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Research article

Laetiporus zonatus: an addition to edible polypore fungi in Pakistan

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Abstract
Laetiporus is a cosmopolitan genus of the "Antrodia clade" in the order Polyporales that causes brown rot in many hardwood trees and some conifers. In the current study, we examined specimens of *Laetiporus zonatus* collected from *Quercus semecarpifolia* (Fagaceae) at three different localities in the district Swat, KP, Pakistan. The specimen's identity was determined through extensive morphoanatomical examination and molecular characterization. For reconstruction of the phylogenetic relationships of the species, the study applied three methods: maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses to the concatenated dataset (ITS + nrLSU + rpb2). The mean character difference approach was utilized to create a phenetic cladogram through morphometric analysis. Our sample sequence grouped together with *Laetiporus zonatus* showing significant support values of 87% for MP, 98% for ML and 1.0 for Bayesian analyses. The morphological data matrix showed a high degree of similarity (Bray-curtis similarity = 0.925, Euclidean distance = 3.81) between our specimen and *L. zonatus*. Our study also provides habitat characteristics and *in vitro* cultural characteristics of the isolates. *Laetiporus zonatus* is characterized by yellow pileal surface, become pale buff to creamy when dry and whitish cream pore surface, distinctly zonate, radially furrowed upper surface and undulating white margin, 2-3 pores mm^{-1}, ellipsoid to ovoid basidiospores (5.6–8.7 × 4.2–5.9 µm) with Q value of 1.16-1.55 and is found exclusively on *Quercus* spp. in temperate forests.

Keywords
brown rot, phylogeny, morphometrics, *Laetiporus zonatus*, edible mushroom

Introduction
Polyporales is an order of the phylum Basidiomycota that includes 285 genera, 18 families, and over 2,500 species (He et al., 2019). However, according to the formal classification by Justo et al. (2017), 37 families within the Polyporales are distributed among 18 distinct clades. One of the largest clades, "Antrodia" was first recognized in the order by Hibbett and Donoghue (2001) and encompasses approximately 26 genera (Ortiz-Santana et al., 2013). Some of the members within the clade are edible and also have medicinal properties referred to as culinary-medicinal mushrooms (Gargano et al., 2020). The species of *Laetiporus sulphureus* (Bull.:Fr.) Murrill complex are highly prized for

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their fleshy textured and brightly colored aromatic fruiting bodies, commonly known as sulfur shelf or chicken of the wood (Turkoglu et al., 2007). Proximate analysis reveals that these mushroooms contain important nutrients, including carbohydrate (particularly polysaccharides), proteins, essential amino acids, minerals, vitamins, polyunsaturated fatty acids and dietary fibre (Martinez et al., 2015; Khatua et al., 2017). Additionally, their secondary bioactive constituents have numerous therapeutic properties including anti-inflammatory, antimicrobial, antioxidant, anti-hyperglycemic, anti-tumor, and immunomodulatory effects (Saba et al., 2015). Ecologically, some *Laetiporus* spp. cause brown rotting in many deciduous and coniferous trees and are pathogenic to living trees in forests (Dai et al., 2007; Banik et al., 2010). Hence, they play a very important role in forest ecosystems.

Laetiporus Murrill 1904 is a cosmopolitan genus belonging to the Laetiporaceae Jülich. The genus was first placed in the "Antrodia clade" of the Polyporales by Hibbett and Donoghue (2001). The genus *Laetiporus* has a total of 40 specific or infraspecific records listed on Index Fungorum (http://www.indexfungorum.org, accessed on March, 2023), while MycoBank database (http://www.mycobank.org, accessed on March, 2023) displays a total of 22 legitimate names, with some of these species having been reclassified under other genera. Currently, 15 well-defined species have been recognized, of which 11 belong only to the *L. sulphureus* complex (Song et al., 2018). The genus has a wide distribution in boreal to subtropical regions and has been reported from Europe, Asia, and North America (Ota and Hattori, 2008). The *Laetiporus* spp. produce annual basidiocarps, which are sessile or laterally substipitate, flabelliform, and yellow in color, with regular round or angular pores ranging from 3–5 mm^{-1}. The hyphal system is dimitic, consisting of septate generative hyphae and binding hyphae, lacking clamp connections. It has pyriform, 4-sterigmate, simple septate basidia that produce broadly ovoid basidiospores and lacks cystidial elements (Gilbertson and Ryvarden, 1986; Ryvarden, 1991; Burdsall and Banik, 2001; Lindner et al., 2008; Ota et al., 2009).

Several taxonomic researches have been conducted on the *Laetiporus* genus using a combination of molecular, morphological, cultural, and ecological methods to determine phylogenetic relationships and interspecific delimitation. Gilbertson (1981) reported that the genus was represented by two species namely *L. sulphureus* and *L. persicinus* (Berk. & M. A. Curtis) Gilb. The latter species was transferred to *Berkcurtia persicina* (Berk. & M.A. Curtis) Robledo & Campi, Lillo (Campi et al., 2022). However, based on the morphological and multilocus molecular data of Paez et al. (2022), *L. persicinus* was recently renamed as *Kusaghiporia persicinus* (Berk. & M.A. Curtis) C.A. Paez, Kraisit. & M.E. Sm. Based on ITS sequence data and ecological relationships with hosts, European isolates of *L. sulphureus* were classified into two major clades (Rogers et al., 1999). Further, mating and molecular studies have been conducted on the genus from North America (Banik, 1999; Banik and Burdsall, 2000; Burdsall and Banik, 2001). A study conducted by Burdsall and Banik (2001) identified six distinct taxa, including *L. cincinnatus* (Morgan) Burds., Banik & Volk, *L. conifericola* Burds. & Banik, *L. gilbertsonii* Burds., *L. gilbertsonii* var. *pallidus* Burds., *L. huroniensis* Burds. & Banik, and *L. sulphureus*. This differentiation was largely based on the attachment of the stipe, the color of the pileal and pore surfaces, and host association. Similarly, Tomsovsky and Jankovsky (2008) added another species *L. montanus* Cerny ex Tomsovsky & Jankovsky from Europe, which was associated with coniferous hosts. Similarly, Banik et al. (2012) described *L. caribensis* Banik & D. L. Lindner, a new species found in the Caribbean basin associated with hardwood hosts. A molecular study from Japan by Ota and Hattori (2008) identified three species of *Laetiporus* including *L. cremeiporus* Y. Ota & T. Hatt., *L. montanus*, and *L. versisporus* (Lloyd) Imazeki, Bull. The phylogenetic relationships among Euro-American and East Asian *Laetiporus* species were studied by Lindner and Banik (2008), Ota et al. (2009), Banik et al. (2010), Song et al. (2014) and Song and Cui (2017). After analyzing Chinese materials Song et al. (2014) described two species, *L. versisporus* and *L. zonatus* B.K. Cui & J. Song, from Southwestern China. Subsequently, Song et al. (2018) described two additional species,

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L. medogensis J. Song & B.K. Cui and *L. xinjiangensis* J. Song, Y.C. Dai & B.K. Cui from the country. A review of the literature showed that several researchers have thoroughly investigated the species from different countries. However, South Asian countries have not been sampled or lack adequate voucher materials for thorough taxonomic and biogeographic analyses of the genus in global context. Therefore, the current study aims to examine collection from three different localities and infer the phylogenetic relationship using both molecular and morphological data.

