Phytotaxa

17

Magnolia Press, Auckland, New Zealand

Vol 317, No 3

18 August 2017

DOI: http://dx.doi.org/10.11646/phytotaxa.317.3

Open Access Subscription or Fee Access

Table of Contents

Article

ISSN 1179-3155 (print); ISSN 1179-3163 (online)

Published by Magnolia Press, Auckland, New Zealand

Phytotaxa 317 (3): 199–210
http://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.317.3.4

Heterobasidion amyloideopsis sp. nov. (Basidiomycota, Russulales) evidenced by morphological characteristics and phylogenetic analysis

CHANG-LIN ZHAO[1,2], MALKA SABA[2,3], ABDUL NASIR KHALID[3], JIE SONG[4] & DONALD H. PFISTER[2]*

[1] *Yunnan Academy of Biodiversity, Southwest Forestry University, Kunming, 650224, P.R. China*
[2]*Farlow Herbarium of Cryptogamic Botany, Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA*
[3]*Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan*
[4]*Institute of Microbiology, P.O. Box 61, Beijing Forestry University, Beijing 100083, P.R. China*
** Corresponding author's e-mail: dpfister@oeb.harvard.edu*

Abstract

Heterobasidion amyloideopsis sp. nov., a new poroid wood-inhabiting species from Pakistan, is introduced based on a combination of molecular evidence and morphological characteristics. We generated sequences from the nuclear internal transcribed spacer regions (ITS) and the large subunit ribosomal RNA gene (LSU), the gene encoding the largest subunit of RNA polymerase II (RPB1) and the second subunit of RNA polymerase II (RPB2), focusing on two specimens from Pakistan. We performed phylogenetic analyses with maximum likelihood, maximum parsimony, and bayesian inference methods on two datasets (RPB1+RPB2 and ITS+nLSU+RPB1+RPB2). Both analyses supported the existence of the new species and showed that it formed a monophyletic group within the *H. insulare* complex as a sister to *H. amyloideum*. In addition to assessing the origin and divergence of this new species, we focused on the RPB1+RPB2 dataset to perform maximum likelihood based estimation and Bayesian binary analyses. *Heterobasidion amyloideopsis* is characterized by an annual habit, pileate basidiomata with a rust colored pileal surface, white, obtuse margin, a dimitic hyphal system with simple septate generative hyphae in the trama and clamp connections present on the contextual hyphae, amyloid skeletal hyphae and broadly ellipsoid, hyaline, fairly thick-walled, and asperulate basidiospores.

Key words: Gene phylogenies, Himalayas, Taxonomy, Wood-inhabiting fungi

Introduction

Heterobasidion annosum (Fries 1821: 373) Brefeld (1888:154) is the type species of *Heterobasidion* Brefeld (1888). It is characterized by annual to perennial, resupinate to pileate basidiomata that are usually imbricate, leathery when young and fresh, and woody and hard when dry. The pileus is semicircular to fan-shaped. Pore surfaces are white to light cream and the pores are round to angular. The context is white to cream. The hyphal system is dimitic with mostly simple septa on generative hyphae with clamp connections in the context. The dextrinoid or amyloid skeletal hyphae predominate in the basidiomata. Basidiospores are broadly ellipsoid to globose, hyaline, thin to slightly thick-walled, asperulate and show no reaction in Melzer's reagent. Members of this genus cause a white rot (Gilbertson & Ryvarden 1986, Dai *et al.* 2007, Dai & Korhonen 2009, Tokuda *et al.* 2009, Otrosina & Garbelotto 2010, Chen *et al.* 2014, Ryvarden & Melo 2014).

Because some species are destructive forest pathogens (Woodward *et al.* 1998), *Heterobasidion* is one of the most intensively studied basidiomycetous genera. Korhonen & Stenlid (1998) mentioned that species are widely distributed and cause root and heart rot in approximately 200 species of primarily coniferous trees. The genus occurs in many areas and is an important ecological player involved in nutrient cycling, forest regeneration and forest succession (Garbelotto 2004).

In phylogenetic analyses of *Heterobasidion* species sequences of the nuclear internal transcribed spacer (ITS) and intergenic spacer (IGS) regions, manganese peroxidase genes and laccase genes have been used (Kasuga & Mitchelson 1993, Maijala *et al.* 2003, Asiegbu *et al.* 2004). Attempts to resolve the taxonomy of the *H. annosum* complex also used fragments of the nuclear genes calmodulin (CAM), elongation factor TEF1-α, glyceraldehyde 3-phosphate

Accepted by Samantha Karunarathna: 4 Aug. 2017; published: 18 Aug. 2017

dehydrogenase (GAPDH) and heat shock protein (HSP) (Johannesson & Stenlid 2003, Ota *et al.* 2006). Linzer *et al.* (2008) employed mitochondrial genes (mitochondrial ATP synthase subunit 6, ATP6) and mitochondrial rDNA regions to investigate this species complex. Studies of the *H. insulare* complex mainly focused on morphology and mating tests (Dai *et al.* 2002, Dai & Korhonen 2009, Tokuda *et al.* 2009). Recently, this genus was analyzed using the largest subunit of RNA polymerase II (RPB1) and the second subunit of RNA polymerase II (RPB2), these studies showed that RPB1 and RPB2 were more sensitive than other genes in distinguishing taxa of the *H. insulare* complex (Chen *et al.* 2014).

Thirteen species have been accepted in *Heterobasidion* worldwide (Brefeld 1888, Buchanan 1988, Niemelä & Korhonen 1998, Ota *et al.* 2006, Dai *et al.* 2007, Dai & Korhonen 2009, Tokuda *et al.* 2009, Otrosina & Garbelotto 2010, Chen *et al.* 2014). Five species are currently recognized in the *H. annosum* complex: *H. abietinum* Niemelä & Korhonen (1998:32), *H. annosum*, *H. parviporum* Niemelä & Korhonen (1998:31), *H. irregulare* Garbelotto & Otrosina (2010: 20) and *H. occidentale* Otrosina & Garbelotto (2010:20). Seven species are recognized in the *H. insulare* complex: *H. amyloideum* Y.C. Dai, Jia J. Chen & Korhonen (2014: 292); *H. australe* Y.C. Dai & Korhonen (Chen *et al.* 2014: 292); *H. ecrustosum* Tokuda, T. Hattori. & Y.C. Dai (2009: 196); *H. insulare* (Murrill 1908: 405) Ryvarden (1972: 237); *H. linzhiense* Y.C. Dai & Korhonen (Dai *et al.* 2007: 141); *H. orientale* Tokuda, T. Hattori & Y.C. Dai (Tokuda *et al.* 2009: 193); *H. tibeticum* Y.C. Dai, Jia J. Chen & Korhonen (Chen *et al.* 2014: 294) and *H. araucariae* P.K. Buchanan (1988:325). There is some debate about the status of two of these species. *Heterobasidion insulare* is incompletely known and *H. ecrustosum* may prove to be a synonym (Chen *et al.* 2015).

During a collecting trip in the western Himalayas, Pakistan, a fungus agreeing with the concept of *Heterobasidion* was found. To confirm the affinity of this species, morphological as well as phylogenetic analyses were carried out using nuclear ITS, LSU, and RPB1 and RPB2 genes.

