



On the taxonomic identity of a fungal morph used in traditional medicine in Kerala State, India

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Abstract

A hypogean fungus, referred to in the local Malayalam language as nilamanga (meaning hypogean mango) is often used by traditional healers and tribal people in Kerala State, India as a cure for an assortment of ailments. Taxonomic identity of this fungal morph has long intrigued mycologists starting from M. J. Berkeley who provisionally named it as *Sclerotium stipitatum* Berk. & Curr. in 1860. Its unique morphology and total lack of spores of any kind defied proper identification. Morphological examinations revealed that a nilamanga specimen that we obtained recently from Kerala was indistinguishable from Berkeley's *Sclerotium stipitatum* currently preserved at Kew herbarium. Molecular phylogenetic methods unequivocally proved that the nilamanga specimen was *Xylaria acuminatilongissima*, a termite associated species first reported from Taiwan. The hypogean origin of nilamanga specimens indicate that they could very well be growing on abandoned subterranean termite nests. The sterile structure can be considered as a morphologically variable, multihyphal aggregated sclerotial stage of the fungus that can remain dormant or quiescent when the environment is unfavourable.

Key words: medicinal mushroom, *Xylaria*, traditional medicine, nilamanga, *Sclerotium stipitatum*

Introduction

A very interesting hypogean fungus, referred to in the local Malayalam language as nilamanga (meaning hypogean mango) and in Tamil as puttumanga (meaning mango of the termitorium) is often used by traditional healers and tribal people in Kerala State, India as a cure for an assortment of ailments especially stomach ailments (Shortt 1867, Balakrishnan & Kumar 2001). It is obtained by chance while digging soil for some other purposes and remarkably, is often obtained from the foundations of old houses when they are dismantled and this feature further added to the mystery surrounding this fungus. The identity of this remarkable medicinal fungus has long intrigued mycologists starting from Berkeley (1860) who examined the material transmitted to him from the former Travancore (southern Kerala) and provisionally named it as *Sclerotium stipitatum* Berk. & Curr. (1860: 93). Currey & Hanbury (1860) and Cooke (1888) supported Berkeley's view that it could be sclerotial stage of some fungus. Dennis (1961) and Rogers *et al.* (2005) indicated that it could be a *Xylaria*. The reason for the inability to identify its exact taxonomic position was the total lack of spores of any kind. Moreover, the sterile morph has a unique morphology quite unlike that of most commonly encountered fungi. Nilamanga specimens are often almost spherical and of the size of a table tennis ball with a smooth blackish rind and a whitish, somewhat hard, homogeneous interior. Owing to this morphology and the hypogean occurrence, they often recall some tuberous roots.

Recently, we obtained specimens of this material from a local healer and also Berkeley's (1860) material (*Sclerotium stipitatum*, holotype: K(M) 125991) on loan from Kew Herbarium. The results of our studies on these materials are presented here.

Materials and Methods

Morphological studies:—Both locally gathered specimens of nilamanga and the type material of Berkeley's (1860) *Sclerotium stipitatum* originally collected from Kerala more than 150 years back and currently preserved at the Kew (Mycology) herbarium were examined in this study. The local material was obtained from a local healer who in turn obtained it from the ruins of an old mansion at Pattambi, Palakkad District, Kerala State, India. Conventional morphology-based taxonomic methods were employed. Cross sections of the stromata were made to examine the stromal interior. Microscopic observations were made on material stained with 1% aqueous solutions of phloxine and Congo red and mounted in 3% aqueous KOH. The examined collections are in Kew (Mycology) Herbarium and the Kew accession numbers (K(M) 191670) are indicated.