Materials and Methods

Sampling and morphological characterization

The present study analyzed voucher specimens collected from the temperate forest of the district Swat, KP, Pakistan, located within the floristically diverse region of the Hindu-Kush mountain range. Dominant tree species in the area are *Picea smithiana* (Wall.) Boiss, *Abies pindrow* Royle, *Cedrus deodara* (Roxb. ex D. Don), G. Don, *Pinus* spp., and *Quercus* spp. Basidiocarps were photographed and collected from three different localities. During the field examination, macro-morphological features such as color changes, bruising, exposure to air and other ephemeral characters were recorded in the field note book. Color terms were adopted from Petersen (1996), and the voucher specimens were dried and deposited in the mycological section of the herbarium at the University of Malakand (BGH), KP, Pakistan.

In the present study, the basidiocarps were subjected to a detailed microanatomical examination. Microscopic techniques and routine notations were performed following the guidelines of Han et al. (2016), Song et al. (2018), and Ji (2022). Freehand anatomical sections were obtained from the hymenial and context layers for microscopic analysis of hyphal system, septal features, hymenial elements, including basidial and sterile elements, and spore characteristics using a camera-equipped binocular microscope. Mounting and staining reagents such as 5% KOH, 2% congo red, lactophenol cotton blue and Melzer's reagent (IKI) were used. Staining was performed for 1–3 hours, and the reactions were classified as follows: amyloid or Melzer's-positive (IKI+) stains blue or black, while inamyloid or Melzer's-negative (IKI-) showed no color change or became faintly yellow to brown. The reaction with lactophenol cotton blue (CB) was used to assess the cyanophily of basidiospore walls, with CB+ denoting cyanophilous and CB- denoting acyanophilous (Banik et al., 2012). On average 32 measurements were taken and rounded to the nearest 0.5 µm. ± standard error, n (a/b) = number of spores (a) measured from given number (b) of specimens (Ji, 2022). L = mean spore length (arithmetic average of all spores), W = mean spore width (arithmetic average of all spores), Q = variation in the L/W ratios between the specimens studied, n = number of spores measured from given number of specimens. The measurements were assessed with Image J (Wayne Rasband, National Institute of Health, USA) software (Tsujikawa et al., 2003).

Morphometrics analyses

The morphological data matrix was generated, comprising of 58 morphological characters obtained from 22 taxa. Some of these characters were binary, while others were multistate, extracted from the original descriptions provided by the authors. Each character state was then scored for the presence or absence, or assigned a specific score for multistate characters. The characters were unweighted and unordered, and the gamma shape was set to 0.5. To show the average difference between the character states of taxa a phenetic cladogram was constructed through the mean character difference method using the neighbor-joining (NJ) algorithm in the software package Paup4 (Swofford, 2002).

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Cultural characterization

The isolation was done from the mycelial tissue (5 × 5 mm) taken from the context portion of the fresh basidiocarps. Culturing techniques were adopted from Lindner and Banik (2011) and Virginia and Catalin (2013). Mycelial tissue was inoculated onto 90 mm potato dextrose agar (PDA) and malt extract agar (MEA) plates and observed after growth under a stereomicroscope for macro-morphological features according to Stalpers (1978). The growth rate and mycelial density were measured and recorded. Microscopic examination was done according to Peintner et al. (2019).

Molecular characterization

DNA extraction

DNA extraction was carried out using CTAB method (Murray and Thompson, 1980; Stirling, 2003) with slight modification. About 25 mg of the material from pore surface was taken and homogenized with multi-beads shocker at 3000 rpm/min using zirconia beads. Homogenization were done in 400 µl of 2% CTAB buffer and incubated at 65 °C for 50 min. The homogenate was added with 350 µl of chloroform:isoamyl alcohol 24:1 for the purification then vortexed for one minute and centrifuged for 20 min at 13,200 rpm at 4 ºC. The S/N or aqueous phase was transferred into another autoclaved microtube. Overnight precipitation was done with 133 µl of ice cold iso-propanol and centrifuged for 20 min at 13,200 rpm at 4 ºC and S/N was discarded. The pellet was washed twice by adding 500 µl of chilled ethanol by vortexing briefly and centrifuged for 3 min. The pellets were air dried and re-suspended in distilled water and stored at -20 ºC.

PCR and Sanger sequencing

Amplification of ITS1-5.8S-ITS2 region of rDNA was carried out using ITS1-ITS4 primer pair (White et al. 1990; Gardes and Bruns, 1993), nrLSU region using LR0R-LR5 primer pair (Vilgalys and Hester, 1990; Moncalvo et al., 2000) and partial region of second largest subunit RNA polymerase-II using RPB2-6R-RPB2-7F primer pair (Liu et al., 1999). Genes were PCR-amplified through PCR (Applied Biosystems Veriti thermal cycler). PCR reaction (20 µl in volume) mixtures contained: 2 µl of gDNA (about 100 ng), 0.5 µl of each primer (10 µl), 14.17 µl of sterile deionized water (Fisher Scientific, Waltham, USA), 26 µl of 2×Taq PCR Mastermix, 0.5 µl Taq DNA polymerase (Takara BIO INC., Shiga, Japan), 2.0 µl $MgCl_2$, 0.5 µl dNTPs (Promega, Madison, Wisconsin). PCR reactions were carried out using following temperature regime: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final extension was kept as 72 °C for 8 min (Schoch et al., 2012). The amplified PCR bands were visualized using 1% agarose gel (Fisher Scientific, Waltham, MA, USA) prepared in 0.5× TAE buffer, stained with ethidium bromide (Stefanova et al., 2022). One Kb ladder (Promega, Durham, NC, USA) was used as standard marker.

Amplicons were purified by using the total fragment DNA purification Kit (MEGA quick-spinTM PLUS (Thermo Fisher Scientific, Seoul, Korea) following manufacturer instructions. The purified samples were sequenced through automated Sanger sequencing by submitting samples to the Macrogen Inc. Seoul (https://dna.macrogen.com) for sequencing facility, South Korea.

