe & Malajczuk, *Sutorius* Halling, Nuhn & N.A. Fechner, *Tylocinum* Yan C. Li & Zhu L. Yang, *Tylopilus* P. Karst., *Veloporphyrellus* L.D. Gómez & Singer, and *Zangia* Yan C. Li & Zhu L. Yang (Halling *et al.,* 2012; Li *et al*., 2011; Li and Yang, 2023). Accurate identification of *Tylopilus* species now requires an integrative approach combining morphological characteristics with multilocus phylogenetic analyses. Molecular markers such as internal transcribed spacer (ITS), nuclear large subunit ribosomal RNA (nrLSU), translation elongation factor 1-alpha (TEF1-α), and RNA polymerase II subunit 2 (RPB2) are widely used to study genetic diversity and phylogenetic relationship within the genus (Li and Yang, 2023).

Genetic diversity refers to the variation in genetic composition among individuals or populations, which provides the basis for adaptation, taxonomy, and conservation. Various molecular techniques have been developed to assess the genetic diversity of species, for example amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), inter simple sequence repeat (ISSR) and simple sequence repeat (SSR). Among these, ISSR technique is widely used because it is simple, cost-effectives, and easy to implement, enabling efficient amplification of genomics regions flanked by simple sequence repeats. ISSR markers have been used to study the genetic diversity in several fungal species, including *Lepista nuda* (Du *et al*., 2018), *Pleurotus eryngii* var. *tuoliensis* (Zhao *et al*., 2013), and *Tricholoma scalpturatum* (Carriconde *et al*., 2008). Compare with other DNA markers, ISSRs are often preferred markers due to high reproducibility across different laboratories and do not require prior sequence information. ISSR primers amplify a DNA segment between identical microsatellite repeats, making them as an useful tools for studies involving genetic diversity, gene tagging, phylogeny, evolutionary biology, and genome mapping.

During field surveys conducted in the rainy seasons of 2023–2025 in *Acacia* hybrid plantations, specimens resembling *Tylopilus* mushrooms commonly collected by local communities as food were gathered. The aim of this study was to reveal previously unknown facts about this wild edible mushroom, clarify its taxonomic position, and evaluate the genetic diversity of specimens collected from different geographical regions of Vietnam using ISSR markers.

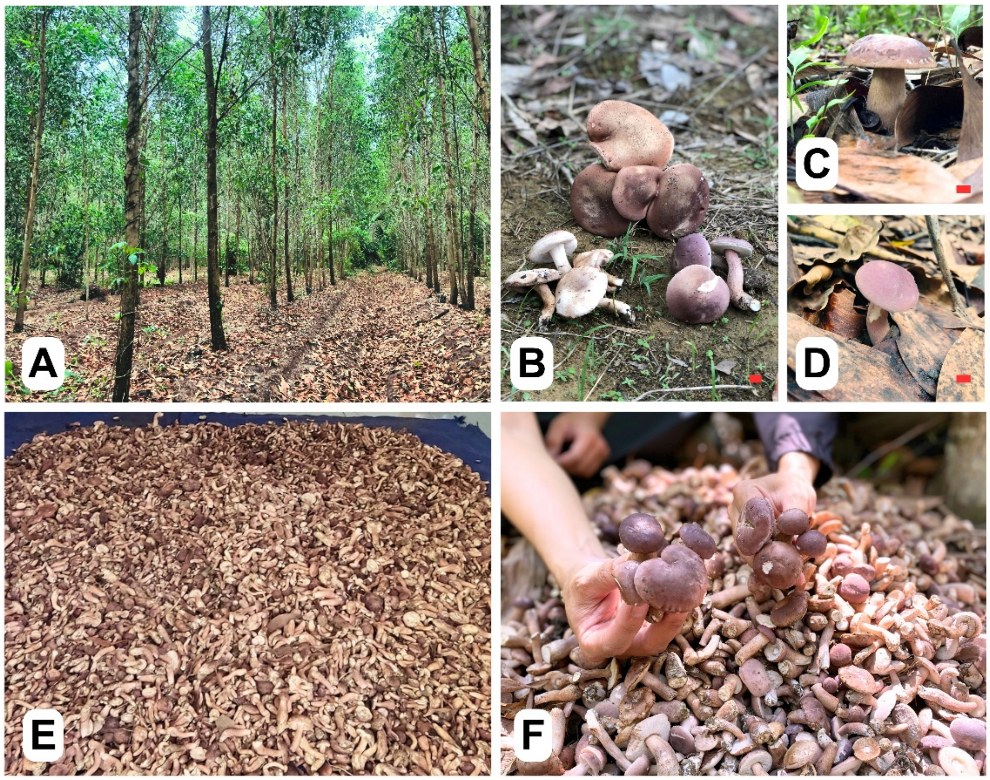


**Figure 1.** Habitat and *Tylopilus* fruiting bodies collected by local people. A: *Acacia* plantation. B-D: Fresh basidiomata exhibiting variable colouration. E-F: *Tylopilus* fruiting bodies collected and sold in the local market. *Bars*: B–D 1 cm.

## Materials and methods

### *Collection site and sampling*

Specimens were collected from four sites across three geographically distinct regions of Vietnam during the 2023–2024 rainy season: Mekong Delta - MD (Phu Quoc National Park, An Giang Province); North Central-NC (Nam Hai Lang Commune, Quang Tri Province); and Southeastern - SE (Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City; Dong Nai Nature and Culture Reserve, Dong Nai Province. The fresh basidiomata were recorded and photographed in the field, and small tissue samples were transferred to cryotubes containing 0.5 mL CTAB buffer (100 mM Tris HCl, pH 8; 1.4 M NaCl; 20 mM $Na_2EDTA$; 2% CTAB). The remaining specimens were dried at 50 °C for 48 h. Voucher specimens (Table 1) were deposited in the herbarium of the Institute of

Life Sciences (VNM), Vietnam Academy of Science and Technology, Vietnam. Herbarium codes follow (Thiers, 2014).