Molecular data:—The partial sequence of the ITS region (ITS1, 5.8S gene, ITS2) was analysed in this study. Genomic DNA was extracted from the dried specimen of the recent Kerala collection employing the protocol described by Izumitsu *et al.* (2012). PCR reactions were performed using primers ITS1 and ITS4 (White *et al.* 1990). The amplification reaction mixture (final volume 30 μ L) contained the following: 15 μ L of EmeraldAmp GT PCR Master Mix, 6 μ L of ddH₂O, 3 μ L of 10 μ M primers (ITS1 and ITS4) and 3 μ L of template DNA. PCR reaction was performed in a GeneAtlas™ Thermal cycler (Astec, Fukuoka, Japan). Thermal profile of PCR was 2' 95°C, 1' 50°C, 1' 72°C; 34 cycles of 30" 94°C, 1' 50°C, 1' 72°C, and a final extension step of 10' 72°C. The PCR product was examined on 1.0% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator. Amplified PCR product was purified using column purification (GeneJet™ PCR purification kit, Thermo Fisher Scientific, Mumbai, India) as per manufacturer's guidelines and was subjected to automated DNA sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, Foster city, California, USA) using the same primers used for PCR. The generated sequence was edited manually using BioEdit sequence alignment editor version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA). The edited sequence was then used separately for BLAST search in the GenBank database (www.ncbi.nlm.nih.gov). The newly generated sequence was deposited in GenBank. As we were not allowed to extract DNA from the holotype of *Sclerotium stipitatum*, it was not included in the phylogenetic analysis.

Phylogenetic analyses:—The newly generated ITS sequence (KP067787; 506 bp) of the present Kerala collection along with those retrieved from GenBank (22 ITS sequences) were aligned using MUSCLE version 3.8.31 (Edgar 2004). A final set of ITS sequences of 23 taxa including the outgroup were aligned (Tab. 1). All sequences were trimmed at the ends to a final length of 438 bp. The sequences of *Xylaria* species from GenBank were selected based on their similarity indices with 0 e-value and >98% sequence identity. The representative sequences of termite associated species of *Xylaria* as well as sequences of other important *Xylaria* species (Hsieh *et al.* 2010, Stadler *et al.* 2014, Visser *et al.* 2009) were also added to the dataset. *Biscogniauxia mediterranea* (De Not. 1851: 96) Kuntze (1891: 398) was selected as outgroup taxon for rooting purpose following Hsieh *et al.* (2010). Maximum Parsimony (MP) analysis was conducted using MEGA version 5 (Tamura *et al.* 2011), with 500 heuristic bootstrap replicates, using random stepwise addition, and holding one tree at each step. Maxtrees were set to 100. TBR branch swapping algorithm was used to assess branch support. Additionally a Maximum Likelihood (ML) analysis was performed using MEGA version 5 with 1000 bootstrap replicates, a Kimura 2-parameter model with gamma distributed rate variation option and the NNI (Nearest-Neighbor-Interchange) heuristic method (Tamura *et al.* 2011). All characters were treated as unordered and gaps were treated as missing data. The aligned sequence data matrix has been deposited in TreeBase (<http://purl.org/phylo/treebase/phyloids/study/TB2:S16743>).

TABLE 1. Species, geographical origin, voucher specimens and GenBank accession numbers of DNA (ITS) sequences used in the molecular analysis. Type specimens are indicated by bold font. Asterisk indicates the sequence of the fungal morph (nilamanga) recently collected from Kerala.

Species	Collection data	GenBank accession no. ITS
<i>Biscogniauxia mediterranea</i> (De Not.) Kuntze	FRANCE. Oloron, Foret de Bugangue, corticated wood of <i>Fagus</i> , 23 April 1995, Candoussau 366 (JDR), Culture accession No.: BCRC34037 (Ju <i>et al.</i> 1998, Hsieh <i>et al.</i> 2005, Hsieh <i>et al.</i> 2010)	EF026134
<i>Xylaria acuminatilongissima</i> Y.M. Ju & H.M. Hsieh	TAIWAN. Tainan Co., Hsinshih, Tan-ting, on ground of bamboo plantation, 8 May–12 July 2006, Chou, K.-H. 95060506 (HOLOTYPE HAST), Culture accession No.: BCRC34211 (Ju & Hsieh 2007)	EU178738

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TABLE 1. (Countined)