Materials and methods

Morphological studies.—Specimens studied are deposited at the Farlow Herbarium, Harvard University (FH) and the Herbarium, University of the Punjab, Lahore, Pakistan (LAH). Macro-morphological descriptions are based on field observations. Color terms followed Petersen (1996). Microscopic measurements were made from slide preparations of dried specimens stained with Cotton Blue and Melzer's reagent by light microscopy following Dai (2010). Sections were examined using an Olympus BX40 compound microscope. In presenting spore size variation, the 5% of extremes of the measurements are given in parentheses. The following abbreviations are used: KOH = 5% potassium hydroxide, CB = Cotton Blue, CB+ = cyanophilous, IKI = Melzer's reagent, IKI– = both non-amyloid and non-dextrinoid, IKI+ = amyloid, L = indicates spore length (arithmetic average of all spores), W = indicates spore width (arithmetic average of all spores), Q = L/W ratio, n (a/b) = number of spores (a) measured from given number of specimens (b).

DNA extraction, amplification, sequencing and phylogenetic analyses.—Genomic DNA was extracted from specimens using the Qiagen DNeasy Plant Mini Kit (cat. no. 69104). 1/10 and 1/100 dilutions of genomic DNA were used for PCR amplification of the ITS, LSU, RPB1 and RPB2. The ITS region was amplified using primer pair ITS5 and ITS4 (White *et al.* 1990). The LSU region was amplified with primer pairs LR0R and LR7 (http://www.biology.duke.edu/fungi/ mycolab/primers.htm). The RPB1 gene was amplified with primer pair RPB1-Af and RPB1-Cf (Matheny *et al.* 2002). The RPB2 gene was amplified with primer pair fRPB2-5F and fRPB2-7cR (Liu *et al.* 1999, Matheny 2005). All PCR reactions were done in a Peltier Thermal cycler PTC-200 (MJ Research, Watertown, MA, USA) and with Econo *Taq* DNA Polymerase (Lucigen, Middleton, WI, USA). PCR amplification, purification, and sequencing followed Zhao *et al.* (2016). The PCR procedure for RPB1 and RPB2 followed Justo & Hibbett (2011) with slight modifications as follows: initial denaturation at 94 °C for 2 min, followed by 10 cycles at 94 °C for 40 s, 60 °C for 40 s, 72 °C for 2 min, then followed by 37 cycles at 94 °C for 45 s, 55 °C for 1.5 min and 72 °C for 2 min, and a final extension at 72 °C for 10 min. All newly generated sequences are deposited in GenBank (Table 1).

Sequencher 4.6 (GeneCodes, Ann Arbor, MI, USA) was used to edit the sequences. Sequences were aligned in MAFFT 6 (Katoh & Toh 2008; http://mafft.cbrc.jp/alignment/server/) using the "G-INS-I" strategy and manually adjusted in BioEdit (Hall 1999). The sequence alignment was deposited in TreeBase (submission ID 18117). RPB1+RPB2 sequences of *Trametes versicolor* (L.) Lloyd from GenBank were used as an outgroup to root the tree following Chen *et al.* (2014). ITS+LSU+RPB1+RPB2 sequences of *Heterobasidion annosum* and *H. parviporum*, obtained from GenBank, were used as outgroups to root the tree.

TABLE 1. A list of species, specimens and GenBank accession number of sequences used in this study.

Species name	Sample no.	Geographic origin	Host	GenBank accessions			
				ITS	LSU	RPB1	RPB2
Heterobasidion abietinum	00057/2	Trentino, Italy	*Abies alba*	KJ651453	KJ651511	KJ651632	KJ651725
Heterobasidion abietinum	00053/1	Trentino, Italy	*Picea abies*	KJ651451	KJ651509	KJ651630	KJ651723
Heterobasidion abietinum	00055/6	Trentino, Italy	*Abies alba*	KJ651452	KJ651510	KJ651631	KJ651724
Heterobasidion amyloideum	Li 1883	Tibet, China	Unknown	KJ651456	KJ651514	KF033156	KF006537
Heterobasidion amyloideum	Li 1878	Tibet, China	Unknown	KJ651455	KJ651513	KF033157	KF006538
Heterobasidion amyloideum	Li 1675	Tibet, China	Unknown	KJ651454	KJ651512	KF033155	KF006534
Heterobasidion amyloideopsis	M 8848	Mansehra, Pakistan	*Pinus wallichiana*	KT598384	KT598386	KT598390	KT598388
Heterobasidion amyloideopsis	M 118	Mansehra, Pakistan	*Pinus wallichiana*	KT598385	KT598387	KT598391	KT598389
Heterobasidion annosum	K 06071/1	Lazio, Italy	*Pinus pinea*	KJ651458	KJ651516	KF453491	KF453497
Heterobasidion annosum	K 06125/2	Krasnoyarsk, Russia	*Pinus sylvestris*	KJ651459	KJ651517	KF453492	KF453498
Heterobasidion annosum	K 06129/6	Krasnoyarsk, Russia	*Pinus sylvestris*	KJ583211	KJ583225	KF453493	KF453499
Heterobasidion araucariae	65008	Queensland, Australia	*Araucaria cunninghamii*	KJ651462	KJ651520	KJ651636	KJ651729
Heterobasidion araucariae	82001	Queensland, Australia	*Araucaria cunninghamii*	KJ651463	KJ651521	KJ651637	KJ651730
Heterobasidion australe	K 04164/3	Anhui, China	*Tsuga* sp.	KJ651464	KJ651522	KF033134	KF006500
Heterobasidion australe	K 04167/4	Zhejiang, China	*Pinus massoniana*	KJ651465	KJ651523	KF033135	KF006502
Heterobasidion australe	K 05172/3	Jiangxi, China	*Tsuga chinensis*	KJ651466	KJ651524	KF033136	KF006504
Heterobasidion australe	K 05175/2	Jiangxi, China	*Tsuga chinensis*	KJ651467	KJ651525	KF033137	KF006506
Heterobasidion ecrustosum	K 05766/7	Fujian, China	*Pinus massoniana*	KJ651469	KJ651527	KF033143	KF006514
Heterobasidion ecrustosum	K 05168/1	Fujian, China	*Pinus massoniana*	KJ651468	KJ651526	KF033142	KF006513
Heterobasidion ecrustosum	K 06123/3	Hubei, China	*Pinus* sp.	KJ651470	KJ651528	KF033144	KF006515
Heterobasidion ecrustosum	K 07103/2	Chongqing, China	*Pinus* sp.	KJ65147	KJ651529	KF033145	KF006517
Heterobasidion ecrustosum	K 08145/2	Hainan, China	Unknown	KJ651472	KJ651530	KF033146	KF006519
Heterobasidion irregulare	57001/VE	North Carolina, USA	*Pinus strobus*	KJ651473	KJ651531	KJ651638	KJ651731
Heterobasidion irregulare	88005	Vermont, USA	*Pinus* sp.	KJ651474	KJ651532	KJ651639	KJ651732
Heterobasidion irregulare	05025/VE	Anzio, Italy	*Pinus pinea*	KJ651478	KJ651536	KJ651643	KJ651736
Heterobasidion irregulare	88010/1	Vermont, USA	*Pinus* sp.	KJ651475	KJ651533	KJ651640	KJ651733
Heterobasidion linzhiense	Cui 7216	Sichuan, China	*Abies* sp.	KJ651480	KJ651538	KF033148	KF006524
Heterobasidion linzhiense	Cui 9645	Tibet, China	*Picea* sp.	KJ651481	KJ651539	KF033147	KF006523
Heterobasidion linzhiense	Cui 9695	Tibet, China	*Abies* sp.	KJ651482	KJ651540	KF033149	KF006525