### *Morphological characterization*

The fresh basidiocarps were used to describe the macroscopic characteristics and color designations followiing Kornerup and Wanscher (1978). The dried basidiocarps were used for micromorphological observation under a BH2 microscope (Olympus, Japan). Sections from dried specimens were mounted in a mixture of 3% KOH and 1% Congo red. Lactophenol cottonblue and Melzer's reagent were used to test the spore wall reactions. The notation [*n*/*m*/*p*] indicates that measurements were made on "*n*" randomly selected basidiospores from "*m*" basidiomes of "*p*" collections. Basidiospore measurements are represented as (a–)b–c(–d), where b–c represents 10 to 90 percentiles, and a or d represents extreme values. The terms denoting the shape of basidiospores proposed by (Largent *et al.,* 1994) will be used. All measurements were obtained using Piximetre version 5.10 (France). The following abbreviations were used: IKI = Melzer's reagent, IKI– = neither amyloid nor dextrinoid, CB = cotton blue, CB+ = cyanophilous, CB– = acyanophilous, L = mean spore length, W = mean spore width, Q = length/width ratio with the extreme values in parentheses, $Q_m$ = mean quotient (length/width ratio) ± standard deviation, *n* = number of spores in the tested specimens.

### *DNA extraction*

Genomic DNA was extracted from approximately 50 mg of fresh tissue using a slightly modified CTAB method (Doyle and Doyle, 1987). The tissue was ground with 3-mm zirconia beads in a BeadBug microtube homogenizer (Benchmark Scientific, USA). Extracted DNA was dissolved in 1× TE buffer and stored at −30 °C until further use. The purity and quality of the genomic DNA were assessed using spectrophotometry.

### *DNA barcode amplification and phylogenetic analysis*

The nuclear ribosomal ITS1-5.8S-ITS2 (ITS) and the nrLSU region were amplified with primer pairs ITS1/ITS4 (White *et al.,* 1990) and LR0R and LR5 (Vilgalys and Hester, 1990), respectively. The PCR reaction was prepared with a final volume of 50 µL, consisting of 2 µL of DNA template, 1.5 µL of each primers, 25 µL of 2× MyTaq HS Mix (Meridian Bioscience, USA), and 20 µL of molecular biology grade water (Corning, USA). PCR amplification thermal cycler protocols for ITS and nrLSU regions followed the protocol described by

Wu *et al.* (2014). The PCR products were sent for sequencing to 1st BASE Laboratories (Selangor, Malaysia). Newly generated sequences have been deposited in GenBank and detailed information on voucher specimens, including GenBank accession numbers, is provided in Table 1.

Raw sequences were edited using Chromas 2.6.6 (Technelysium, Australia) and compared with sequences deposited in GenBank using the BLASTN algorithm. The ITS and nrLSU datasets were assembled following the procedures of previous studies (Li and Yang, 2023; Wu *et al.,* 2016). Sequences alignment and manual adjustment were performed using AliView 1.30 (Larsson, 2014). Ambiguously aligned positions and highly divergent regions were removed by Gblocks with the least stringent settings (allow gap positions within the final blocks) (Talavera and Castresana, 2007). Phylogenetic analyses were conducted based on the combined ITS+nrLSU dataset. Maximum likelihood (ML) was inferred in MEGA 12, and Maximum Likelihood bootstrap percentage (MLB) was obtained using nonparametric bootstrapping with 1000 replicates with the T93+G+I model (Kumar *et al.,* 2024). *Porphyrellus porphyrosporus* voucher MB97-023 and *P. porphyrosporus* voucher DJM1332 were used as outgroups. Details of all sequences included in the phylogenetic analyses are presented in Table 1. Sequence alignment and phylogenetic tree were deposited at Figshare (DOI: 10.6084/m9.figshare.30405652). Phylogenetic trees were visualized and edited using Canva (*https://www.canva.com/*).

***ISSR amplification, electrophoresis and phylogeny***

The design of ISSR primers was based on sequences published by Columbia University. A total of 10 primers that produced clearly distinguishable and reproducible fragments were selected and used in this study (Table 2). PCR reactions were performed in a final volume of 25 µL, containing 1 µL of DNA template, 0.5 µL of each primer, 12.5 µL of 2× MyTaq HS Mix (Meridian Bioscience, USA), and 11 µL of molecular biology–grade water (Corning, USA). ISSR amplification was carried out in 35 cycles under the following thermal profile: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at 45.4~50.2 °C for 90 s, and extension at 72 °C for 35 s, with a final extension step at 72 °C for 5 min.

For electrophoresis, 4 µL of 6× GelRed loading buffer (ABT, Vietnam) was mixed with 10 µL of each ISSR amplification product. Samples were resolved on a 1.2 % agarose gel prepared in 0.5× TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM $Na_2EDTA$; pH 8) at a constant voltage of 100 V for 90 min using a Mupid EXu electrophoresis system (Mupid, Japan). DNA fragments were visualised under ultraviolet illumination with a UV transilluminator

(Maestrogen, Taiwan), and fragment sizes were estimated by comparison with the GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, USA).

Electrophoretograms were used to define the size and the appearance of the bands. The amplification products generated by each ISSR primer were manually scored as binary data, indicating the presence (1) or absence (0) of each band. The binary matrix combined all data obtained from all primers was used to calculate Jaccard's pairwise similarity coefficients. The unweighted pair-group method with arithmetic average (UPGMA) dendrogram was constructed to depict the genetic relationship using the Numerical Taxonomy and Multivariate Analysis System Program (NTSYSpc 2.1) (Rohlf, 2009). To determine the efficiency of ISSR marker, Polymorphism Information Content (PIC) values were calculated using the common equation:

$$PIC = 2f\,(1 - f)$$

where $f$ is the frequency of present bands, and 1-f is the frequency of absent bands in the electrophoresis gel.

## Results

### *Phylogenetic analysis*

In total, 19 ITS sequences and 19 nrLSU sequences derived from basidiocarps were obtained. The combined ITS+nrLSU dataset comprised sequences from 51 specimens representing 10 taxa, including 48 ITS and 34 LSU sequences. The final aligned dataset contained 1,241 characters, of which 863 were constant and 360 were parsimony-informative. The best-fit substitution model selected by ModelTest in MEGA version 12 was T93+G+I.

In the phylogenetic tree, the sequences of *Tylopilus* clustered together in a monophyletic group, clearly separated from the outgroup *Porphyrellus* (Figure 2). All 19 Vietnamese basidiomata of *T. griseipurpureus* clustered with specimens of *T. griseipurpureus* from China, Malaysia, and Thailand (bootstrap support = 100%). This clade was a sister clade to the New Zealand specimens of *T. formosus*, also with strong support (bootstrap support = 99%). The sequences labelled as *T. felleus* were distributed among three distinct clades, indicating taxonomic complexity within that species name.