Species	Collection data	GenBank accession no. ITS
<i>Xylaria acuminatilongissima</i> Y.M. Ju & H.M. Hsieh*	INDIA. Kerala State, hypogaeal, K(M) 191670 (K(M))	KP067787
<i>X. adscendens</i> (Fr.) Fr.	THAILAND. Nakorn Nayok, Wangtakrai, on wood, 7 July 1996, Bandoni, R. J., Bandoni, A. A. & Flegel, T. W. 12017 (JDR) (Hsieh <i>et al.</i> 2010)	GU322432
<i>X. allantoidea</i> (Berk.) Fr.	TAIWAN. I-lan Co., Yuan-shan, Fu-shan, on trunk, 29 April 2005, Ju, Y.-M. & Hsieh, H.-M. 94042903 (HAST) (Hsieh <i>et al.</i> 2010)	GU324743
<i>X. atrodivaricata</i> Y.M. Ju & H.M. Hsieh	TAIWAN. Tainan Co., Hsin-shih, Tan-ting, on ground of mango orchard, 20 May 2006, Ju, Y.-M. & Hsieh, H.-M. 95052001 (HOLOTYPE HAST), Culture accession No.: BCRC34212 (Ju & Hsieh 2007)	EU178739
<i>X. cirrata</i> Pat.	TAIWAN. Tainan Co., Hsinshih, Tan-ting, on ground of vegetable farm, 4 May 2006, Chou, K.-H. 95050402 (EPITYPE HAST), Culture accession No.: BCRC34214 (Ju & Hsieh 2007)	EU179863
<i>X. cubensis</i> (Mont.) Fr.	USA. North Carolina, Great Smoky Mountains National Park, Big Creek, on wood, 9 September 2005, Rogers, J. D. (JDR) (Hsieh <i>et al.</i> 2010)	GU991523
<i>X. culleniae</i> Berk. & Broome	THAILAND. On pod, Whalley, M. F. NH9 (JDR) (Hsieh <i>et al.</i> 2010)	GU322442
<i>X. digitata</i> (L.) Grev.	UKRAINE. Kharkov District, Zmiev Area, National Nature Park “Gomolshanskie Lesa”, vicinity of Velicaya Gomolsha Village, on wood, 13 July 2007, Prilutsky, O. (CWU [Myc] AS2438, HAST) (Hsieh <i>et al.</i> 2010)	GU322456
<i>X. escharoidea</i> (Berk.) Sacc.	TAIWAN. Tainan Co., Shen-hua, I-min-liao, on ground of mango orchard, 18 July 2006, Chou, K.-H. 95071801 (EPITYPE HAST), Culture accession No.: BCRC34215 (Ju & Hsieh 2007)	EU179864
<i>X. feejeensis</i> (Berk.) Fr.	TAIWAN. Ping-tung Co., Heng-chun, Ken-ting, on bark, 20 September 2003, Ju, Y.-M. & Hsieh, H.-M. 92092013 (HAST) (Hsieh <i>et al.</i> 2010)	GU322454
<i>X. grammica</i> (Mont.) Mont.	TAIWAN. Nan-tou Co., Lu-ku, Shi-tou, on wood, September 2005, Chen, G.-T. (HAST) (Hsieh <i>et al.</i> 2010)	GU300097
<i>X. griseosepiacea</i> Y.M. Ju & H.M. Hsieh	TAIWAN. Tainan Co., Hsinshih, Tan-ting, on ground of mango orchard, 20 May 2006, Ju, Y.-M. & Hsieh, H.-M. 95052008 (HOLOTYPE HAST), Culture accession No.: BCRC34216 (Ju & Hsieh 2007)	EU179865
<i>X. hypoxylon</i> (L.) Grev.	SWEDEN. Uppland. Stockholm, Va'llingby. Grimsta Nature Reserve near Lake Ma'laren, <i>Sorbus aucuparia</i> , 21 October 2007, leg. A.-L. & A. Anderberg G07-1 (S-F72430). Culture accession No.: STMA 07069 (also deposited as CBS 122620) (Peršoh <i>et al.</i> 2009)	AM993141
<i>X. ianthinovelutina</i> (Mont.) Mont.	FRENCH WEST INDIES. Martinique, Forêt de Montravail, on fallen fruit of <i>Swietenia macrophylla</i> , 7 December 2005, Lechat, C. CLL5599 (HAST, JF) (Hsieh <i>et al.</i> 2010)	GU322441
<i>X. multiplex</i> (Kunze) Fr.	USA. Hawaii, Kole Kole Beach Park, on wood of <i>Hibiscus tiliaceus</i> , 8 September 1989, Hemmes, D. E. Xy-7 (JDR) (Hsieh <i>et al.</i> 2010)	GU300099
<i>X. nigripes</i> (Klotzsch) Cooke	TAIWAN. Tainan Co., Shen-hua, I-min-liao, on ground of mango orchard, 30 May–28 June 2005, Chou, K.-H. 94053001 (HAST), Culture accession No.: BCRC34219 (Ju & Hsieh 2007)	GU324755

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TABLE 1. (Countined)