...continued on the next page

TABLE 1. (Continued)

Species name	Sample no.	Geographic origin	Host	GenBank accessions			
				ITS	LSU	RPB1	RPB2
Heterobasidion linzhiense	Cui 9707	Tibet, China	*Pinus* sp.	KJ651483	KJ651541	KF033150	KF006526
Heterobasidion linzhiense	Dai 5408	Tibet, China	*Picea* sp.	KJ651484	KJ651542	KF033154	KF006533
Heterobasidion occidentale	98005/VE	Oregon, USA	*Picea engelmannii*	KJ651489	KJ651547	KJ651649	KJ651742
Heterobasidion occidentale	98003/1	Oregon, USA	*Picea engelmannii*	KJ651487	KJ651545	KJ651647	KJ651740
Heterobasidion occidentale	79034/VE	Oregon, USA	*Abies magnifica*	KJ651485	KJ651543	KJ651645	KJ651738
Heterobasidion orientale	K 97011/7	Jilin, China	*Abies* sp.	KJ651490	KJ651548	KF033141	KF006512
Heterobasidion orientale	K 03293/2	Liaoning, China	*Pinus tabulaeformis*	KJ651493	KJ651551	KF033140	KF006510
Heterobasidion orientale	K 00085/2	Heilongjiang, China	*Abies* sp.	KJ651492	KJ651550	KF033139	KF006508
Heterobasidion orientale	K 00082/6	Heilongjiang, China	Unknown	KJ651491	KJ651549	KF033138	KF006507
Heterobasidion parviporum	K 04121/3	Artjärvi, Finland	*Picea abies*	KJ583212	KJ583226	KF453493	KF453499
Heterobasidion parviporum	K 08021/7	Krasnoyarsk, Russia	*Picea abies*	KJ651498	KJ651556	KF453494	KF453500
Heterobasidion parviporum	K 08123VE	Irkutsk, Russia	*Picea abies*	KJ651500	KJ651558	KF453495	KF453501
Heterobasidion tibeticum	Dai 5468	Tibet, China	*Pinus* sp.	KJ651505	KJ651563	KF033151	KF006527
Heterobasidion tibeticum	Dai 5534	Tibet, China	*Pinus* sp.	KJ651506	KJ651564	KF033152	KF006529
Heterobasidion tibeticum	Dai 5537	Tibet, China	*Pinus* sp.	KJ651507	KJ651565	KF033153	KF006531
Trametes versicolor	Cui 9310	Tibet, China	*Platycladus* sp.	—	—	KF453496	KF453502

Most parsimonious phylogenies were inferred from the RPB1+RPB2 and the multi-gene dataset of ITS+nLSU+RPB1+RPB2. Their combinability was evaluated with the incongruence length difference (ILD) test (Farris *et al.* 1994) implemented in PAUP* 4.0b10 (Swofford 2002), under heuristic search and 1,000 homogeneity replicates giving a P value of 1.000, much greater than 0.01, which means there is no discrepancy among the RPB1+RPB2 and ITS+LSU+RPB1+RPB2 in reconstructing the phylogenetic trees. Approaches to phylogenetic analyses followed Zhao & Cui (2014) and the tree construction procedure was performed in PAUP* version 4.0b10 (Swofford 2002). All characters were equally weighted and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Max-trees were set to 5000, branches of zero length were collapsed and all parsimonious trees were saved. Clade robustness was assessed using a bootstrap (BT) analysis with 1,000 replicates (Felsenstein 1985). Descriptive tree statistics, tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for each Maximum Parsimonious Tree (MPT) generated. Sequences were also analyzed using Maximum Likelihood (ML) with RAxML-HPC2 with GTR+G+I model of site substitution including estimation of Gamma-distributed rate heterogeneity and a proportion of invariant sites on Abe through the CipresScience Gateway (www.phylo.org; Miller *et al.* 2009). Branch support for ML analysis was determined by 1000 bootstrap replicates (Hillis & Bull 1993).

MrModeltest2.3 (Posada & Crandall 1998, Nylander 2004) was used to determine the best-fit evolution model for each data set for Bayesian inference (BI). Bayesian inference was calculated with MrBayes3.1.2 with a general time reversible (GTR) model of DNA substitution and a gamma distribution rate variation across sites (Ronquist & Huelsenbeck 2003). Four Markov chains were run for 2 runs from random starting trees for 1 million generations for the [RPB1+RPB2] dataset and for 1 million generations for [ITS+nLSU+RPB1+RPB2]. Trees were sampled every 100th generation. The first quarter of the generations were by default discarded as burn-in. A majority rule consensus tree of all remaining trees was calculated. Branches that received bootstrap support for maximum likelihood (BS), maximum parsimony (MP) and Bayesian posterior probabilities (BPP) greater than or equal to 75 % (MP) and 0.95 (BPP) were considered as significantly supported.

Fossils are commonly used to estimate divergence times when reconstructing the evolutionary history of species (Dalman *et al*. 2010). The fossil *Paleopyrenomycites devonicus* Taylor, Hass, Kerp, Krings, & Hanlin (2005: 270) was used for calibration by Chen *et al.* (2015), who proposed that *Heterobasidion* was inferred to originate 19.72 Mya (11.82–28.33 Mya, 95% HPD; million years ago) by using the RPB1-RPB2 data. Based on this inferred divergence for *Heterobasidion* we applied a normal distribution by setting the mean and the standard deviation to 19.72 and 2.1, respectively. The combined RPB1+RPB2 datasets were used to assess the divergence and biogeography of *Heterobasidion* lineage listed in Table 1.

The BEAST 1.8.0 software package on Abe through the Cipres Science Gateway (www.phylo.org; Miller *et al.* 2009) was used to estimate divergence times. We first generated xml files executable in BEAST using BEAUti. RPB1 and RPB2 datasets set as two partitions, with substitution and molecular clock models unlinked while trees were linked. For both partitions, the GTR+G model was chosen as the best substitution model by MrModelTest and a relaxed lognormal model was employed for molecular clock analysis (Drummond *et al*. 2006). Tree prior was set to Yule speciation. The xml files were then executed in BEAST. For each analysis, two independent runs were conducted for 50 million generations. Log files of the two runs were combined using Log Combiner by setting the 10% logs as burn-ins and then analyzed in Tracer 1.5 (Rambaut & Drummond 2007). The resulting trees were also combined and then interpreted in TreeAnnotator and viewed in FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Molecular phylogeny

The RPB1+RPB2 dataset included sequences from 43 specimens representing 13 taxa. The dataset had an aligned length of 2534 characters, of which 1686 characters are constant, 529 parsimony-uninformative and 319 parsimony-informative. Two equally parsimonious trees (TL = 1134, CI = 0.843, HI = 0.157,RI = 0.916, RC = 0.772) were derived from the MP analysis. The best model for RPB1+RPB2 alignment estimated and applied in the BI was as follows: GTR+I+G, lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1). Bayesian analysis and ML analyses resulted in a topology similar to the MP analysis, with an average standard deviation of split frequencies = 0.003039. The phylogeny (Fig. 1) inferred from RPB1+RPB2 sequences demonstrated that the two specimens from Pakistan fell into the *H. insulare* complex and then formed a well-supported lineage (99% BS, 99% BP, 1.00 BPP) and grouped with *H. amyloideum*.