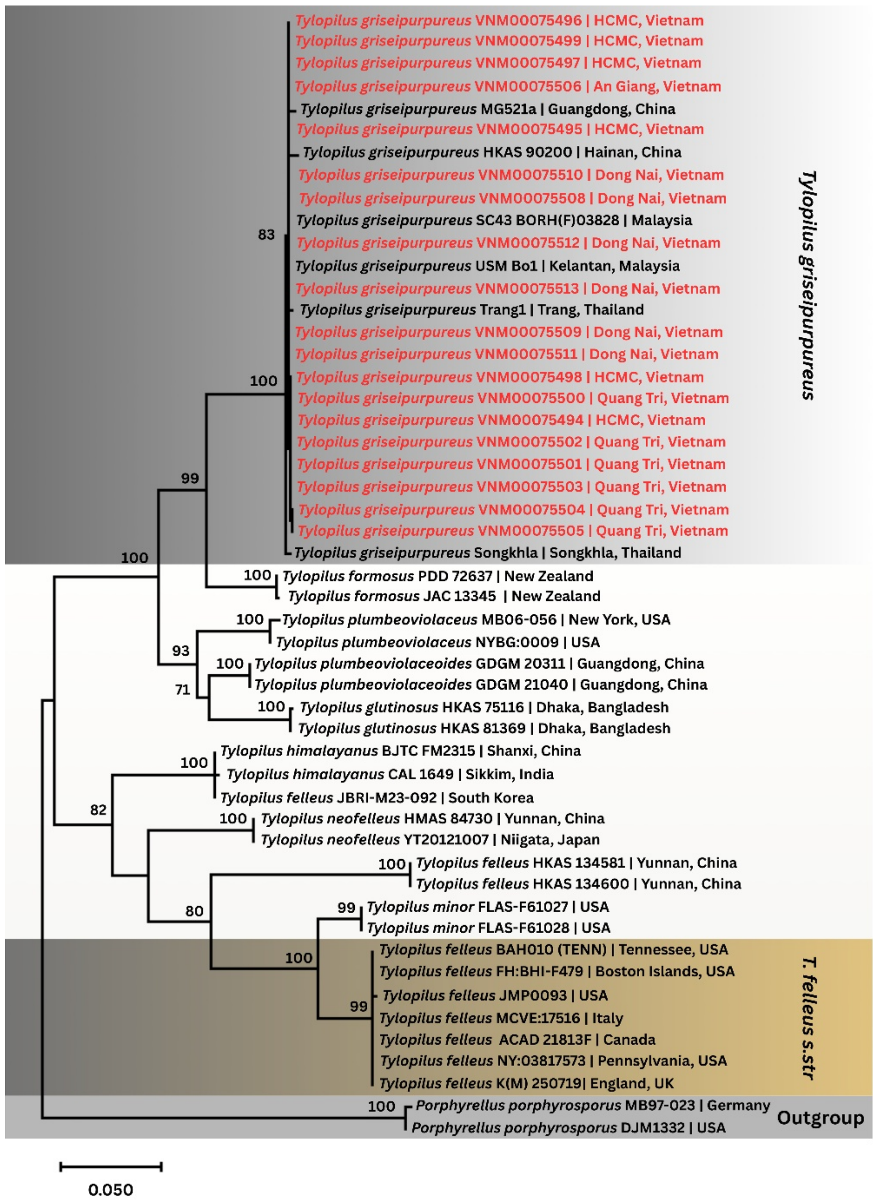


**Figure 2.** Phylogenetic tree generated from Maximum Likelihood (ML) analysis using the T93+G+I substitution model based on the combined ITS+nrLSU dataset. *Porphyrellus porphyrosporus* was used as an outgroup taxon. Bootstrap values (≥70%) are shown at supported branches. Specimens used in this study are highlighted in bold red

***Taxonomy***

***Tylopilus griseipurpureus*** (Corner) E. Horak, Malayan Forest Records 51: 132 (2011)
Mycobank: MB 624089; Indexfungorum: IF 624089
≡ *Boletus griseipurpureus* Corner, Boletus in Malaysia (Singapore): 168 (1972)
Vietnamese name: nấm Tràm

Basidiomata small to medium-sized, annual. Pileus convex when young, becoming plano-convex to nearly flat at maturity; 1.2–9.0 cm diameter; surface dry, somewhat velvety, becoming slightly sticky, glabrous when moist; light-lilac (15A5) to greyish-rose (12B3) when young, reddish-grey (10B2) to grey (9B1) with age; margin entire. Pores surface white (1A1) to greyish-white (1B1), then pinkish-white (7A2) with age; turning brown (7E8) when bruised; pores angular, 2–3 pores/mm. Hymenophore tubulate, adnate to subdecurrent. *Tube* up to 6.2 mm deep, greyish-white, brownish when bruised. Stipe 3.0–7.5 × 1.2–1.8 mm, cylindrical to subclavate, straight or curved; solid; concolorous with the pileus; surface pruinose; basal covered with white mycelial tomentum. Context firm when young, then soft with age, up to 15 mm thick; white to pinkish-white, unchanging when cut. Odor and Taste: odor mild, taste very bitter. Macrochemical reaction: yellowish-white (4A2) in guaiacol, olive-brown (4D4) in 10% $NH_4OH$, brownish-grey (4F2) in $FeSO_4$, and greyish-yellow (4C4) in 5% KOH.

Basidiospores [200/4/4] (7.2–)8.1–11.2(–11.6) × (2.9–)3.0–4.0(–4.5) µm, L=9.6 µm, W=3.6 µm, Q= (2–)2.2–3.2(–3.5), $Q_m$=2.7 ± 0.28; subfusiform and inequilateral to ellipsoid and usually with one end tapering at maturity in side view; smooth, hyaline in water, yellowish in 3% KOH, IKI–, CB–; 1-3 oil droplets (Figure 3L–M). Basidia 18.8–25.1 × 6.9–11.6 µm (n=15); clavate to elongated clavate, predominantly 4-sterigmate, sometimes 2-sterigmate; hyaline, thin-walled; containing straw-yellow oil guttules in 3% KOH (Figure 3I). Basidioles subcylindrical to clavate, similar in size to basidia. Cheilocystidia 17.7–19.2 × 3.8–5.3 µm (n=15), subcylindrical to subfusiform; smooth, hyaline, thin-walled. Pleurocystidia 26.4–44.8 × 7.7–13.5 µm (n=15), subfusiform, fusiform to fusoid-ventricose, smooth, hyaline, thin-walled, abundant (Figure 3H). Pileipellis a trichodermium up to 80 µm thick, composed of yellowish-brown interwoven filamentous hyphae; terminal elements 14.9–25.6 × 2.4–4.3 µm, cylindrical to subfusoid (Figure 3G). Stipipellis up to 100 µm thick, composed of yellowish-brown hyphae, thin-walled, parallel to subparallel, and running longitudinally along the stipe. Hymenophoral trama bilateral divergent (boletoid); hyphae septate, unbranched, 1.4–3.6 µm wide; hyaline to pale yellowish in 3% KOH (Figure 3E). Clamp connection absent in all tissues.