Species	Collection data	GenBank accession no. ITS
<i>X. ochraceostroma</i> Y.M. Ju & H.M. Hsieh	TAIWAN. Tainan Co., Shenhua, I-min-liao, on ground of mango orchard, 31 July 2004, Chou, K.-H. 93073101 (HOLOTYPE HAST), Culture accession No.: BCRC34220 (Ju & Hsieh 2007)	EU179869
<i>X. polymorpha</i> (Pers.) Grev.	USA. West Virginia, Tucker Co., Blackwater Falls State Park, on wood, 26 August 2008, Rogers, J. D. (HAST, JDR) (Hsieh <i>et al.</i> 2010)	GU322460
<i>X. regalis</i> Cooke	INDIA. Maharashtra, Western Ghats, Pune, on log of <i>Ficus racemosa</i> , 22 October 2007, Gailawad, S. AMH 9204 (HAST) (Hsieh <i>et al.</i> 2010)	GU324745
<i>X. scruposa</i> (Fr.) Fr.	FRENCH WEST INDIES. Martinique, Prêcheur, Anse Couleuvre, on dead wood, 19 August 2005, Lechat, C. CLL5025 (HAST, JF) (Hsieh <i>et al.</i> 2010)	GU322458
Xylariaceae sp.	SOUTH AFRICA. Pretoria University campus, fungus comb from fungus-growing termite nest, 518.F8 (Visser <i>et al.</i> 2009)	FJ425673

JDR refers to the personal herbarium of Dr. Jack D. Rogers, Pullman, WA, USA; JF: Jonkershoek Forestry Research Centre, Western Cape Province, Stellenbosch, South Africa; HAST: Biodiversity Research Center, Academia Sinica, Taipei, Taiwan; BCRC: Bioresource Collection and Research Center, Hsin-chu, Taiwan; CBS: Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.

Results

Morphological observations:—The results of our morphological examination of ‘*Sclerotium stipitatum*’ and the nilamanga material recently collected from Kerala are given below:

‘*Sclerotium stipitatum*’ Berk. & Curr. (1860: 93) (Fig. 1. C–D)

The material based on which Berkeley proposed *Sclerotium stipitatum* and which is currently preserved in Kew (Mycology) Herbarium consists of five dried stromata glued on paper and is in excellent condition. Three of these stromata are rather fusoid and reminiscent of *Xylaria* stromata while the remaining two are rather subglobose, all rather hard, unbranched, with a black, wrinkled, somewhat glabrous surface lacking perithecial mounds, covered with a thick, black, rather carbonous outer skin; interior solid, cream-colored, soft, sterile, composed of thick-walled, interwoven hyphae with a narrow lumen. Perithecia, asci, ascospores, and paraphyses not observed.

Specimen examined:—“INDIA, Travencore, in the ground, Date: 1860, Coll. Dr E, J, Waring (dedit D. Wamburg)”, K(M) 125991, holotype.

The nilamanga specimen recently collected from Kerala (Fig. 1. A–B) showed the following features: Stroma hypogaeal, rather short, 30 × 36 mm; globose, with a pedicel-like appendage up to 50 mm long, rather hard, unbranched, with a black, wrinkled, somewhat glabrous surface lacking perithecial mounds, covered with a thick, black, rather carbonous outer skin; interior solid throughout, cream-colored, soft, sterile, composed of thick-walled, interwoven hyphae with a narrow lumen. Perithecia, asci, ascospores, and paraphyses not observed.

Specimen examined:—INDIA. Kerala State, Palakkad District, Pattambi: obtained from the mud foundation of an old, dismantled building (date of collection and name of collector not recorded), K(M) 191670.

As can be seen from the descriptions given above, morphologically the present collection of nilamanga is indistinguishable from ‘*Sclerotium stipitatum*’ as both have stromata with similar color and texture, a thick, black, rather carbonous outer skin and a cream-colored, soft and solid stromatal interior composed of sterile, thick-walled, interwoven hyphae with a narrow lumen. Also, both are sterile lacking perithecia, asci, ascospores, and paraphyses.

Phylogenetic analysis:—Based on a megablast search of NCBI's GenBank nucleotide database, the closest hits using the ITS sequence were *Xylaria acuminatilongissima* Y.M. Ju & H.M. Hsieh (2007: 938) (GenBank EU178738; Identities = 436/440 (99 %), Gaps = 0/440 (0 %)) made from termite nests, Tainan County, Taiwan and a collection labelled as ‘Xylariaceae species’ (GenBank FJ425673; Identities = 389/390 (99 %), Gaps = 0/390 (0 %)) made from termite nests, University of Pretoria campus, South Africa (Visser *et al.* 2009). Subsequent analysis yielded a phylogenetic tree that showed the placement of the fungal morph (nilamanga) collected from Kerala.