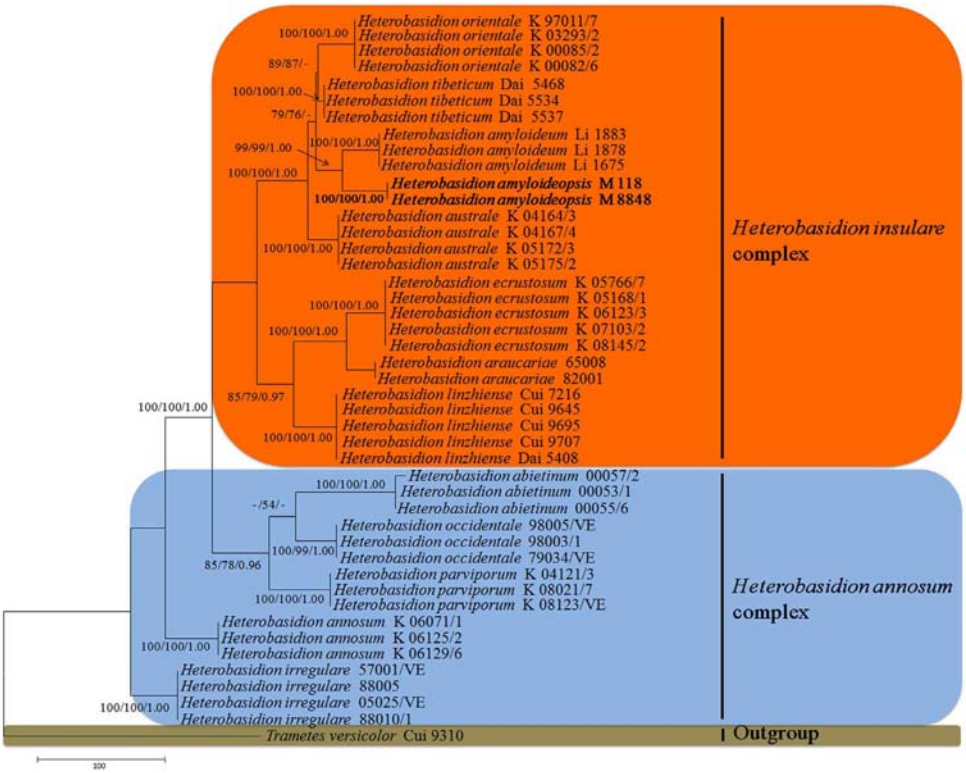

FIGURE 1. Maximum Parsimony strict consensus tree illustrating the position of *Heterobasidion amyloideopsis* and related species based on the combined *RPB1+RPB2* sequences. Branches are labeled with maximum likelihood bootstrap higher than 70%, parsimony bootstrap proportions higher than 50% and Bayesian posterior probabilities more than 0.95 respectively.

The four gene sequence dataset (ITS+LSU+RPB1+RPB2) did not show any conflicts in tree topology for the reciprocal bootstrap trees, P value much greater than 0.01, therefore, there is no discrepancy among the four loci in reconstructing phylogenetic trees. This allowed us to combine them. The combined dataset included sequences from 28 specimens representing 9 species. The dataset had an aligned length of 3784 characters, of which 3529 characters are constant, 58 are variable and parsimony-uninformative, and 197 are parsimony-informative. The maximum parsimony analysis yielded one equally parsimonious tree (TL = 319, CI = 0.862, HI = 0.138, RI = 0.958, RC = 0.826). The best model for the combined ITS+LSU+RPB1+RPB2 estimated and applied in the Bayesian analysis: GTR+I+G, lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1). The bayesian analysis and ML analyses resulted in a topology similar to the MP analysis, with an average standard deviation of split frequencies = 0.002007.

A phylogeny (Fig. 2) inferred from multiple genes of the combined ITS+LSU+RPB1+RPB2 sequences was obtained for additional representative taxa in *Heterobasidion insulare* complex and demonstrated that the undescribed species formed a monophyletic lineage with strong support (100% BS, 99% BP, 1.00 BPP) and then grouped with *H. amyloideum.*

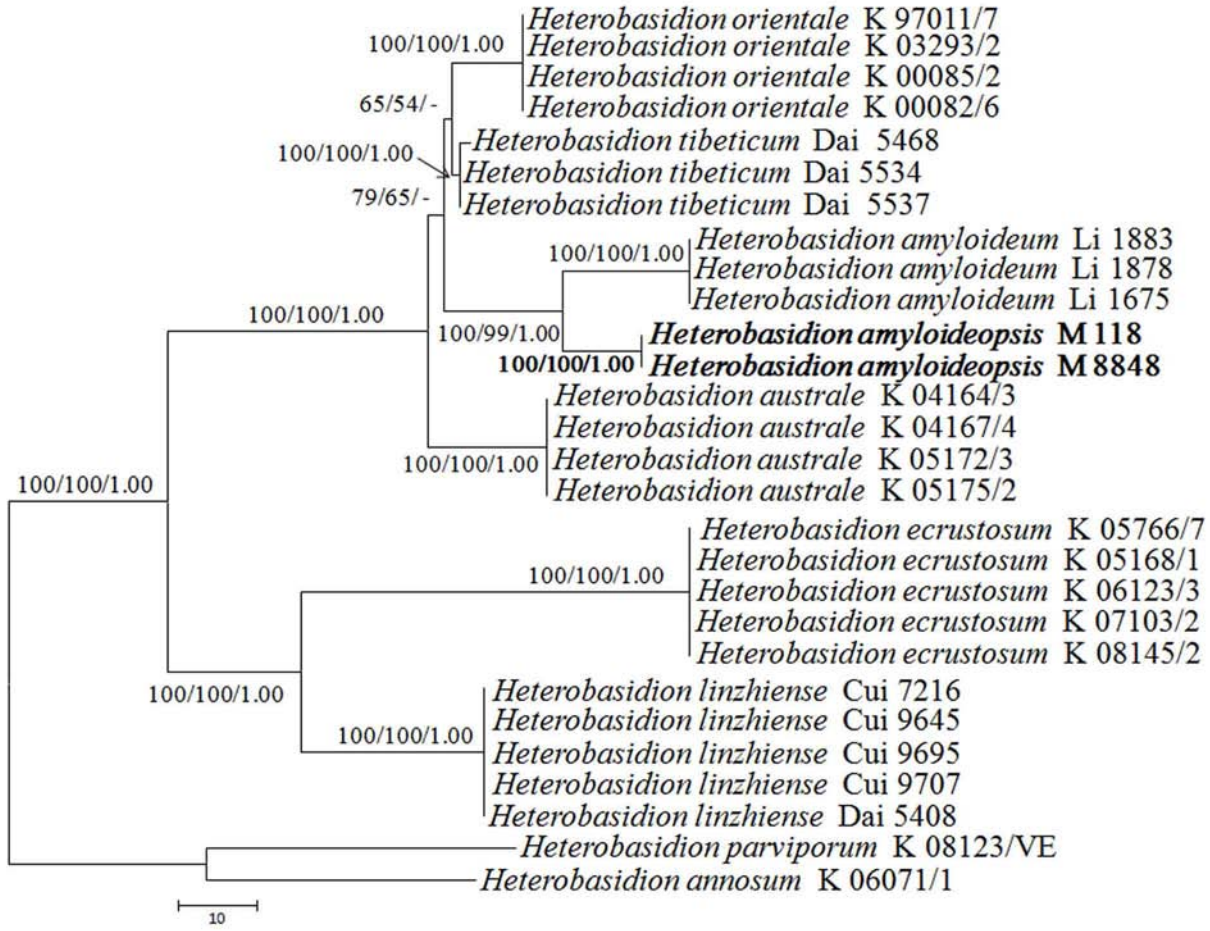

FIGURE 2. Maximum Parsimony strict consensus tree illustrating the position of *Heterobasidion amyloideopsis* and related species in the *H. insulare* complex based on the combined ITS+LSU+*RPB1*+*RPB2* sequences. Branches are labeled with maximum likelihood bootstrap higher than 70%, parsimony bootstrap proportions higher than 50% and Bayesian posterior probabilities more than 0.95 respectively.