Phylogenetic analyses

To verify identity level, query sequences were blasted in Basic Local Alignment Search Tool (BLAST) in NCBI-GenBank (Altschul et al., 1990). For correct identification and to retrieve reference sequences, protocols recommended by Nilsson et al. (2012) and Schoch et al. (2014) were followed. Furthermore, the taxonomic annotations of the included sequences were carefully checked to ensure they were authentic and published, while uncultured and environmental sequences were omitted. The query coverage was set at 80% and sequence similarity at 97–100%, according to Raja et al.

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(2017). The sequences, based on vouchered herbarium samples, are available in GenBank under the specified accession numbers in the Table 1. The sequences were aligned using the MAFFT 7 online multiple sequence alignment program (https//mafft.cbrc.jp/ alignment/server/; Katoh et al., 2019). The alignment was reviewed and manual adjustments were made at misaligned sites. Ambiguous sites at both ends were excluded using BioEdit 7.2.5 (Hall et al., 2011). Most of the gaps were removed and the remaining gaps were treated as missing data in all analyses (Shen et al., 2002). *Wolfiporia dilatohypha* (FP72162) was used as the outgroup (Song et al., 2014). Three different phylogenetic analyses were performed on the combined ITS + nLSU + rpb2 data set. The best-fit substitution model was found using jModelTest2 (Darriba et al., 2012) based on different criteria.

The parsimony analyses (MP) were conducted using PAUP v.4.0.b10 (Swofford, 2002) with equal weighting. The analysis involved 1,000 heuristic search replicates, utilizing random taxon addition searches and tree-bisection-reconnection (TBR) branch swapping, with an unrestricted number of trees. A 50% majority-rule consensus tree was generated from the remaining trees, and the tree's topology was evaluated by calculating various indices such as tree length, consistency index (CI), homoplasy index (HI), and retention index (RI) (Justo and Hibbett, 2011). The Maximum Likelihood (ML) estimates were computed on the three partitioned data sets (ITS, nLSU, and rpb2) using IQ-TREE version 1.6.12 (Nguyen et al., 2015), with the substitution models of TIM2ef+G, TrN+I, and TrN+I selected for the respective partitions (Chernomor et al., 2016). The branch support was determined through bootstrapping with 1,000 replicates (Hoang et al., 2018). Bayesian Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) inference was performed on the same three partitioned data sets using MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003). The substitution models for each partition were specified as nst = 6, rates = gamma (ITS) and nst = 6, rates = propinv (nLSU and rpb2). The base frequencies, substitution rates, gamma shape, and p.inv. parameters were set based on the best-fit models obtained through the Akaike Information Criterion (AIC) as determined by JModelTest2. The analysis was run for 2 million generations with four chains and trees were sampled every 100 generations. The first 5,000 trees (25% of the total) were excluded as burn-in and were not used in constructing the consensus tree. The stop rule was set at stopval = 0.01 (Lindner and Banik, 2008). The trees were analyzed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and later edited. The optimal topologies from maximum likelihood (ML) analyses were displayed and validated based on the ML-BS score (≥75), MP-BS score (≥50), and BPPs (≥0.95) (Ji, 2022).

Results

In the current study, morpho-anatomical characteristic of the three specimens (MUBS80, MUSL21-44, MUMnd301) showed resemblance with *L. zonatus*, particularly in the color of pore and pileal surface, pore size, thickness of contextual binding hyphae, basidiospore dimension, spore quotient, and host plants. Similarly, the phylogenetic assessment of concatenated dataset from three different genetic markers (ITS, nrLSU, rpb2) including sequence data from the voucher MUBS80 indicated that our sample sequence clustered together with *L. zonatus*, with highly significant support values. Additionally, a morphometric analysis yielded similar results, corroborating the resemblance between our specimen and the *L. zonatus* described by Song et al. (2014).

Taxonomy

Laetiporus zonatus B.K. Cui & J. Song, Mycologia 106 (5): 1042 (2014)
MB#808204

Basidiocarps large sized, annual, solitary pileate or imbricate, flabellate or dimidiate to applanate, sessile to laterally substipitate, thick fleshy textured, become crumbly and lightweight when dry,

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having a distinct pungent odor and acidic flavor. Pilei projecting 8-15 cm., 10–20 cm. wide, 2–3 cm. thick at the base; upper surface orange yellow, become pale buff to creamy when dry, distinctly zonate, concentric zonation in the growing margin are distinct, deeply furrowed along the radii, completely glabrous become dull brown or buff brown when dried. Margin: whitish or pale, sterile, smoothly rounded and blunt and undulating or profusely wavy, up to 3–4 mm thick, fading to brownish colored when dry. Stipe almost absent but stout or robust attachment to the bark can be observed. Pore surface whitish cream or light yellow when fresh, brown when become dried; pores mostly angular and irregularly distributed. Pore lining rounded later become angular when dry, number of pores 2–4 or usually 3 per mm; dissepiment thin uniform in thickness 0.1–0.2 mm, and usually lacerate. Tube layer is tightly affixed to the context, whitish to pale colored about 1–3.5 (2) mm in thickness, concolorous with pore surface and context which become distinct in color when dry. Context is differentiated from the upper pileal cover whitish cottony becoming light brown and spongy on drying, 3-7 mm at the widest point, context to tube layer ratio is 2-5.5:1 (Fig. 1).

Hyphal system dimitic, skeletal hyphae in the tube layer and binding hyphae from the context layer dissolve in 2% KOH. The contextual binding hyphae are dominant characterized by light yellow, usually branched, laterally interwoven, septate (wavy septae) hyphae showing parallel or subparallel arrangement and give up frequently lateral tapering branches. Hyphal thickness range 7.5–20 (15) µm with wall thickness 0.6–3 µm, while the lateral hyphae vary in thickness from 3–4.2 µm. Generative hyphae rarely observed in the context. Tramal layer dimetic consisting of predominantly generative hyphae, profusely septated, 2.5–8.7 (5.0) µm in diameter, thin walled, regularly arranged, yellowish in color. Skeletal hyphae from the trama (subhymenium) rare, usually hyaline, thick walled less frequently branched and septated. Basidia are clavate, with short sterigmata and a simple septum at the base without clamp connection, 11.5–21.6 (15.6) × 5.5–8.2(6.4) µm, containing a large sized guttule. Basidioles frequent small sized, pyriform to globose shaped 7–10(8.5) × 5.5–6.8(5.7) µm. Cystidial elements are absent. Large sized diffused crystals were also observed. Basidiospores are ellipsoid to ovoidal shaped, thin walled, non-dixtrinoid, acyanophilous, 5.6–8.7 × 4.2–5.9 µm, L = 6.5 ± 0.21 µm, W = 5.0 ± 0.14 µm, Q = 1.16-1.55 (n = 32/3) (Fig. 2).