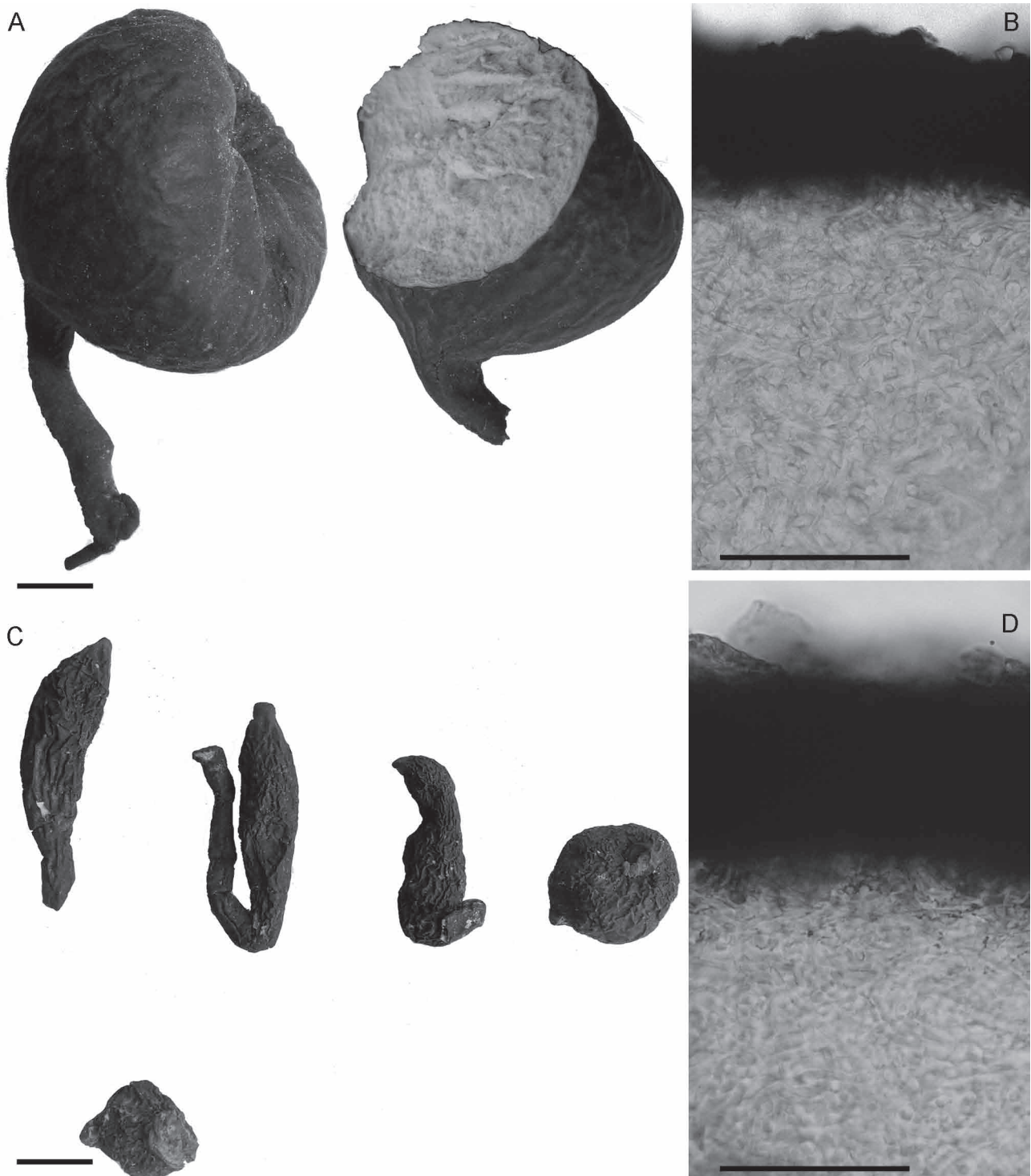


FIGURE 1. A, B: The fungal morph (nilamanga) recently collected from Kerala; A. Stromata; B. Section showing the stromal interior. C, D: *Sclerotium stipitatum*; C. Holotype (K(M) 125991); D. Section showing the stromal interior. Scale bars: A, C=10 mm; B, D=10 µm.