Chronographic analysis using 19.72 Mya as the calibration point for the origin of *Heterobasidion* is shown in Fig. 3.The estimated divergence times for *Heterobasidion* spp. lineages were summarized in Table 2. The date indicated the divergence of two *Heterobasidion* species: *H. amyloideum* and *H. amyloideopsis* occurred 3.35 ± 1.22 Mya mainly during the Pliocene (1.27–5.87 Mya, 95% HPD).

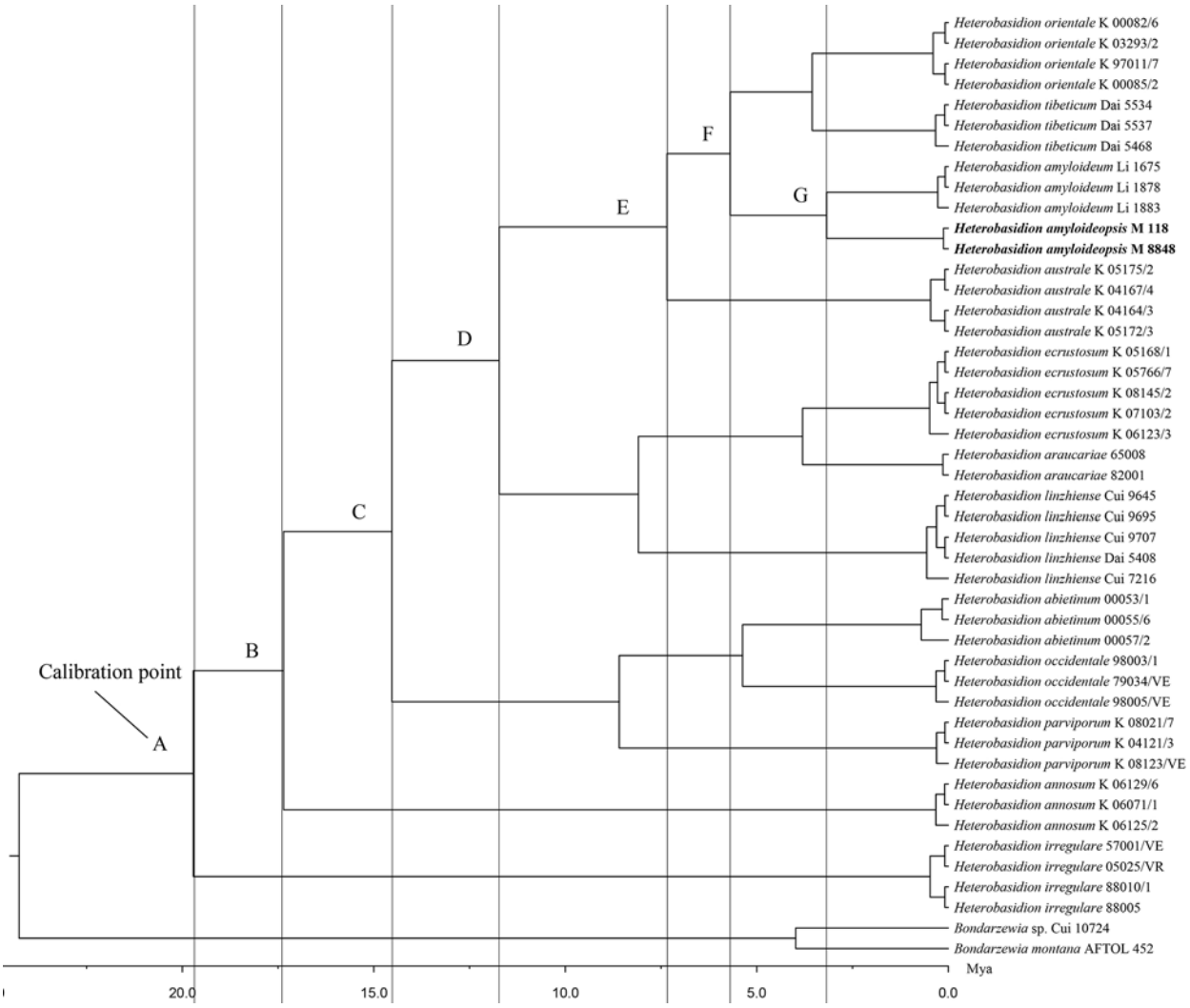

FIGURE 3. Chronogram and estimated divergence times of *Heterobasidion* spp. generated from molecular clock analysis using the *RPB1-RPB2* data. Chronogram obtained using the *Heterobasidion* divergence time of 19.72 Mya (Chen *et al*. 2015) as the calibration point is shown. The calibration point and objects of this study are marked in the chronogram. The geological time scale is in millions of years ago (Mya). Divergence times of *H. amyloideopsis* highlighted in bold.

TABLE 2. Estimated divergence times of each lineage.

Node	Mean ± standard error	95% HPD
*A: *Heterobasidion*	19.71 ± 0.21	19.31–20.13
B: II/III/IV/V/VI/VII/VIII/IX/X/XI/XII/XIII	17.02 ± 1.82	13.44–19.81
C: III/IV/V/VI/VII/VIII/IX/X/XI/XII/XIII	14.37 ± 1.97	10.57–18.06
D: *Heterobasidion insulare* complex	11.70 ± 1.96	7.98–15.60
E: IX/X/XI/XII/XIII	7.46 ± 1.82	4.06–11.09
F: X/XI/XII/XIII	5.84 ± 1.57	3.00–8.98
G: X/XI	3.35 ± 1.22	1.27–5.87

* Calibration point

I: *H. irregulare*; II: *H. annosum*; III: *H. parviporum*; IV: *H. occidentale*; V: *H. abietinum*; VI: *H. linzhiense*; VII: *H. araucariae*; VIII: *H. ecrustosum*; IX: *H. australe*; X: *H. amyloideopsis*; XI: *H. amyloideum*; XII: *H. tibeticum*; XIII: *H. orientale*

Taxonomy

Heterobasidion amyloideopsis M. Saba, C.L. Zhao, Khalid & Pfister, *sp. nov.* (Figs. 4, 5)

MycoBank no.: MB 821895

Diagnosis:—Basidiomata annual, pileate. Pileal surface rust, margin white, blunt. Hyphal system dimitic; generative hyphae simple septate in trama and clamp connections present in context; contextual skeletal hyphae amyloid. Basidiospores broadly ellipsoid, hyaline, fairly thick-walled, asperulate, IKI–, CB+.