Materials examined: PAKISTAN, KP PROVINCE: (i) *Laetiporus zonatus* voucher no. MUBS80, Sailand, district Swat, (34º59’45’’ N and 72º11’08’’, 2797 m asl), mixed coniferous forest on living trees and dead tree stumps of *Quercus semecarpifolia,* August, 2020, (BGH000303; BGH); (ii) *Laetiporus zonatus* voucher no. MUSL21-44, Lalku, district Swat, (34º09’41’’ N and 72º25’03’’ E 2755 m asl), in mixed coniferous forest on *Q. semecarpifolia,* September, 2021, (BGH000304; BGH); (iii) *Laetiporus zonatus* voucher no. MUMnd301, Miandam, district Swat, (35º01’14’’ N and 72º35’06’’ E 2876 m asl), in mixed coniferous forest on living trees of *Quercus* sp.*,* September, 2020, (BGH000305; BGH).

Rot type: brown rotting

Cultural characteristics (Fig. 3): mycelial mat was irregular to circular shaped, consisting of sparsely and slow grown interwoven hyphae. Mat was raised with abundant aerial hyphae, showed azonate growth and slowly becoming moderately dense at the center; advancing edge: raised, uneven or irregular margin with sparse hyphal extension; colony odour: fruit or mushroom like; exudates: not found; front color: white to brown yellowish; obverse color: pale whitish; *in vitro* teleomorph formations: not found; KOH test: brown; growth rate: the average GR was measured as 4.7 (± 0.5) mm day^{-1}. The colony achieved the total radial growth of 42.7 mm on day 10 at 25 °C; hyphal characteristics: the aerial hyphae were usually thick, pale yellow, branched, non-clamped, septate (ring like), and diameter was 3–8.4 µm, producing lateral branches (dia. 2.5-3 µm). The conidiophores were showing

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racemose branching terminated on oval, spherical or nearly lemon shaped dense blastoconidia (8.5–10 × 10–12.5 µm) which are separated by double or single septum. Submerged hyaline hyphae were branched and septate lacking clamp connection. Spherical or subglobose shaped chlamydospores were frequently observed. Comments: colony initiation and establishment was slightly slow. Success rate of isolation was high on both on PDA (4/5) and MEA (4/5). Culture viability lost after few sub-culturing.

Fig. 1 - Basidiocarps of *Laetiporus zonatus* (a,b,c,f) from natural habitat focusing pileal and pore surfaces, margin; d) pores surface (fresh); e) pore surface (dried); g) cross section of fresh specimen; h) cross section of dried specimens. Bars: a,b = 3 cm, c,f = 2 cm, d,e,g,h = 3 mm.

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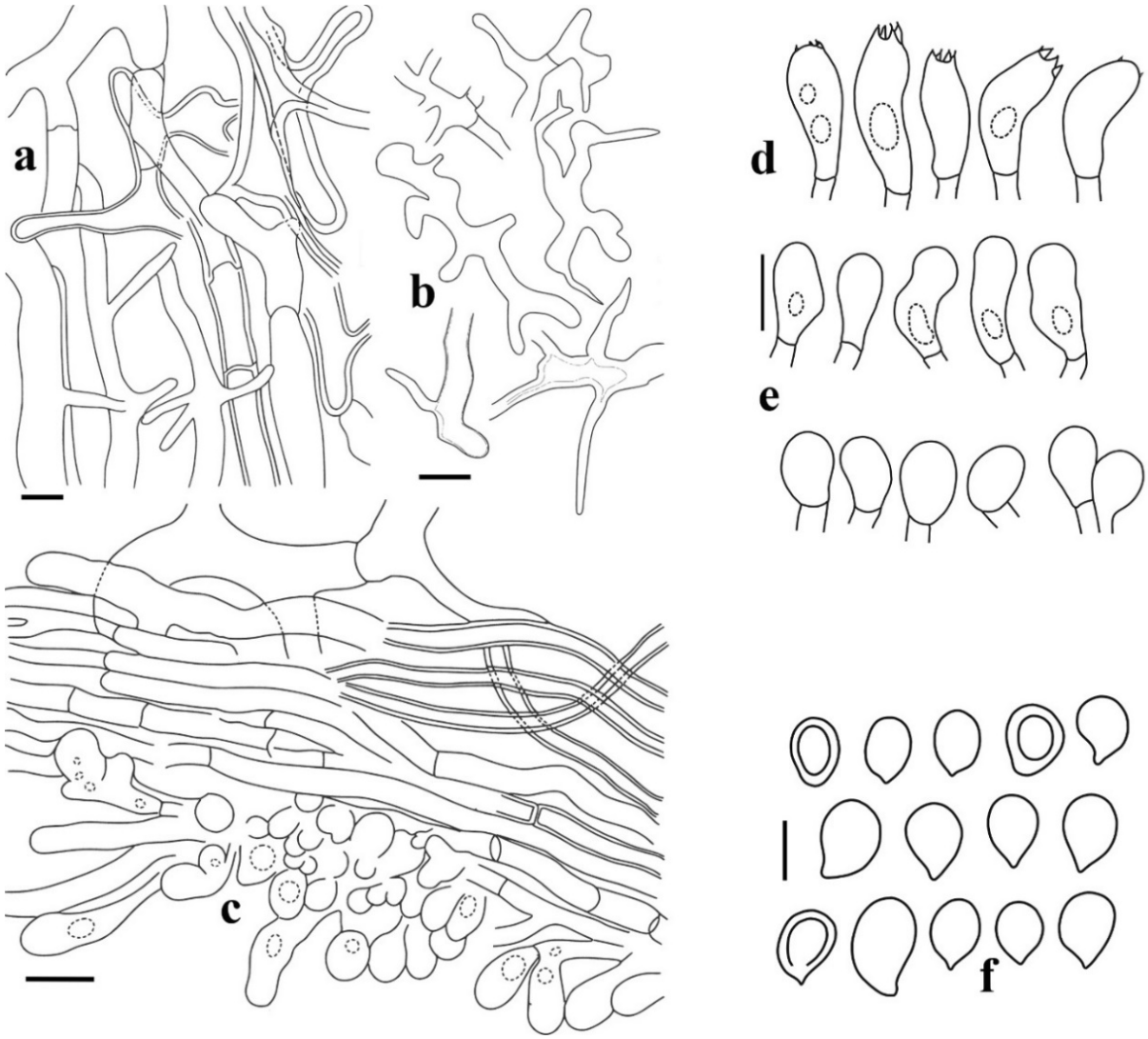

Fig. 2 – Microscopic structures drawn from voucher specimens of *Laetiporus zonatus*. a, b) binding hyphae of context showing branches; c) generative and skeletal hyphae from trama and hymenium structure; d) basidia; e) basidioles; f) basidiospores. Scale bars: a,b,c,d,e = 10 µm f = 5 µm.