The Maximum Parsimony (MP) analysis resulted in a single tree (Fig. 2) that revealed a monophyletic clade comprising the present Kerala collection of nilamanga and eight species of termite associated *Xylaria*. Within this clade, the present Kerala collection of nilamanga, the ‘Xylariaceae species’ and *X. acuminatilongissima* formed a distinct clade with 100% bootstrap support. The Maximum likelihood (ML) analysis (Fig. 3) also supported the placement of the present Kerala collection of nilamanga within the monophyletic clade of termite-associated *Xylaria* species. In ML analysis, the present Kerala collection of nilamanga, the ‘Xylariaceae species’ and *X. acuminatilongissima* formed a discrete clade with 98% bootstrap support. *Xylaria acuminatilongissima* from Taiwan, the South African collection ‘Xylariaceae species’ and the present Kerala collection showed 99% sequence similarity on pair wise alignment and therefore these three collections seems to be conspecific. In both phylogenetic trees, *Xylaria nigripes* (Klotzsch 1832:

203) Cooke (1883: 89) formed a clade sister to the nilamanga clade. Also, in both analyses, *X. escharoidea* (Berk. 1843: 385) Fr. (1851: 128) showed no close relation to the present Kerala collection of nilamanga.

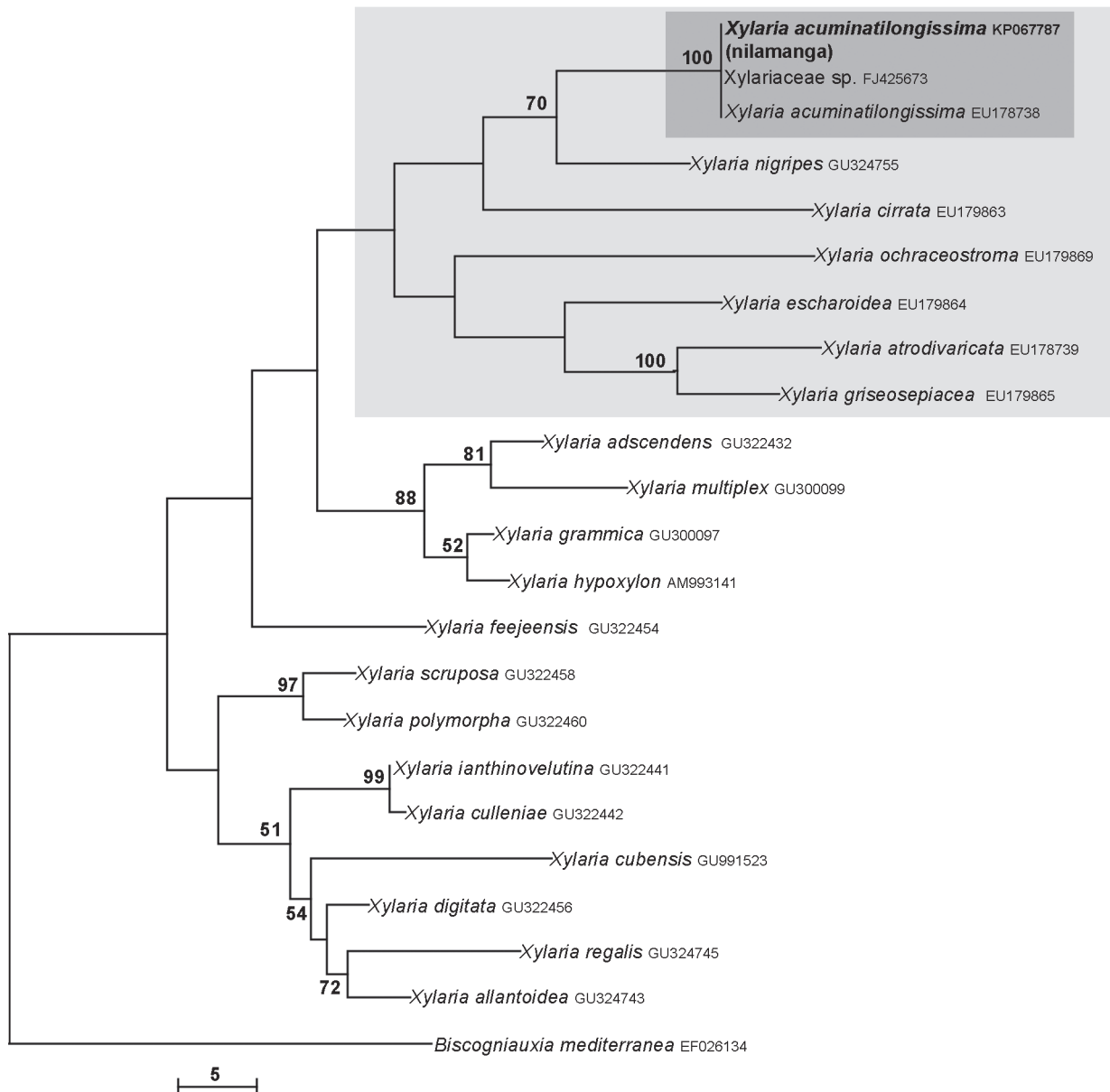