Type:—PAKISTAN. Khyber Pakhtoonkhwa: Mansehra, Chattar Plain, elev. 520 m, on stump of *Pinus wallichiana* A. B. Jacks., 22 November 2013, *Malka Saba 8848* (holotype, FH!).

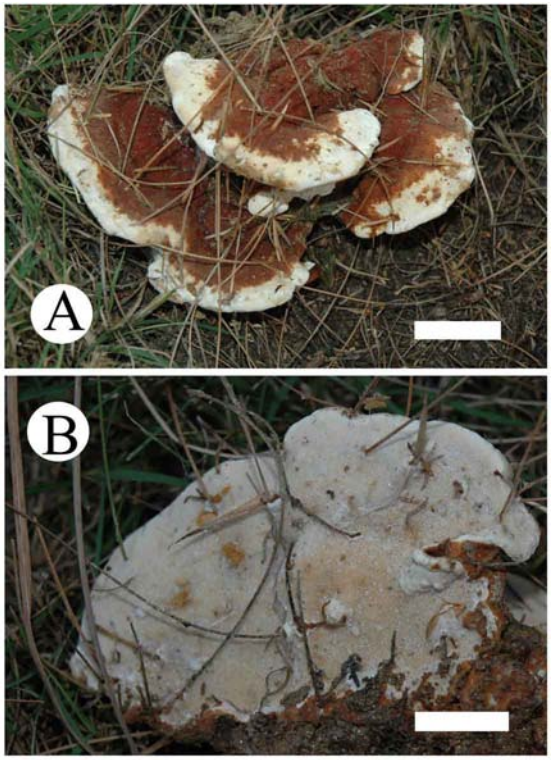

FIGURE 4. Basidioma of *Heterobasidion amyloideopsis* (holotype). Scale bars: 2 cm.

Etymology:—amyloideopsis (Lat.): referring to the similarity to *Heterobasidion amyloideum.*

Basidiomata annual, pileate, usually imbricate, leathery and without odor or taste when fresh, becoming woody and hard upon drying. *Pileus* semicircular to fan-shaped, projecting up to 3 cm, 6 cm wide, and 1 cm thick at base. *Pileal surface* rust when young, becoming deep rust to dark brown with age, crustose, azonate; margin white, blunt. *Pore surface* white when fresh, pinkish buff when dry, not shiny; pores round, 2–4 per mm; dissepiments thin, entire. *Context* white to cream, woody hard when dry, azonate, homogeneous up to 4 mm thick, without a thin black line under the pileus surface. *Tubes* cream, hard and corky, up to 6 mm long.

Hyphal structure dimitic; simple septate generative hyphae in trama, clamp connections present in contextual generative hyphae; tramal skeletal hyphae dextrinoid, CB+; contextual skeletal hyphae IKI+, CB+, hyphae unchanged in KOH (not dissolving).

Context generative hyphae frequently present, hyaline, thick-walled, with a wide lumen, occasional clamp connections and branching, 3.5–6 µm in diameter; skeletal hyphae dominant, hyaline, thick-walled with a wide to narrow lumen, unbranched, regularly arranged, 4.5–7 µm in diameter. Hyphae at crust mostly hyaline, thick-walled with distinct lumens.

Tube trama generative hyphae occasionally seen, hyaline, thin-walled, simple septate and unbranched, 3–5 µm in diam; skeletal hyphae dominant, hyaline, thick-walled with a wide to narrow lumen, unbranched, approximately parallel, 4–6 µm in diam. Cystidia not seen, but fusoid cystidioles present, hyaline, thin-walled, 10–12 × 2–3 µm; basidia short clavate to barrel-shaped, with a simple basal septum and four sterigmata, 13–15 × 5–6 µm; basidioles dominant, similar to basidia, but slightly smaller.

Basidiospores broadly ellipsoid, hyaline, fairly thick-walled, asperulate, IKI–, CB+, (4.7–)4.9–6.2(–6.6) × (3.7–)4–5.2(–5.4) µm, L = 5.6 µm, W = 4.6 µm, Q = 1.18–1.24 (n = 60/2).

Additional specimen examined:—PAKISTAN. Khyber Pakhtoonkhwa: Mansehra, Chattar Plain, elev. 520 m, on stump of *Pinus wallichiana*, 22 November 2013, *Malka Saba 118* (LAH!)