Phylogenetic analyses

The phylogenetic relationships of the species were reconstructed by utilizing three methods: MP, ML, and Bayesian analyses. The concatenated dataset comprised of 75 sequence variants, including 29 ITS, 28 nrLSU, and 19 RPB2 sequences, including our subject sequences from the representative voucher sample (MUBS80) (Table 1). The topology of the ML analysis depicting the bootstrap proportion (MP), bootstrap maximum likelihood (ML), and Bayesian posterior probability (BPP) values, was shown in Fig. 4. Topologically, the inferred trees of MP, ML and Bayesian analyses on each region were more or less congruent, hence combined matrix was developed to undertake the analyses. A total of 13 species were included in the matrix i.e., *L. cincinnatus*, *L. conifericola*, *L. gilbertsonii*, *L. huroniensi*, *L. sulphureus*, *L. versisporus*, *L. ailaoshanensis*, *L. zonatus*, *L. cremeiporus*, *L. caribensis*, *L. medogensis*, *L. montanus,* and *Wolfiporia dilatohypha* Ryvarden & Gilb. (as outgroup). The aligned data matrix consisted of 1,547 nucleotide characters, including gaps, of which 86.3% were constant sites, 4.91% were variable characters that were parsimoniously uninformative, and 8.8% were parsimoniously informative characters with 222 distinct patterns.

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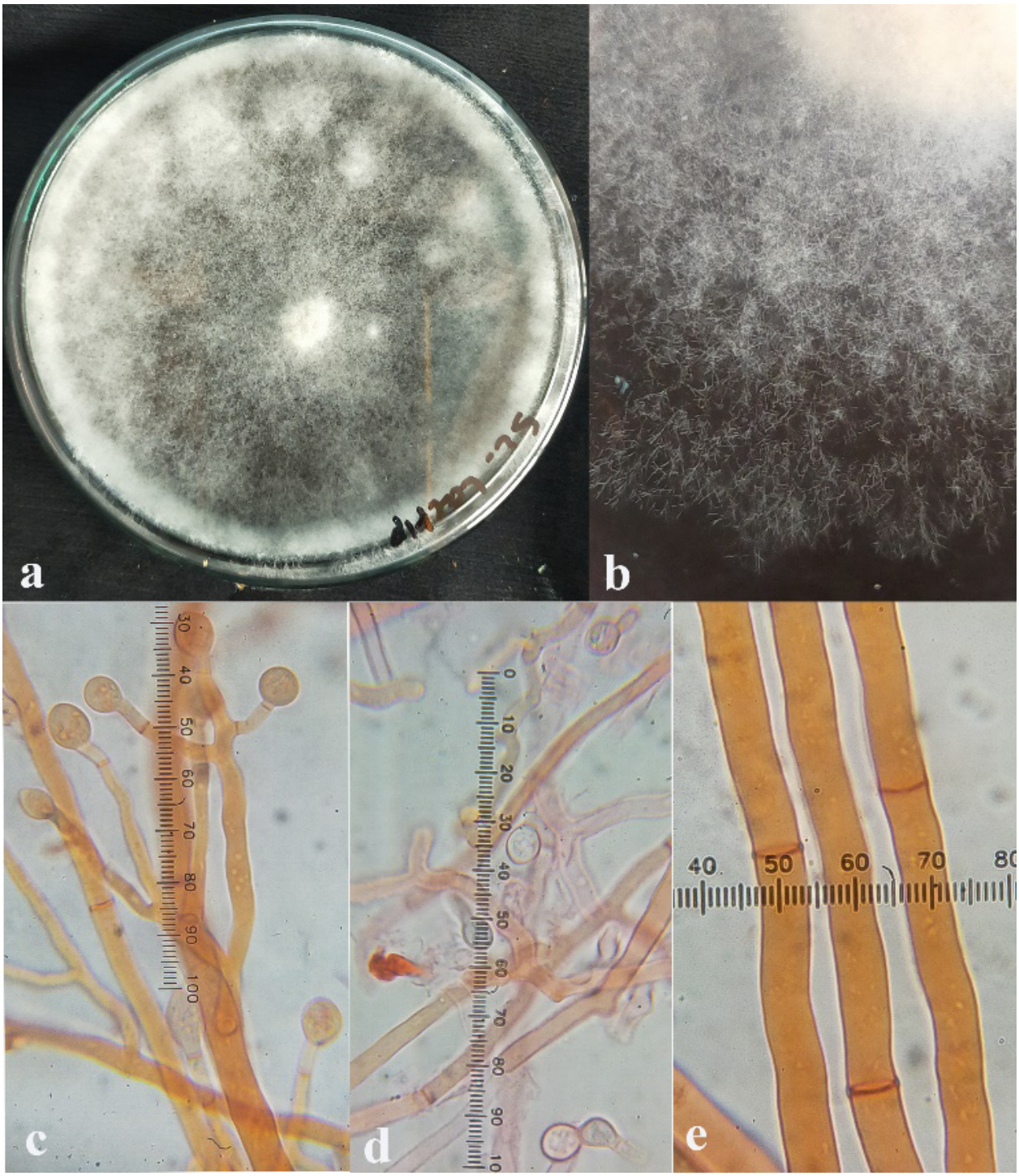

Fig. 3 - Cultural characteristics; a) *Laetiporus zonatus* MEA grown culture (90 mm); b) margin of distant and branched hyphal extension; c) conidiophore producing terminal blastoconidia; d) submerged hyaline hyphae; e) aerial hyphae. Scale in c,d,e. 1 division = 1.16 µm (100×).