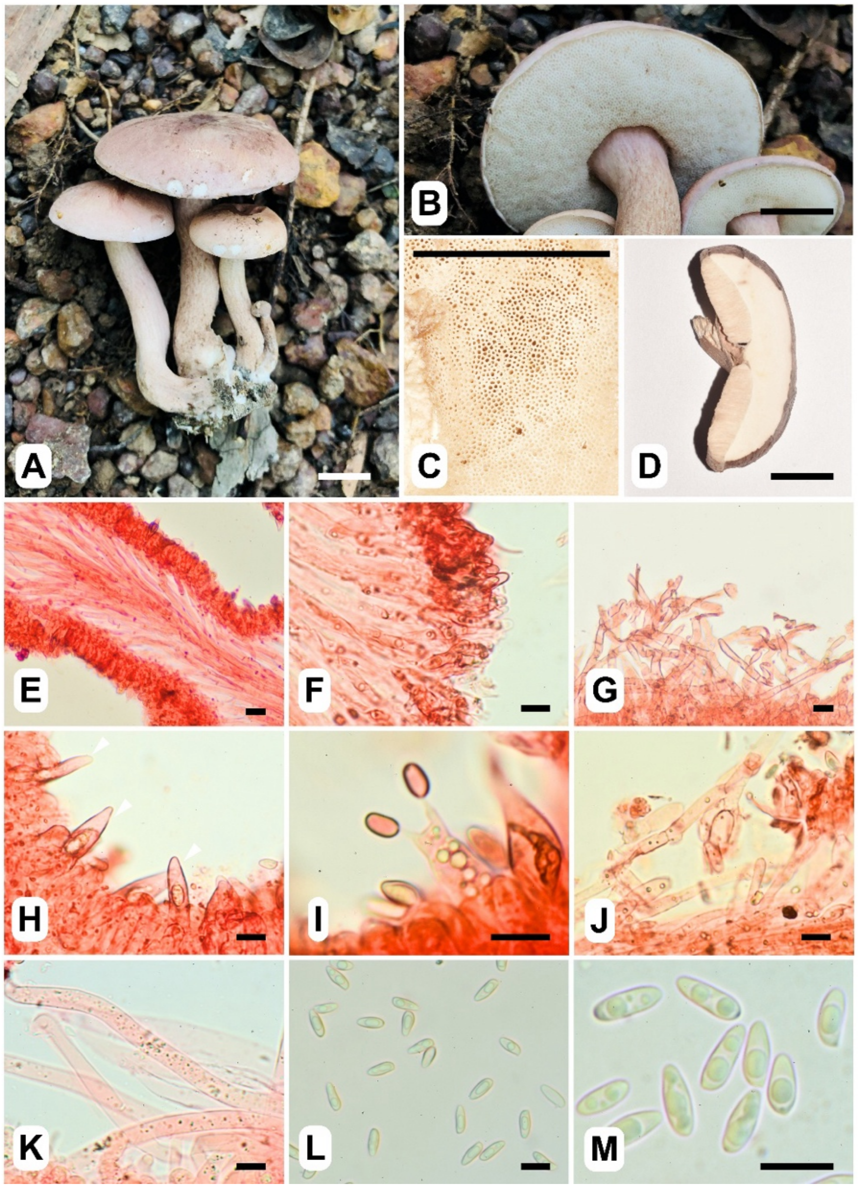


**Figure 3.** Morphological characteristics of *T. griseipurpureus*: A: Fresh basidiomata. B: Upper layer of basidiomata. C: Pore surface. D: Cross section of basidiome. E: Cross section of hymenophoral trama. F: Tube edge. G: Pileipellis. H: Pleurocystidia (arrow). I: Basidium. J: Stipipellis. K: Hyphae of hymenophoral trama. L-M: Basidiospores. *Bars*: A–D 1 cm; E–M 10 µm

**Specimens examined:** VIETNAM. Dong Nai Province: Dong Nai Nature and Culture Reserve, Ma Da commune, alt. 73 m, 03 May 2024, Nhan LT & Hung LT (VNM00075508, VNM00075512). Ho Chi Minh City: Binh Chau – Phuoc Buu Nature Reserve, Xuyen Moc commune, alt. 60 m, 25 May 2024, Nhan LT & Hung LT (VNM00075495, VNM00075497). Quang Tri Province: Nam Hai Lang commune, alt. 23 m, 18 Jun 2024, Nhan LT & Hung LT (VNM00075500, VNM00075502). An Giang Province: Phu Quoc Island, alt. 5 m, 14 Jun 2023, Nhan LT & Hung LT (VNM00075506, VNM00075507).

**Habitat and distribution**: scattered or clustered on the ground in *Acacia* plantation. *Tylopilus griseipurpureus* has been widely reported in tropical areas Australia, China, Malaysia, Thailand (Aungaudchariya *et al.,* 2010; Horak, 2011; Lee and Mohammad, 2020; Leonard, 2015; Wu *et al.,* 2016). This mushroom has been reported as being ectomycorrhizal with *Acacia auriculiformis*, *A. magium* (Aungaudchariya *et al.,* 2010; Seehanan and Petcharat, 2008); *Eucalyptus maculatab* (Leonard, 2015); fagaceous plants (Wu *et al.,* 2016); *Meulaleuca cajubuti*, *M. leucadendron* (Aungaudchariya *et al.,* 2010; Lee and Mohammad, 2020).

**Edibility**: an edible mushroom with a distinctly bitter taste.

**Notes:** This species was originally described by (Corner, 1972) from Singapore under the name *Boletus griseipurpureus*. It is reported here as a new record for the mycobiota of Vietnam.