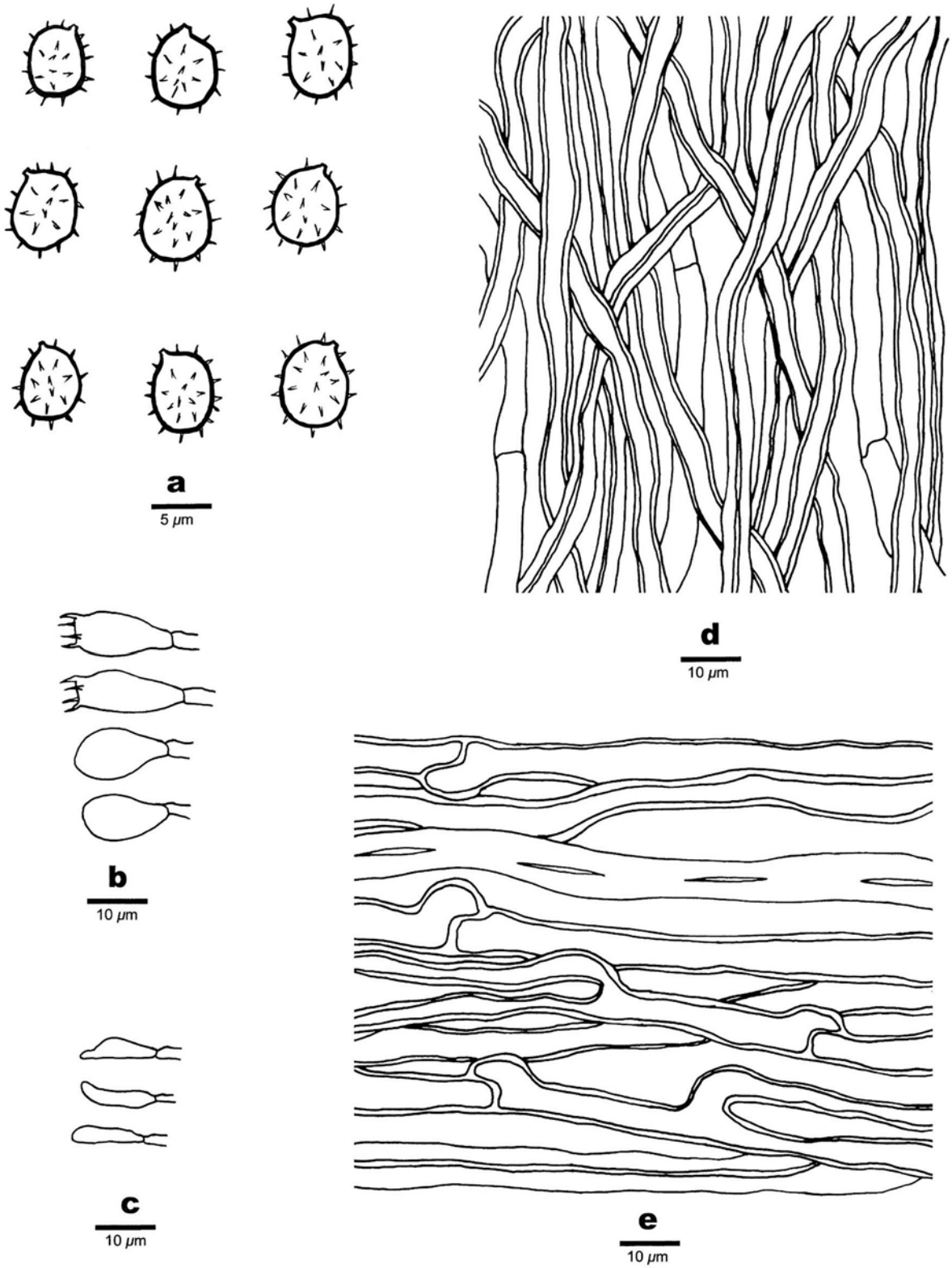

FIGURE 5. Microscopic structures of *Heterobasidion amyloideopsis* (drawn from the holotype). a. basidiospores. b. basidia and basidioles. c. cystidioles. d. hyphae from trama. e. hyphae from context. Bars: a–5 µm; b–e–10 µm.

Discussion

Phylogenetic analyses demonstrated that the two samples of *Heterobasidion amyloideopsis* form a monophyletic lineage with high support (Figs. 1, 2). It is most closely related to *H. amyloideum*. Morphologically, *H. amyloideum* differs from *H. amyloideopsis* in its cream to greyish pileal surface (reddish at base) and sharp margin and smaller pores (4–6 per mm), narrower, interwoven skeletal hyphae in the trama and presence of encrusted cystidia (Chen *et al.* 2014).

Morphologically, *Heterobasidion tibeticum* may be confused with *H. amyloideopsis* because of its pileate basidiomata with white to cream margin, a dimitic hyphal system with amyloid skeletal hyphae in the context and similar basidiospores (4.5–6×3.6–5.3 µm). However, *H. tibeticum* differs in having perennial basidiomata with relatively smaller pores (3–6 per mm) and simple septate generative hyphae in the context and by the presence of cystidia (Chen *et al.* 2014). Additionally, these two species are not closely related in the phylogenetic trees (Figs. 1, 2).

Stochastic changes in temperature and aridification in the Miocene may have led to population expansions and range contractions into small regions (refugia) (Yang 2005). Ota *et al.* (2006) proposed that the population of the *Heterobasidion insulare* complex migrated to more suitable refugia because of climate change. On the basis of the additional significant increases in the elevation of the Himalayan Mountains during the late Miocene (8–10Mya) the climate of Asia was significantly affected by these changes in the extent of the Tibetan Plateau and height of the Himalayan Mountains (Kutzbach *et al.* 1993). *Heterobasidion amyloideum* is mainly restricted to the temperate regions of East Asia and probably originated from the eastern Himalayas in the late Miocene during the global increase in the plant biomass (6–8 Mya, Cerling *et al.* 1997). *Heterobasidion amyloideopsis* was collected in the western Himalayas and our analyses indicate that *H. amyloideum* and *H. amyloideopsis* share the same origin and diverged 3.35 MYA. Thus, the divergence of these species may have been the result of climate change and changes in the uplift of the mountains in the region and plateau development.

Acknowledgements

The work was supported by a grant from the Higher Education Commission (HEC) Pakistan under indigenous PhD fellowships Program (Phase II, Batch I) and International Research Support Initiative Program.

References

Asiegbu, F.O., Abu, S., Stenlid, J. & Johansson, M. (2004) Sequence polymorphism and molecular characterization of laccase genes of the conifer pathogen *Heterobasidion annosum*. *Mycological research* 108: 136–148. https://doi.org/10.1017/S0953756203009183

Brefeld, O. (1888) Basidiomyceten III. Autobasidiomyceten. *Untersuchungen aus dem Gesammtgebiete der Mykologie* 8:1–184.

Buchanan, P.K. (1988) A new species of *Heterobasidion* (Polyporaceae) from Australasia. *Mycotaxon* 32: 325–337.

Cerling, T.E., Harris, J.M., MacFadden, B.J., Leakey, M.G., Quade, J., Eisenmann, V. & Ehleringer, J.R. (1997) Global vegetation change through the Miocene/Pliocene boundary. *Nature* 389: 153–158. https://doi.org/10.1038/38229

Chen, J.J., Korhonen, K., Li, W. & Dai, Y.C. (2014) Two new species of the *Heterobasidion insulare* complex based on morphology and molecular data. *Mycoscience* 55: 289–298. https://doi.org/10.1016/j.myc.2013.11.002

Chen, J.J., Cui, B.K., Zhou, L.W., Korhonen, K. & Dai, Y.C. (2015) Phylogeny, divergence time estimation, and biogeography of the genus *Heterobasidion* (Basidiomycota, Russulales). *Fungal Diversity* 71: 185–200. https://doi.org/10.1007/s13225-014-0317-2

Dai, Y.C. (2010) Hymenochaetaceae (Basidiomycota) in China. *Fungal Diversity* 45: 131–343. https://doi.org/10.1007/s13225-010-0066-9

Dai, Y.C., Yu, C.J. & Wang, H.C. (2007) Polypores from eastern Xizang (Tibet), western China. *Annales Botanici Fennici* 44: 135–145.

Dai, Y.C. & Korhonen, K. (2009) *Heterobasidion australe*, a new polypore derived from the *Heterobasidion insulare* complex. *Mycoscience*

50: 353–356.
https://doi.org/10.1007/S10267-009-0491-3

Dai, Y.C., Vainio, E.J., Hantula, J., Niemelä, T. & Korhonen, K. (2002) Sexuality and intersterility within the *Heterobasidion insulare* complex. *Mycological research* 106: 1435–1448.
https://doi.org/10.1017/S0953756202006950

Dalman, K., Olson, A. & Stenlid, J. (2010) Evolutionary history of the conifer root rot fungus *Heterobasidion annosum sensu lato*. *Molecular ecology* 19: 4979–4993.
https://doi.org/10.1111/j.1365-294X.2010.04873.x

Drummond, A.J., Ho, S.Y.W., Phillips, M.J. & Rambaut, A. (2006) Relaxed phylogenetics and dating with confidence. *PLoS Biology* 4: 88.
https://doi.org/10.1371/journal.pbio.0040088

Farris, J.S., Källersjö, M., Kluge, A.G. & Bult, C. (1994) Testing significance of incongruence. *Cladistics* 10: 315–319.
https://doi.org/10.1111/j.1096-0031.1994.tb00181.x

Felsenstein, J. (1985) Confidence intervals on phylogenetics: an approach using bootstrap. *Evolution* 39: 783–791.
https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Fries, E.M. (1821) *Sytema mycologicum*, vol. 1. Berlingiana, Lund, pp. i–lvii, 1–520.

Garbelotto, M. (2004) Root and butt rot diseases. *In*: Burley, J., Evans, J. & Youngquist, J.A. (Eds.) *The encyclopedia of forest sciences*. Elsevier, Oxford, pp. 750–758.

Gilbertson, R.L. & Ryvarden, L. (1986) *North American polypores 1. Abortiporus-Lindtneria.* Fungiflora, Oslo.

Hall, T.A. (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98.