The heuristic search of MP analysis resulted in 100 equally parsimonious trees on one island using tree-bisection-reconnection (TBR) branch swapping. A 50% majority rule consensus parsimonious tree was produced with the with tree description: tree length = 330, consistency index = 0.709, retention index = 0.773, rescaled consistency index = 0.548, and homoplasy index = 0.2994. The ML analysis

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was conducted on a three-partition dataset (ITS + nLSU + RPB2) with an initial log-likelihood of -3955.6186. The best evolutionary models were found to be time-independent model (TIM2ef) +G (with estimated gamma shape 0.26721) for the ITS region, Tamura-Nei (TrN)+I (with estimated p-invariance 0.894899) for the LSU region, and TrN+I (with estimated p-invariance 0.796262) for the RPB2 region. A Bayesian analysis with lset nst = 6 was applied to all three partitions, converging at ngen=20,000,000 with a standard deviation of split frequencies of 0.048481. The results of the Bayesian and likelihood analysis showed a similar topology and clade distribution. The data set revealed lineages mostly supporting species-level clades. Our sample sequence grouped together with *L. zonatus*, showing significant support values (87% MP, 98% ML and 1.00 BPP) showed sister relationship with *L. ailaoshanensis*, *L. medogensis* (branches were poorly resolved in this group) and distantly related to *L. cincinnatus*, *L. sulphureus* and *L. cremeiporus* (Fig. 4).

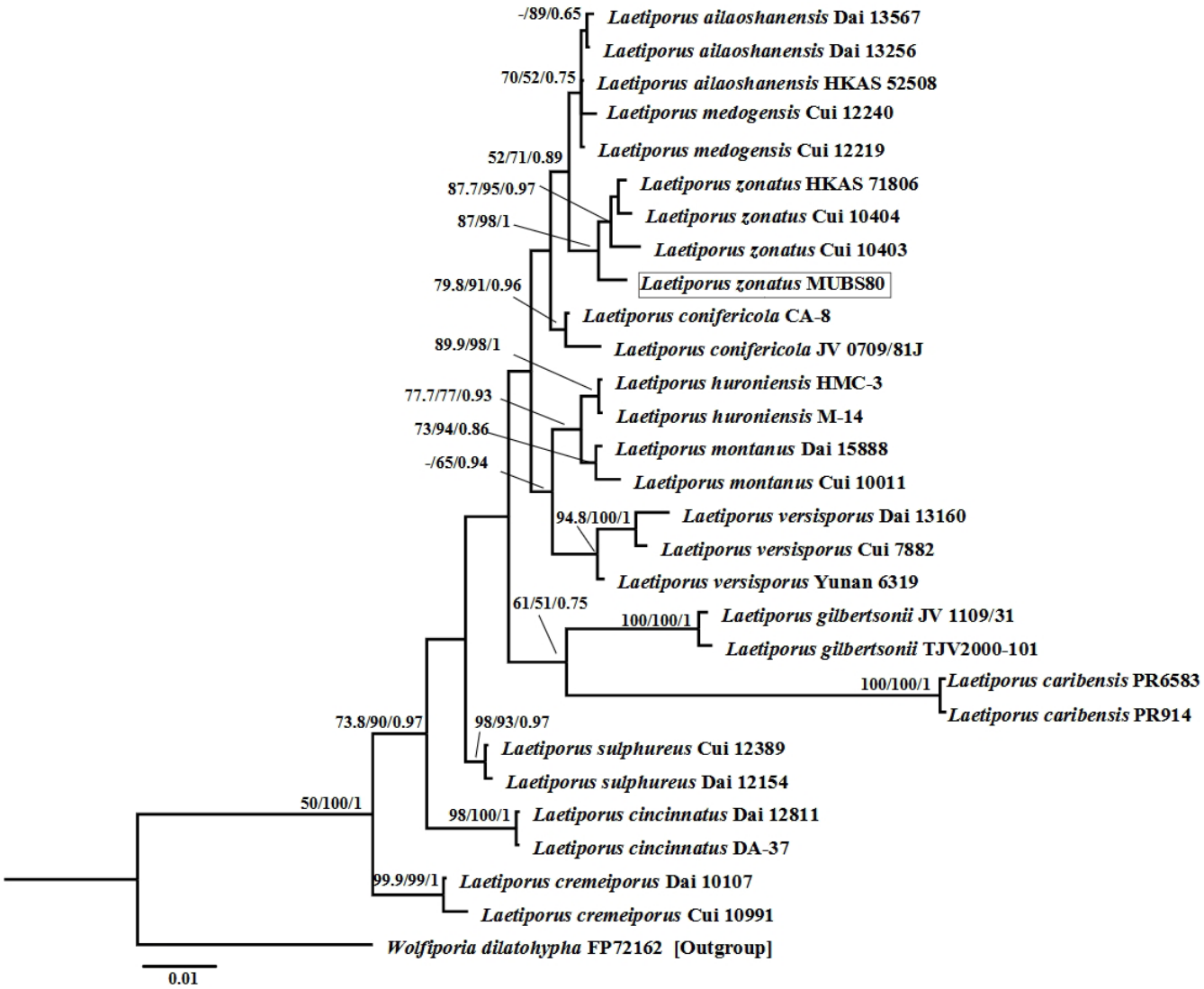

Fig. 4 - The strict consensus tree determined through maximum likelyhood depicts the phylogenetic relationships among *Laetiporus* spp., (including *Laetiporus zonatus* placed in box) inferred from ITS + nLSU + RPB2 sequences. Branch support values indicated before the corresponding node bootstrap MP/ bootstrap ML/ posterior probabilities BPP. Tip labels after taxa name designate voucher specimen number.

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Morphometric analyses

Analysis of 58 morphological characters from 22 taxa, using Euclidean distance and other indices of similarity showed that our specimens were clustered with *L. zonatus* B.K. Cui and J. Song (Bray-curtis similarity = 0.925, Euclidean distance = 3.81). By comparing morphological characters, the specimens shared several characteristics with other *Laetiporus* spp., especially *L. zonatus* and as well as with the *L. xinjiangensis*, *L. versisporus* and *L. medogenesis* (Fig. 5). Resemblance was found particularly in color of pore surface (white cream), and pileal surface, pore size, contextual binding hyphae thickness, basidial length, basidiospore size, spore quotient (Q) value (1.2–1.5), distribution (temperate, high altitude forest), and host plants (associated with *Quercus* spp. only).

The morphological character-matrix revealed that all of the described species exhibited a high degree of morphological similarity (Supplementary Material S1). The largest basidiocarps were observed in the *L. cremeiporus*, *L. sulphureus*, and *L. persicinus* (length > 25 cm). All species were flabelliform, dimidiate shaped except for *L. versisporus*, *L. discolour* var. *pallidus*, *L. persincinus*, which appeared in rosette or singly. *Laetiporus cincinnatus* and *L. persincinus* were observed to produce centrally or excentrically stipitate basidiocarps, while the other species were sessile to laterally substipitate. Similarly, *L. persincinus* and *L. cincinnatus* also had hyphal extension or tomentose pileal surfaces, whereas the other species were glabrous. *Polyporus rubricus* Berk. [syn. *L. sulphureus*], *L. discolor* var. *diffluens*, and *L. miniatus* had an azonate surface, while the other species exhibited some degree of zonation. *Laetiporus persincinus* and *P. rubricus* had a brown pileal surface, while *L. versisporus*, *L. discolour* var. *pallidus*, *L. discolor* var. *brunnescens* had a white surface, and *L. medogensis* and *L. xinjiangensis* had a pink-buff or pale-buff pileal surface. The remaining species had pileal surfaces in shades of yellow, orange, ochraceous, sulfur buff, and cream. Further, the pore surface of *L. zonatus* and *L. persincinus* was white cream-colored, while the other species had pore surfaces in shades of sulfur, yellow, orange, ochraceous, and pale when fresh.