### *ISSR analysis*

Table 1 summarised the number of bands generated by each ISSR primer. The banding pattern varied among primers and specimens, producing between 6 and 16 bands per primer. A total of ten primers produced 546 scorable bands ranging from 600 to 4000 bp in size for all 19 *Tylopilus* specimens.

In total, 101 distinct DNA fragments were generated, resulting in 100% polymorphism and an average of 10.1 amplicons per primer (Table 3). Primer P817 produced the highest number of polymorphic bands (16) followed by primers P828, P825 and P889, each generating 12 bands. The lowest number of bands (6) was observed in the primer P55. Among the 19 *Tylopilus* specimens, the maximum number of bands (39) was obtained in specimens VNM00075512, VNM00075513, VNM00075498 and VNM00075499, whereas the minimum number of bands (14) was recorded for specimen VNM00075503. About one in ten specimens showed no visible amplification band, with different proportions depending on the primer used (data not shown). Though primer P816 produced only seven DNA fragments, it exhibited the highest PIC value (0.41), followed by primers P889 (0.40), P817 (0.39), and P55 (0.38) with 12, 16 and 6 bands, respectively. The lowest PIC value (0.23) was recorded in primer P812 (Table 2).

**Table 1.** Reference sequence from NCBI

| Taxa | Voucher | Locality | Habitat | Genebank accession no. | | References |
|---|---|---|---|---|---|---|
| | | | | ITS | nrLSU | |
| *Porphyrellus porphyrosporus* | MB97-023 | Germany: Walhalla, Bavaria | *Picea abies* | DQ534563 | DQ534643 | Binder & Hibbett, 2006 |
| *P. porphyrosporus* | DJM1332 | USA: Oregon | - | JN021085 | HQ161850 | Dentinger *et al.*, 2011 |
| *Tylopilus felleus* | K(M):250714 | United Kingdom: England, South Hampshire | - | MZ159717 | - | GeneBank |
| *T. felleus* | MCVE:17516 | Italy | - | JF908787 | - | Osmundson *et al.*, 2013 |
| *T. felleus* | NY:03817573 | USA: Pennsylvania, | - | MW899062 | - | GeneBank |
| *T. felleus* | FH:BHI-F479 | USA: Boston Islands | - | MF161285 | - | Haelewaters *et al.*, 2018 |
| *T. felleus* | BAH010 (TENN) | USA: Tennessee | - | MF773588 | - | GeneBank |
| *T. felleus* | JMP0093 | USA | - | EU819449 | - | Palmer *et al.*, 2008 |
| *T. felleus* | ACAD 21813F | Canada: Kejimkujik National Park | - | PV248587 | - | GeneBank |
| *T. felleus* | JBRI-M23-092 | South Korea | - | OR852505 | - | GeneBank |
| *T. felleus* | HKAS 134581 | China: Yunnan | - | PP806336 | - | GeneBank |
| *T. felleus* | HKAS 134600 | China: Yunnan | - | PP806355 | - | GeneBank |
| *T. formosus* | PDD 72637 | New Zealand | - | HM060320 | HM060319 | GeneBank |
| *T. formosus* | JAC 13345 | New Zealand | *Fuscospora cliffortioides* | OP141386 | OP141535 | GeneBank |
| *T. glutinosus* | HKAS 81369$^{T}$ | Bangladesh: Dhaka | *Shorea robusta* | MZ351438 | MZ351442 | Hosen, 2021 |
| *T. glutinosus* | HKAS 75116 | Bangladesh: Dhaka | *Shorea robusta* | MZ351437 | - | Hosen, 2021 |

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|---|---|---|---|---|---|---|
| *T. griseipurpureus* | HKAS 90200 | China: Hainan | tropical forests dominated by fagaceous plants | - | KT990624 | Wu *et al.,* 2016 |
| *T. griseipurpureus* | MG521a | China: Guangdong, Nan'ao island | - | KM975484 | KM975493 | Gelardi *et al.,* 2021 |
| *T. griseipurpureus* | USM Bo1 | Malaysia: Kelantan | *Melaleuca leucadendra* | KF442405 | - | GeneBank |
| *T. griseipurpureus* | SC43 BORH(F)03828 | Malaysia | - | PX658178 | PX658179 | This study |
| *T. griseipurpureus* | Trang 1 | Thailand: Trang | *Melaleuca leucadendron* | JQ726596 | - | Aung-aud-chariya *et al*., 2012 |
| *T. griseipurpureus* | Songkhla | Thailand: Songkhla | *Acacia mangium* | JQ726597 | - | Aung-aud-chariya *et al*., 2012 |
| *T. griseipurpureus* | ABI-V-F402 (VNM00075508) | Dong Nai Nature and Culture Reserve, Dong Nai Province | *Acacia* hybrid | PX238742 | PX233188 | This study |
| *T. griseipurpureus* | ABI-V-F403 (VNM00075509) | Dong Nai Nature and Culture Reserve, Dong Nai Province | *Acacia* hybrid | PX238750 | PX233189 | This study |
| *T. griseipurpureus* | ABI-V-F404 (VNM00075510) | Dong Nai Nature and Culture Reserve, Dong Nai Province | *Acacia* hybrid | PX238751 | PX233190 | This study |
| *T. griseipurpureus* | ABI-V-F407 (VNM00075511) | Dong Nai Nature and Culture Reserve, Dong Nai Province | *Acacia* hybrid | PX238747 | PX233191 | This study |

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|---|---|---|---|---|---|---|
| *T. griseipurpureus* | ABI-V-F412 (VNM00075512) | Dong Nai Nature and Culture Reserve, Dong Nai Province | *Acacia* hybrid | PX238752 | PX233192 | This study |
| *T. griseipurpureus* | ABI-V-F414 (VNM00075513) | Dong Nai Nature and Culture Reserve, Dong Nai Province | *Acacia* hybrid | PX238753 | PX233193 | This study |
| *T. griseipurpureus* | ABI- V-BC20 (VNM00075497) | Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City | *Acacia* hybrid | PX238754 | PX233187 | This study |
| *T. griseipurpureus* | ABI-V-BC22 (VNM00075498) | Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City | *Acacia* hybrid | PX238743 | PX233199 | This study |
| *T. griseipurpureus* | ABI-V-BC23 (VNM00075499) | Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City | *Acacia* hybrid | PX238755 | PX233201 | This study |
| *T. griseipurpureus* | ABI-V-BC27 (VNM00075495) | Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City | *Acacia* hybrid | PX238756 | PX233194 | This study |
| *T. griseipurpureus* | ABI-V-BC28 (VNM00075496) | Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City | *Acacia* hybrid | PX238749 | PX233195 | This study |
| *T. griseipurpureus* | ABI-V-BC29 (VNM00075494) | Binh Chau – Phuoc Buu Nature Reserve, Ho Chi Minh City | *Acacia* hybrid | PX238744 | PX233196 | This study |