FIGURE 2. The phylogenetic tree obtained from the MP analysis using ITS sequence data. Values in the branches indicate the BS support of the clades. BS values greater than 50% are shown. GenBank accession numbers are given after the name of each taxon.

Discussion

Based on morphology, the present collection of nilamanga is indistinguishable from *Sclerotium stipitatum*. Unfortunately, as we were not permitted to extract DNA from the type material of *S. stipitatum*, we could not prove the conspecificity of the two collections based on molecular methods. Molecular phylogenetic methods, however, unequivocally prove that the nilamanga material that we obtained from Kerala is a *Xylaria* species sharing 99% sequence similarity with *X. acuminatilongissima*, a termite associated species first reported from Taiwan. Remarkably, in our analyses, the present Kerala collection of nilamanga was found to be distinct from both *X. nigripes* that Dennis (1961) thought what *Sclerotium stipitatum* really was and *X. escharoidea* that is often confused with *X. nigripes* (Rogers *et al.* 2005). The hypogean origin of nilamanga specimens indicate that they could very well be growing on abandoned subterranean termite nests. The diagnostic features of the genus *Xylaria* are the upright, cylindrical to clavate, multiperitheciate stromata and geniculosporium-like conidiophores that are arranged into dense palisades, cylindrical asci with an apical apparatus