The morphological analysis further revealed that *L. zonatus* and *L. xinjiangensis* produced the largest pores, measuring up to 3 pores mm^{-1}, while smaller pores were recorded for *L. cincinnatus*, *L. sulphureus* (E), *L. ailaoshanensis*, *L. caribensis*, and *L. versisporus*, with a measurement of up to 6 pores mm^{-1}. The smallest pore size, measuring up to 7 pores mm^{-1}, was recorded for *L. discolour*. The data matrix also showed that the color of the tube layer was different from the pore surface in two species, *L. cremeiporus* and *L. versisporus*, while the others were concolorous. The contextual binding hyphae thickness was higher than 15 µm in species such as *L. cremeiporus*, *L. montanus*, *L. discolour* var. pallidus, *L. discolor* var. *brunnescens*, *L. versisporus*, and *L. sulphureus* (E). The study observed that three species, *L. discolour* var. *pallidus*, *L. discolor* var. *diffluens*, and *P. miniatus*, had tubular-shaped basidia, while the others possessed clavate basidia. The morphological analysis revealed that *L. montanus*, *L. discolour* var. pallidus, *L. sulphureus* (E), *L. medogensis*, *L. xinjiangensis*, *L. persincinus*, and *L. zonatus* possessed basidia with lengths exceeding 20 µm, while the others were less than 20 µm in length. Furthermore, *L. huroniensis* had the highest spore quotient (Q = 1.5–2) among the studied species, while the quotients of *L. conifericola*, *L. cremeiporus*, *L. gilbertsonii* var. *gilbertsonii*, *L. gilbertsonii* var. *pallidus*, *L. montanus*, *L. sulphureus* (A), *L. sulphureus* (E), and *L. discolour* spp. ranged from Q = 1.2–>1.4.

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Table 1 - List of species specimens and accession of GenBank used in the phylogenetic analyses

Species	Voucher	Country	ITS	nLSU	RPB2	Reference
L. versisporus	Yuan 6319	China	KX354475	KX354503	KX354670	Song et al. 2018
L. versisporus	Cui 7882	China	KF951269	KF951323	KT894783	Song et al. 2014
L. versisporus	Dai 13160	China	KF951266	KF951320	KT894785	Song et al. 2014
L. ailaoshanensis	Dai 13567	China	KX354470	KX354498	KX354665	Song et al. 2018
L. ailaoshanensis	Dai 13256	China	KF951289	KF951317	KT894786	Song et al. 2018
L. ailaoshanensis	HKAS 52508	China	NR154610	NG059499	-	Song et al. 2014
L. zonatus	HKAS 71806	China	KF951284	KF951310	KT894796	Song et al. 2014
L. zonatus	Cui 10403	China	KF951282	KF951307	ON424779	Song et al. 2014
L. zonatus	Cui 10404	China	KF951283	KF951308	-	Song et al. 2014
L. conifericola	CA-8	USA	EU402575	EU402523	-	Lindner and Banik 2008
L. conifericola	JV 0709/81J	China	KF951292	KF951327	KX354683	Song et al. 2018
L. huroniensis	HMC-3	USA	EU402571	EU402540	-	Lindner and Banik 2008
L. huroniensis	MI-14	China	EU402573	EU402539	-	Song et al. 2018
L. sulphureus	Cui 12389	China	KR187106	KX354487	KX354653	Song et al. 2018
L. sulphureus	Dai 12154	China	KF951295	KF951302	KX354655	Song et al. 2014
L. cincinnatus	Dai 12811	China	KF951291	KF951304	KT894788	Song et al. 2014
L. cincinnatus	DA-37	USA	EU402557	EU402521	-	Lindner and Banik 2008
L. cremeiporus	Dai 10107	China	KF951281	KF951301	KX354650	Song et al. 2014
L. cremeiporus	Cui10991	China	KF951279	KF951298	KX354679	Song et al. 2014
L. gilbertsonii	JV 1109/31	China	KF951293	KF951306	KX354671	Song et al. 2014
L. gilbertsonii	TJV2000-101	USA	EU402553	EU402528	-	Lindner and Banik 2008
L. caribensis	PR6583	USA	JN684766	-	-	Lindner et al. 2012
L. caribensis	PR914	USA	JN684762	EU402526	-	Lindner et al. 2012
L. medogensis	Cui 12219	China	KX354472	KX354500	KX354667	Song and Cui 2017
L. medogensis	Cui 12240	China	KX354473	KX354501	KX354668	Song and Cui 2017
L. montanus	Dai 15888	China	KX354466	KX354494	KX354662	Song and Cui 2017
L. montanus	Cui 10011	China	KF951274	KF951315	KT894790	Song and Cui 2017
L. zonatus	MUBS80	Pakistan	OQ300108	OQ300110	OQ320751	This study
Wolfiporia dilatohypha	FP72162	USA	EU402556	EU402517	-	Lindner et al. 2012