| | | | | | | |
|---|---|---|---|---|---|---|
| *T. griseipurpureus* | ABI-V-QT10.1 (VNM00075500) | Nam Hai Lang commune, Quang Tri Province | *Acacia* hybrid | PX238738 | PX233185 | This study |
| *T. griseipurpureus* | ABI-V-QT10.2 (VNM00075501) | Nam Hai Lang commune, Quang Tri Province | *Acacia* hybrid | PX238739 | PX233186 | This study |
| *T. griseipurpureus* | ABI-V-QT13.1 (VNM00075504) | Nam Hai Lang commune, Quang Tri Province | *Acacia* hybrid | PX238740 | PX233198 | This study |
| *T. griseipurpureus* | ABI-V-QT13.2 (VNM00075505) | Nam Hai Lang commune, Quang Tri Province | *Acacia* hybrid | PX238741 | PX233197 | This study |
| *T. griseipurpureus* | ABI-V-QT14.1 (VNM00075502) | Nam Hai Lang commune, Quang Tri Province | *Acacia* hybrid | PX238745 | PX233202 | This study |
| *T. griseipurpureus* | ABI-V-QT14.2 (VNM00075503) | Nam Hai Lang commune, Quang Tri Province | *Acacia* hybrid | PX238746 | PX233203 | This study |
| *T. griseipurpureus* | ABI-V-PQ4 (VNM00075506) | Phu Quoc National Park, An Giang Province | *Acacia* hybrid | PX238748 | PX233200 | This study |
| *T. himalayanus* | CAL 1649$^{T}$ | India: Sikkim | *Pinus* sp. | MG799322 | MG799328 | Chakraborty *et al.*, 2018 |
| *T. himalayanus* | BJTC FM2315 | China: Shanxi Province, Yuncheng City | China, Shanxi Province, Yuncheng City | OR655170 | OR655216 | Mao et al., 2023 |
| *T. minor* | FLAS-F-61027 | USA: Florida, Putnam County | *Quercus* dominated hardwood forest | MH211698 | - | GeneBank |
| *T. minor* | FLAS-F-61028 | USA: Florida, Putnam County | *Quercus* dominated hardwood forest | MH211699 | - | GeneBank |

| | | | | | | |
|---|---|---|---|---|---|---|
| *T. neofelleus* | HMAS 84730$^T$ | China: Yunnan, Simao | | KM975485 | KM975494 | Gelardi *et al*., 2021 |
| *T. neofelleus* | YT20121007 | Japan: Niigata, Higashikanbara-gun | *Pinus densiflora* | KM975488 | KM975496 | Gelardi *et al*., 2021 |
| *T. plumbeoviolaceoides* | GDGM 20311$^T$ | China: Guangdong, Guangzhou | Fagaceae | KC984859 | KC984858 | |
| *T. plumbeoviolaceoides* | GDGM 21040 | China | - | - | MT154720 | Li & Yang, 2021 |
| *T. plumbeoviolaceus* | MB06-056 | USA: New York, Chestnut Ridge Park | - | - | KF030350 | Nuhn *et al*., 2013 |
| *T. plumbeoviolaceus* | NYBG:0009 | USA: Missouri | - | KY432830 | KY432825 | GeneBank |

Note: T−holotype specimen.

**Table 2.** Similarity Matrix based on Jaccard's Coefficient for ISSR

| **Vouchers** | **VNM00075508** | **VNM00075509** | **VNM00075510** | **VNM00075511** | **VNM00075512** | **VNM00075513** | **VNM00075497** | **VNM00075498** | **VNM00075499** | **VNM00075495** | **VNM00075496** | **VNM00075494** | **VNM00075501** | **VNM00075500** | **VNM00075503** | **VNM00075502** | **VNM00075504** | **VNM00075505** | **VNM00075506** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **VNM00075508** | 1.00 | | | | | | | | | | | | | | | | | | |
| **VNM00075509** | 0.84 | 1.00 | | | | | | | | | | | | | | | | | |
| **VNM00075510** | 0.68 | 0.70 | 1.00 | | | | | | | | | | | | | | | | |
| **VNM00075511** | 0.70 | 0.80 | 0.58 | 1.00 | | | | | | | | | | | | | | | |
| **VNM00075512** | 0.57 | 0.63 | 0.63 | 0.63 | 1.00 | | | | | | | | | | | | | | |
| **VNM00075513** | 0.69 | 0.81 | 0.57 | 0.89 | 0.60 | 1.00 | | | | | | | | | | | | | |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **VNM00075497** | 0.66 | 0.74 | 0.82 | 0.60 | 0.63 | 0.61 | 1.00 | | | | | | | | | | | | |
| **VNM00075498** | 0.67 | 0.75 | 0.63 | 0.71 | 0.58 | 0.74 | 0.71 | 1.00 | | | | | | | | | | | |
| **VNM00075499** | 0.69 | 0.77 | 0.67 | 0.65 | 0.66 | 0.70 | 0.77 | 0.76 | 1.00 | | | | | | | | | | |
| **VNM00075495** | 0.82 | 0.88 | 0.70 | 0.68 | 0.57 | 0.71 | 0.78 | 0.77 | 0.79 | 1.00 | | | | | | | | | |
| **VNM00075496** | 0.76 | 0.76 | 0.64 | 0.66 | 0.53 | 0.63 | 0.76 | 0.75 | 0.65 | 0.82 | 1.00 | | | | | | | | |
| **VNM00075494** | 0.83 | 0.75 | 0.67 | 0.65 | 0.52 | 0.62 | 0.71 | 0.70 | 0.58 | 0.75 | 0.91 | 1.00 | | | | | | | |
| **VNM00075501** | 0.62 | 0.56 | 0.70 | 0.49 | 0.51 | 0.50 | 0.70 | 0.53 | 0.55 | 0.60 | 0.68 | 0.69 | 1.00 | | | | | | |
| **VNM00075500** | 0.64 | 0.58 | 0.70 | 0.52 | 0.51 | 0.51 | 0.68 | 0.57 | 0.53 | 0.62 | 0.66 | 0.69 | 0.94 | 1.00 | | | | | |
| **VNM00075503** | 0.60 | 0.56 | 0.68 | 0.50 | 0.50 | 0.50 | 0.66 | 0.53 | 0.51 | 0.56 | 0.66 | 0.69 | 0.92 | 0.94 | 1.00 | | | | |
| **VNM00075502** | 0.63 | 0.53 | 0.65 | 0.46 | 0.43 | 0.45 | 0.67 | 0.50 | 0.50 | 0.57 | 0.71 | 0.74 | 0.87 | 0.87 | 0.91 | 1.00 | | | |
| **VNM00075504** | 0.61 | 0.55 | 0.61 | 0.48 | 0.43 | 0.49 | 0.65 | 0.52 | 0.52 | 0.57 | 0.69 | 0.70 | 0.91 | 0.91 | 0.93 | 0.94 | 1.00 | | |
| **VNM00075505** | 0.62 | 0.60 | 0.66 | 0.52 | 0.50 | 0.53 | 0.68 | 0.55 | 0.53 | 0.60 | 0.68 | 0.71 | 0.92 | 0.92 | 0.94 | 0.89 | 0.93 | 1.00 | |
| **VNM00075506** | 0.50 | 0.55 | 0.57 | 0.50 | 0.50 | 0.49 | 0.57 | 0.47 | 0.50 | 0.51 | 0.55 | 0.54 | 0.63 | 0.63 | 0.67 | 0.60 | 0.62 | 0.65 | 1.00 |