Hillis, D.M. & Bull, J.J. (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* 42: 182–192.
https://doi.org/10.1093/sysbio/42.2.182

Johannesson, H. & Stenlid, J. (2003) Molecular markers reveal genetic isolation and phylogeography of the S and F intersterility group of the wood-decay fungus *Heterobasidion annosum*. *Molecular Phylogenetics and Evolution* 29: 94–101.
https://doi.org/10.1016/S1055-7903(03)00087-3

Justo, A. & Hibbett, D.S. (2011) Phylogenetic classification of *Trametes* (Basidiomycota, Polyporales) based on a five-marker dataset. *Taxon* 60: 1567–1583.

Kasuga, T. & Mitchelson, K. (1993) Determination of the DNA sequence of the 5.8S ribosomal gene of *Heterobasidion annosum* and *Heterobasidion araucariae*. *Nucleic Acids Research* 21: 1320.
https://doi.org/10.1093/nar/21.5.1320

Katoh, K. & Toh, H. (2008) Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* 9: 286–298.
https://doi.org/10.1093/bib/bbn013

Korhonen, K. & Stenlid, J. (1998) Biology of *Heterobasidion annosum*. *In:* Woodward, S., Stenlid, J., Karjalainen, R. & Hüttermann, A. (Eds.) *Heterobasidion annosum: biology, ecology, impact and control*. CAB International, Wallingford, pp. 43–70.
http://dx.doi.10.1086/648215

Kutzbach, J.E., Prell, W.L. & Ruddiman, W.F. (1993) Sensitivity of Eurasian climate to surface uplift of the Tibetan Plateau. *The Journal of Geology* 101: 177–190.
https://doi.org/10.1086/648215

Linzer, R.E., Otrosina, W.J., Gonthier, P., Bruhn, J., Laflamme, G., Bussières, G. & Garbelotto, M. (2008) Inferences on the phylogeography of the fungal pathogen *Heterobasidion annosum*, including evidence of interspecific horizontal genetic transfer and of human-mediated, long range dispersal. *Molecular Phylogenetics and Evolution* 46: 844–862.
https://doi.org/10.1016/j.ympev.2007.12.010

Liu, Y.L., Whelen, S. & Hall, B.D. (1999) Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* 16: 1799–1808.
https://doi.org/10.1093/oxfordjournals.molbev.a026092

Maijala, P., Harrington, T.C. & Raudaskoski, M. (2003) A peroxidase gene family and gene trees in *Heterobasidion* and related genera. *Mycologia* 95: 209–221.
https://doi.org/10.1080/15572536.2004.11833106

Matheny, P.B. (2005) Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*; Agaricales). *Molecular Phylogenetics and Evolution* 35: 1–20.
https://doi.org/10.1016/j.ympev.2004.11.014

Matheny, P.B., Liu, Y.J., Ammirati, J.F. & Hall, B.D. (2002) Using RPB1 sequences to improve phylogenetic inference among mushrooms (*Inocybe*, Agaricales). *American Journal of Botany* 89: 688–698.
https://doi.org/10.3732/ajb.89.4.688

Miller, M.A., Holder, M.T., Vos, R., Midford, P.E., Liebowitz, T., Chan, L., Hoover, P. & Warnow, T. (2009) The CIPRES Portals. CIPRES. Available from: http://www.phylo.org/sub_sections/portal (accessed 4 August 2009) [Archived by WebCite(r) at: http://www.webcitation.org/5imQlJeQa]

Murrill, W.A. (1908) Additional Philippine polypores. *Bulletin Torrey Botanical Club* 35: 391–416.
https://doi.org/10.2307/2479285

Niemelä, T. & Korhonen, K. (1998) Taxonomy of the genus *Heterobasidion*. *In*: Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A. (Eds.) *Heterobasidion annosum: biology, ecology, impact and control*. CAB International, Wallingford, pp. 27–33.

Nylander, J.A.A. (2004) *MrModeltest v2. Program distributed by the author*. Evolutionary Biology Centre, Uppsala University.

Ota, Y., Tokuda, S., Buchanan, P.K. & Hattori, T. (2006) Phylogenetic relationships of Japanese species of *Heterobasidion–H. annosum sensu lato* and an undetermined *Heterobasidion* sp. *Mycologia* 98: 717–725.
http://dx.doi.10.3852/mycologia.98.5.717

Otrosina, W.J. & Garbelotto, M. (2010) *Heterobasidion occidentale* sp. nov. and *Heterobasidion irregulare* nom. nov.: a disposition of North American *Heterobasidion* biological species. *Fungal Biology* 114: 16–25.
https://doi.org/10.1016/j.mycres.2009.09.001

Petersen, J.H. (1996) Farvekort. *The Danish Mycological Society´s colour-chart*. Foreningen til Svampekundskabens Fremme, Greve, 6 pp.

Posada, D. & Crandall, K.A. (1998) Modeltest: Testing the model of DNA substitution. *Bioinformatics* 14: 817–818.
https://doi.org/10.1093/bioinformatics/14.9.817

Rambaut, A. & Drummond, A.J. (2007) Tracer version v1.5. Available from: http://beast.bio.ed.ac.uk (accessed 1 August 2017)

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes3: bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.
https://doi.org/10.1093/bioinformatics/btg180

Ryvarden, L. (1972) A critical checklist of Polyporaceae in tropical East Africa. *Norwegian Journal of Botany* 19: 229–238.

Ryvarden, L. & Melo, I. (2014) Poroid fungi of Europe. *Synopsis Fungorum* 31: 1–455.

Swofford, D.L. (2002) *PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10*. Sinauer Associates, Massachusetts.

Taylor, N.T., Hass, H., Kerp, H., Krings, M. & Hanlin, R.T. (2005) Perithecial ascomycetes from the 400 million year old Rhynie chert: an example of ancestral polymorphism. *Mycologia* 96: 1403–1419.

Tokuda, S., Hattori, T., Dai, Y.C., Ota, Y. & Buchanan, P.K. (2009) Three species of *Heterobasidion* (Basidiomycota, Hericiales), *H. parviporum*, *H. orientale* sp. nov. and *H. ecrustosum* sp. nov. from East Asia. *Mycoscience* 50: 190–202.
https://doi.org/10.1007/S10267-008-0476-7

White, T.J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (Eds.) *PCR Protocols: A guide to methods and applications*. Academic Press, San Diego, pp. 315–322.
https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Woodward, S., Stenlid, J., Karjalainen, R. & Hüttermann, A. (1998) Preface. *In*: Woodward, S., Stenlid, J., Karjalainen, R. & Huttermann, A. (Eds.) *Heterobasidion annosum: biology, ecology, impact and control*. CAB International, Wallingford, pp. xi–xii.

Yang, Z.L. (2005) Diversity and biogeography of higher fungi in China. *In*: Xu, J.P. (Ed.) *Evolutionary genetics of fungi*. Horizon Bioscience, Norfolk, pp. 35–62.

Zhao, C.L. & Cui, B.K. (2014) Phylogeny and taxonomy of *Ceriporiopsis* (Polyporales) with descriptions of two new species from southern China. *Phytotaxa* 164: 17–28.
https://doi.org/10.11646/phytotaxa.164.1.2

Zhao, C.L., Xu, F. & Pfister, D.H. (2016) Morphological and molecular identification of a new species of *Truncospora* (Polyporales, Basidiomycota) in North America. *Phytotaxa* 257: 89–97.
https://doi.org/10.11646/phytotaxa.257.1.7