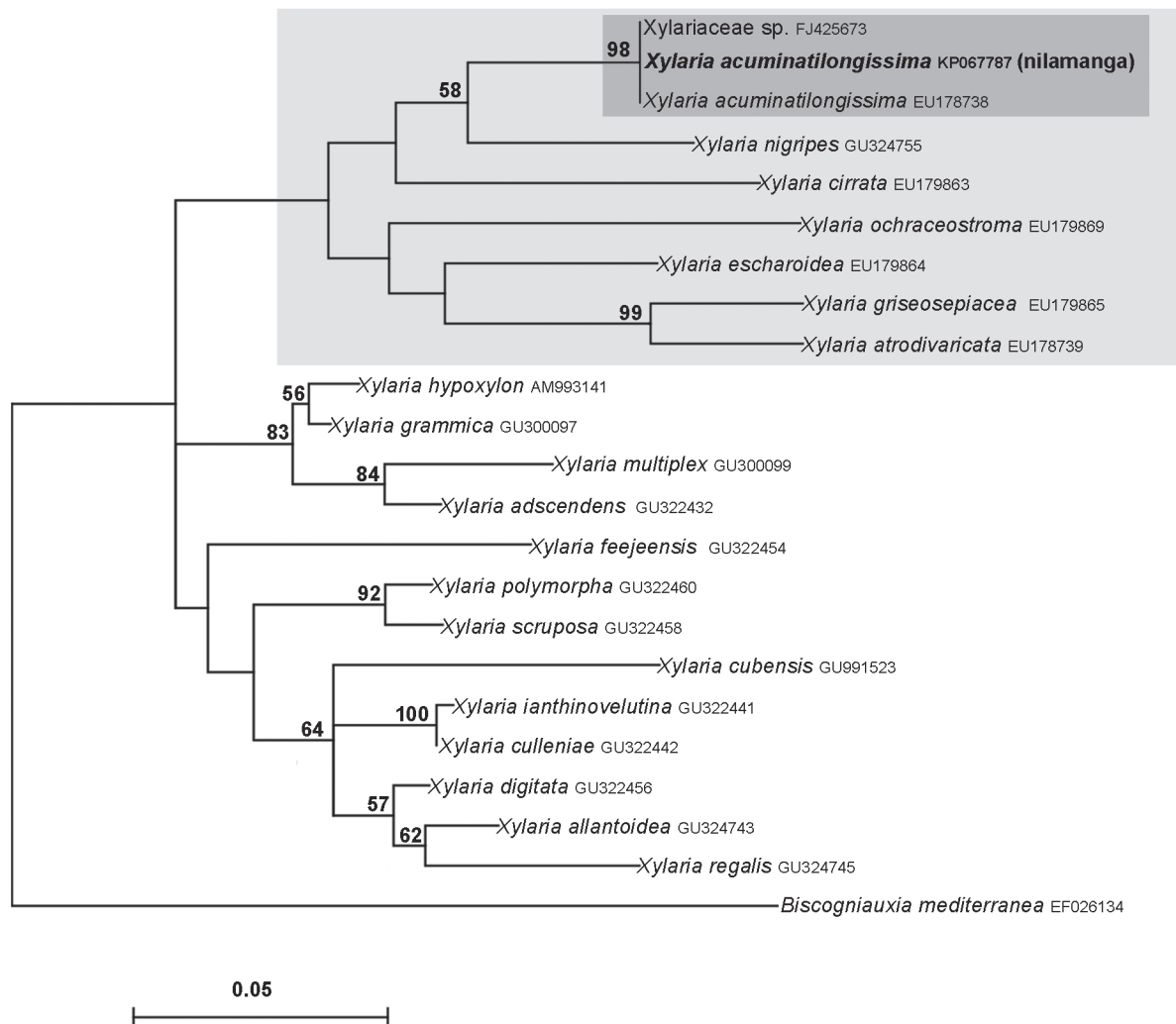


FIGURE 3. The phylogenetic tree obtained from the ML analysis using ITS sequence data. Values in the branches indicate the BS support of the clades. BS values greater than 50% are shown. GenBank accession numbers are given after the name of each taxon.

bluing in an iodine solution, and dark, unicellular ascospores with a slit-like germination site (Rogers & Samuels 1986). Although the sterile morphs of nilamanga do not exhibit any of these features, DNA analysis now reveals them to be *Xylaria*. They can be considered as a morphologically variable, multihyphal aggregated sclerotial stage of the fungus that can remain dormant or quiescent when the environment is unfavourable.

Apart from species of the agaric genus *Termitomyces*, other taxonomic groups also are associated with termite nests (Ju & Hsieh 2007). Stromata of the ascomycetous genus *Xylaria* frequently are found to emerge from abandoned termite nests (Petch 1906, 1913, Batra & Batra 1979). Unlike *Termitomyces* species that are well accepted as symbionts of termites, *Xylaria* species are considered as saprobes, invading comb material and competing with *Termitomyces* for nutrients (Sands 1969, Heim 1977, Wood & Thomas 1989). About 20 described *Xylaria* species are undoubtedly or likely associated with termite nests (Rogers *et al.* 2005, Ju & Hsieh 2007).

Many of the *Xylaria* species found on termite nests are reported to have potential medicinal properties (Wu 2001, Ma *et al.* 1989, Lu *et al.* 1993, Li & Wen 2008, Ko *et al.* 2011, Song *et al.* 2011, Zhao *et al.* 2014) and these reports are in support of the attributed medicinal values of the present fungal morph.

Conclusions

Morphological examinations reveal that the nilamanga specimen that we obtained recently from Kerala is indistinguishable from *Sclerotium stipitatum* described by Berkeley (1860) based on a collection from Kerala. Molecular

phylogenetic methods unequivocally prove that the recently collected nilamanga specimen is *X. acuminatilongissima* (Xylariaceae, Xylariales, Ascomycota, Fungi), a termite associated species.

Acknowledgments

We are thankful to Dr Firdous Iqbal of Changampally Vaidya Bhavan, Tirunavaya, Kerala for providing specimens of nilamanga for this study. We are also thankful to the staff of Kew (Mycology) Herbarium for arranging loan of holotype of *Sclerotium stipitatum*. KPDL acknowledges support from the Kerala State Council for Science, Technology and Environment (KSCSTE) in the form of a PhD fellowship (Grant No. 001/FSHP/2011/CSTE).

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