**Table 3.** ISSR primer and their sequences used in this study

| Primer no. | Sequence (5' → 3') | $T_a$ value (°C) | Size of fragment range (bp) | TB | PB | PPL (%) | PIC value |
|---|---|---|---|---|---|---|---|
| P5 | GGATGCA$(AC)_6$ | 50.2 | 600-2500 | 10 | 10 | 100 | 0.27 |
| P55 | KKRBRBR$(AC)_6$ | 44.5 | 900-3000 | 6 | 6 | 100 | 0.38 |
| P810 | $(GA)_8$T | 47.8 | 1000-3000 | 9 | 9 | 100 | 0.28 |
| P812 | $(GA)_8$A | 47.8 | 900-2500 | 9 | 9 | 100 | 0.23 |
| P816 | $(CA)_8$T | 47.8 | 700-2200 | 7 | 7 | 100 | 0.41 |
| P817 | $(AG)_8$G | 50.2 | 600-4000 | 16 | 16 | 100 | 0.39 |
| P825 | $(AC)_8$T | 47.8 | 600-4000 | 12 | 12 | 100 | 0.28 |
| P828 | $(TG)_8$A | 47.8 | 700-3000 | 12 | 12 | 100 | 0.30 |
| P829 | $(CA)_8$ | 47.2 | 600-2500 | 8 | 8 | 100 | 0.30 |
| P889 | DBD$(AC)_7$ | 45.4 | 700-2500 | 12 | 12 | 100 | 0.40 |

*Note: B = (C, G, T), D = (A, G, T), K =(G, T), R = (A, G), Y = (C, T). TB = Total bands. PB = Polymorphic bands. PPL = Percentage of polymorphic bands. PIC: Polymorphic information content.*

The similarity indices among all specimens ranged from 0.43 to 0.94, with an average of 0.66, indicating substantial genetic variation within the 19 genotypes (Table 2). The highest similarity index was recorded between specimens VNM00075500 and VNM00075501, VNM00075500 and VNM00075503, VNM00075504 and VNM00075502, and VNM00075505 and VNM00075503. The lowest similarity values were recorded between VNM00075504, VNM00075502 and VNM00075512. The coefficient of similarity among all of the specimens ranged from 0.58 to 0.94, with an average of 0.76 A UPGMA dendrogram constructed from the ISSR data separated 19 specimens into three major clades, which revealed the proximity of their genetic distance (Figure 4). Clade I was the largest group, consisting of 12 specimens, including VNM00075508, VNM00075509, VNM00075510, VNM00075511, VNM00075512, VNM00075513, VNM00075497, VNM00075498, VNM00075499, VNM00075495, VNM00075496, and VNM00075494. Clade II and clade III were showing more genetic similarity among themselves, in which VNM00075500, VNM00075501, VNM00075504, VNM00075505, VNM00075502, and VNM00075503 were grouped to form clade II, while clade III was comprised of only one specimen, VNM00075506.

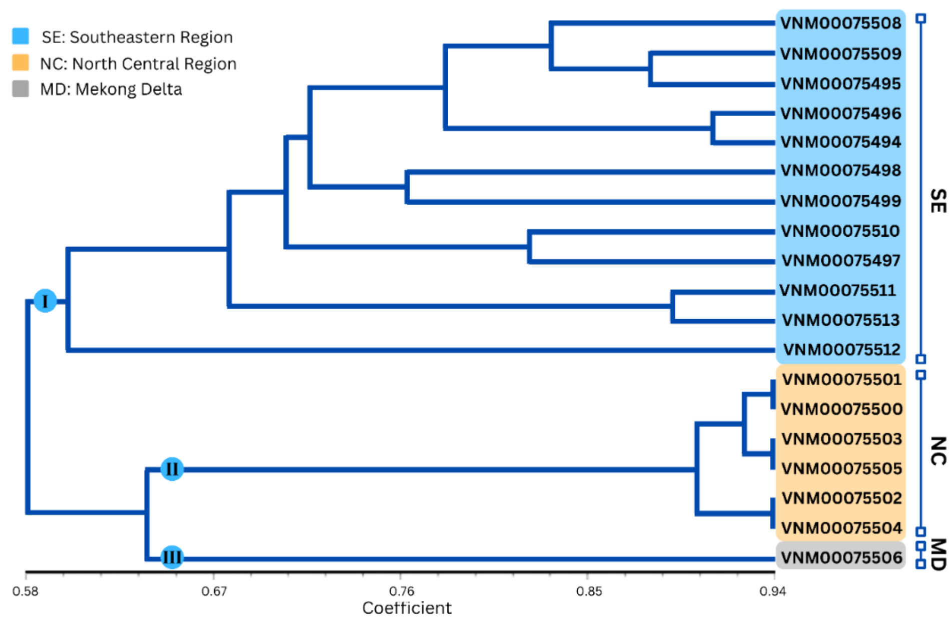


**Figure 4.** A dendrogram was constructed using the UPGMA method and Jaccard similarity index for 19 vouchers of *T. griseipurpureus*, based on the combined band pattern data obtained from ten ISSR primers

## Discussion

Based on both morphological and molecular phylogeny analyses, the Vietnamese '*Tylopilus felleus'* (locally known as Nấm Tràm) is confirmed to be distinct from the *T. felleus sensu stricto.* and is conspecific with *T. griseipurpureus*. Although the name '*T. felleus*' has been widely applied to the Vietnamese species of 'Nấm Tràm' in recent decades, it represents a misapplied taxon. The epithet *T. felleus* was first used by (Pham, 1972) for specimens collected in Saigon. Since then, *T. felleus* has since been widely adopted as the binomial for 'Nấm Tràm' in numerous studies (Anh, 2003; Kiet, 2013).