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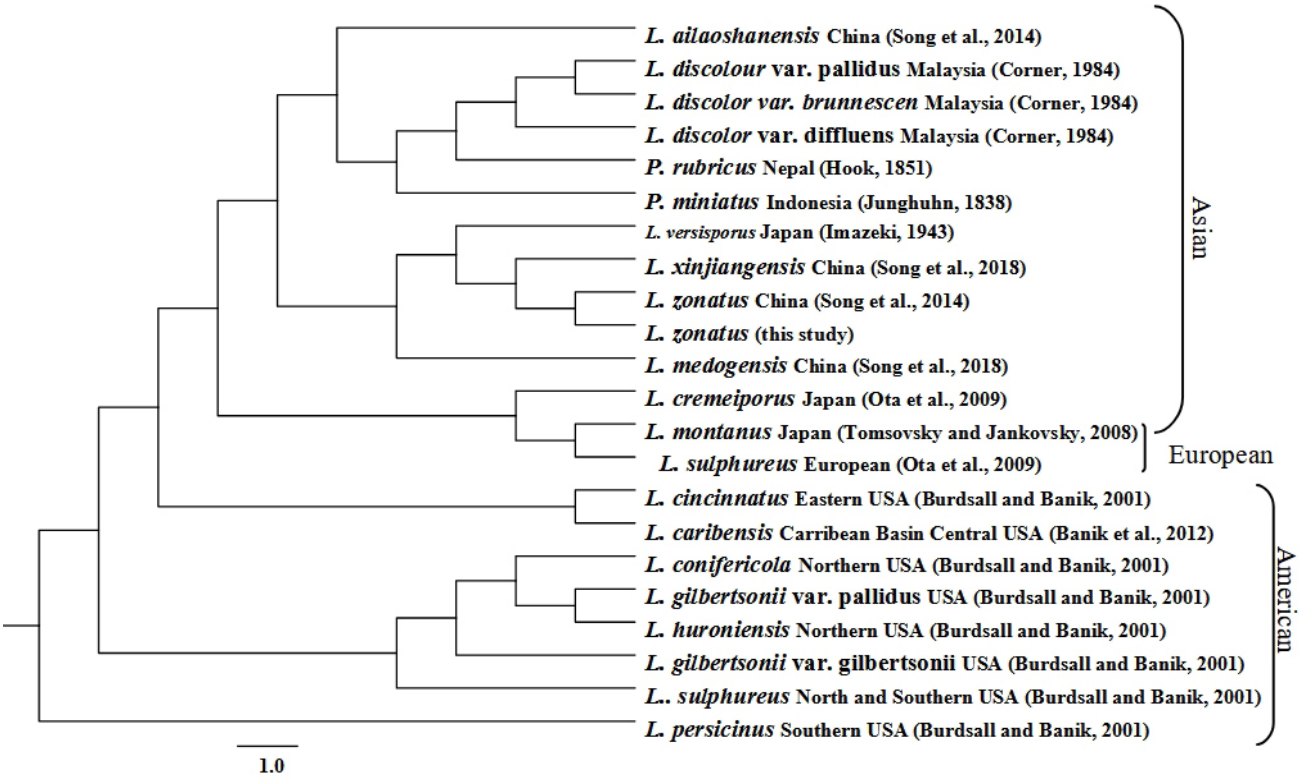

Fig. 5 - Phenetic cladogram calculated through the mean character difference method (characters = unweighted, gamma shape = 0.5 using NJ algorithm) using Paup4 showing relation between different species in *Laetiporus* based on the degree of similarity in their morphological inferred from the morphological data matrix created from original species description

Discussion

Taxonomically, the genus *Laetiporus* has been the subject of much revision in the past (Ota and Hattori, 2008; Ota et al., 2009; Banik et al., 2012; Pires et al. 2016; Song et al., 2018; Paez et al., 2022). Lack of distinctive morphological characters, many of those which are overlapping and confusing to hinder the classification of the genus (Lindner and Banik, 2011). In the current study we created replicable data set of the morphological characters based on the original descriptions to establish phenetic relationships among the species. The morphological character-matrix revealed a high degree of morphological similarity among the described species (Lindner and Banik, 2011). It seems reasonable that inference based on this morphological data set was found to be concordant to some extent with the molecular data. However, it was recognized that some of the species were showing minimal description or lacking some important morphological characters and was assigned as missing data. The morphoanatomical characteristics of three specimens of *L. zonatus* were found similar to majority of other species in the genus, with the highest level of similarity (Bray-Curtis similarity = 0.925) noted between *L. zonatus* from Southwestern China reported by Song et al. (2014). Additionally, this study has shown some interesting anamorphic characteristics not previously documented (Song et al., 2014).

In terms of host association, similar results were observed with those of the previous studies (Burdsall and Banik, 2001; Tomsovsky and Jankovsky, 2008; Ota et al., 2009). Unlike, *L. ailaoshanensis* recorded from subtropical *Lithocarpus* spp. (Fagaceae), *L. zonatus* in this study and Song et al. (2014) were collected from high-altitude temperate forests associated with *Quercus* spp.

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(Fagaceae). When considering basidiocarp size, pores size and spore dimension the *L. zonatus* is more closely related to the *L. xinjiangensis*, *L. versisporus* and *L. medogenesis* than to *L. conifericola*, and *L. montanus* (Song et al., 2014). However, comparing stipe attachment, context thickness, pore size, decurrent pores, fruiting behaviors *L. caribensis, L. cincinnatus, L. gilbertsonii, L. cremeiporus*, and *L. huroniensis* were distantly related and belong to the geographically different region (Burdsall and Banik, 2001; Ota et al., 2009; Banik et al., 2012). *L. persicinus* was found to be entirely different in many characteristics and greatly differ in ITS sequence (Burdsall and Banik, 2001).

The outcomes of the phylogenetic analysis were consistent with previous studies conducted by Lindner and Banik (2008), Ota et al. (2009), and Banik et al. (2010) and Song et al. (2014). The current analysis failed to properly resolve the species complex of *L. huroniensis* and *L. medogenesis*. Hence, further research is required to clarify the species complex in the genus as identified by Lindner and Banik (2008). The rest of the species were successfully assigned into their respective position in phylogenetic tree. The clade of *L. zonatus* was strongly supported by high level of bootstraps and Bayesian posterior probability values (MP 87.7 - ML 95 - BPP 0.97) and was determined to be distinct from other species. *L. versisporus* and *L. montanus* formed a strongly supported clade (MP 77.7 - ML 77 - BPP 0.9). The phylogenetic study showed that *L. zonatus* appear to be significantly different from other species, such as *L. versisporus*, *L. cremeiporus*, *L. caribensis*, *L. gilbertsonii*, *L. cincinnatus*, and *L. sulphureus*, which are otherwise well-supported clades.

ITS based sequence data is best to determine species delimitation in the genus *Laetiporus* (Banik et al., 2010). The genus is believed to possess distinct ecological, morphological and also several cryptic species (Burdsall and Banik, 2001; Ota et al., 2009). Many of which were identified from Chinese, Japanese and American collections by Tomsovsky and Jankovsky (2008), Ota et al. (2009) and Song et al. (2014). However, recent studies showed that there is a high degree of genetic diversity within the genus. Many populations showing significant genetic variation possibly due to intragenomic and intraspecies variation within the ITS region even at the level of local population. This issue presents a formidable challenge for correct species identification and phylogenetic analyses in the genus (Lindner and Banik, 2011). There is a significant amount of diversity that has yet to be described and resolved. To fully comprehend the relationships, it is crucial to conduct more in-depth studies that use a multiple phylogenetic approach and a larger sample size from a broader geographical area.

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