*Tylopilus griseipurpureus* was originally described from Singapore by Corner (1972) as *Boletus griseipurpureus* and later transferred to the genus *Tylopilus* by Horak (2011) based on the color of the hymenophore. The morphology of the Vietnamese specimens examined in this study is consistent with the descriptions of the holotype (Corner, 1972; Horak, 2011), as well as with specimens reported from Australia (Leonard, 2015) and China (Wu *et al*., 2016).

Morphologically, *T. griseipurpureus* resembles several other species, including *T. felleus*, *T. glutinosus*, *T. neofelleus*, *T. plumbeoviolaceus*, and *T.*

*plumbeoviolaceoides*. These species share similar macroscopic traits such as purple to dark purplish pilei and hymenophore that is initially white but later turns pinkish with age, often leading to misidentification among morphologically similar taxa. However, *T. felleus* can be distinguished by its gray to grayish-brown pileus and larger basidiospores [11–15 × 4–4.5 µm] (Watling, 2008). *Tylopilus glutinosus* differs in having a glutinous pileus when moist, with coloration ranging from dark purple to pale pinkish purple, and broader basidiospores [(8.5–)9.0–10.0(–11.0) × (4.0–)4.4–4.8(–5.2) µm; $Q_m$ = 2.16 ± 0.11] and is known to be associated with *Shorea robusta* forests (Hosen, 2021). *Tylopilus neofelleus* (= *T. microsporus*) is separable by its brownish to pale ochraceous-brown pileus, smaller basidiospores [(7.0–)8.3 ± 0.6(–10.3) × (2.0–)3.7 ± 0.2(–4.4) µm], and association with *Pinus* or members of Fagaceae (Gelardi *et al.,* 2015). *Tylopilus plumbeoviolaceoides* differs by its dark violaceous to brown-vinaceous pileus and slightly narrower basidiospores [(7.5–)8.5–10.5(–12.0) × (2.5–)3.0–3.8(–4.2) µm] (Li *et al.,* 2002). In contrast, *T. plumbeoviolaceus*, originally described from North America, possesses a deep violet-purplish to violaceous-brown pileus, a distinctly reticulate purplish-violaceous stipe, and broader basidiospores [10–13(–14) × 3–4(–5.5) µm; Qm = 2.8] (Singer, 1947; Snell and Dick, 1941).

In the phylogenetic tree, *Tylopilus griseipurpureus* forms a sister relationship with *T. formosus*, a species originally described from New Zealand. *Tylopilus formosus* differs from *T. griseipurpureus* in its chocolate-brown to dark brownish-black pileus and stipe, longer basidiospores [9.8–14.2(–15) × 4–5.3 µm], and its association with *Leptospermum* and *Nothofagus* (Stevenson, 1962).

ISSR is a DNA marker technique developed based on microsatellites regions and does not require prior genomic sequence information. It is characterized by clear and distinct banding patterns, simple laboratory procedures, and high reproducibility. Therefore, ISSR markers have become widely used for detecting interspecific genetic variation and are widely applied in species identification and genetic diversity research of edible and medicinal fungi (Du *et al*., 2018; Du *et al*., 2011; Li *et al*., 2024; Wang *et al*., 2019; Zhao *et al*., 2013). High levels of genetic variation are commonly observed in wild fungal populations that reproduce sexually, have broad ecological niches, or have wide geographical distributions (James *et al*., 1999). Among commonly used metrics, the percentage of polymorphic bands (PPB) is important parameter for assessing genetic variation at the species level. In this study, ISSR markers were used to investigate the genetic diversity of 19 *Tylopilus griseipurpureus* specimens collected from four plantations representing three distinct geographical regions of Vietnam. The ten ISSR primers revealed a high level of genetic variation among the specimens. A total of 101 DNA fragments were generated across the four populations, all of which were polymorphic, resulting in a percentage of polymorphic bands (PPB) of 100%. Comparable levels of polymorphism have been reported in other edible mushrooms, including 96.32% in *Pleurotus eryngii* var. *tuoliensis* (Zhao *et al.,* 2013), 99.8% in *Auricularia polytricha* (Du *et al*., 2011), and 94.4% in *Lepista nuda* (Du *et al*., 2018).

The UPGMA dendrogram based on the Jaccard coefficients distributed 19 accessions into three main clades corresponding to their geographical origins. Clade I included specimens collected from two plantations in the Southeastern region (SE), indicating close genetic relationships. Clade II consisted of specimens from the North Central region (NC), while Clade III comprised a single specimen from the Mekong Delta region (MK), which was clearly separated from the other two clades. These findings suggest that geographical separation contributed to genetic differentiation of *T. griseipurpureus* populations, potentially driven by environmental adaptation and host plant associations. Therefore, the ISSR markers employed in this study proved effective for evaluating the genetic diversity of *T. griseipurpureus* in relation to its geographical distribution. Previous biogeographical studies indicate that ecological factors such as natural geographic barriers, including mountain ranges and river systems, can promote population differentiation even at microgeographic scale (Cozzolino *et al*., 2003; Gascon *et al*., 2000).

This study is limited by the relatively small sample size and the sole use of ISSR markers, which may not capture the full extent of genetic variation in *T. griseipurpureus*. In addition, ecological factors such as host plant associations were not assessed. Future research should incorporate broader sampling and integrate analyses of genetic diversity, geographical distribution, and host plant composition to obtain a more comprehensive understanding of this valuable fungal species.

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## Conflict of interest

The authors declare no conflict of interest.

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