Identification and comparison of *Xylaria curta* and *Xylaria* sp. from Western Ghats-Courtallum Hills, India

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Xylaria curta and *Xylaria* sp., originating from evergreen forests of Courtallum Hills, Western Ghats Tamil Nadu, India were identified based on 18S rRNA gene sequence comparisons and morphological characteristics. These two species nested within a subclade that also contained *X. curta* from Thailand and *X. longipes* from Spain.

Key words – Ascomycetes **–** MEGA 5 **–** Molecular phylogeny **–** Neigbour-joining method **–** Nuclear small subunit 18s RNA

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Introduction

Traditional classification and identification of fungi has relied upon microscopic features, colony characteristics on artificial media and biochemical reactions (Sutton & Cundell 2004). Such methods have served in the past but they have major drawbacks as they cannot be applied to non-cultivatable organisms and occasionally biochemical characteristic of some organisms do not fit into the patterns of any known genus and species. Amplification and sequencing of target regions within the ribosomal DNA gene complex has emerged as a useful adjunctive tool for the identification of fungi and does not depend on fungus sporulation for identification (Buzina et al. 2001, Iwen et al. 2002, Rakeman et al. 2005, Schwarz et al. 2006). The internal transcribed spacer (ITS) regions 1 and 2 located between the highly conserved small (18S) and large (28S) ribosomal subunit genes in the rRNA operon are known to have sufficient sequence variability to allow identification to the species level for many fungi (Brandt et al. 2005, White et al. 1990). Nucleic acid sequences from small subunit ribosomal RNAs (18s rRNAs) have proved useful for phylogenetic analysis in eukaryotes. Because of their ubiquity and evolutionary conservation these molecules are useful for inferring distant phylogenetic relationships providing a means of assessing relationship between organisms which lack any informative homologous morphological or developmental traits (Sogin et al. 1977, Woese 1987, Field et al. 1988).

The Xylariaceae is a large and relatively well-known ascomycete family found in most countries (Whalley 1996), and it contains 35 genera (Eriksson & Hawksworth 1993). It is characterized by perithecial ascocarps bearing paraphyses and periphyses

that are embedded in a stroma. The asci of most species bear a ring at the apex that appears as a characteristic amyloid ascal plug when stained with iodine. Many species actively decay wood of living or dead angiosperms and are known to be saprobic in most cases (Rogers 1979). *Xylaria* is a large and the first described genus of the Xylariaceae (Martin 1970). *Xylaria* species are saprobic or sometimes weakly to strongly parasitic on woody plants and usually have erect elongated stromata. Although they are found mostly on wood, some species are found on sawdust, leaf, dung or soil.

Species of *Xylaria* are difficult to identify and classify especially as the stromata of a given species often vary greatly in colour, size and sometimes in general shape (Whalley 1996). Until now, identification of fungal species has been mainly done on the basis of morphological and microscopically characteristics but this is not suitable for differentiating closely related species of *Xylaria*.

In the present study, *Xylaria curta* and *Xylaria* sp.R005 are described for the first time from southern Tamil Nadu, India based on molecular analysis as well as morphological characteristics.

Materials and methods

Fungal isolates

Fruiting bodies of the two *Xylaria* strains were isolated from the soil and decomposing wood bark in tropical evergreen forest of Southern Western Ghats of Courtallum Hills, Tamil Nadu. The fruiting bodies were cleaned, air dried and stored at room temperature in paper bags. The studied specimens were deposited at the Department of Botany, VHNSN College, Virudhungar, Tamil Nadu. Cultures were initiated from perithecial contents or mycelial plugs of freshly collected stromata, propagated and studied as described by Stadler et al. (2005) on potato dextrose agar (PDA) medium at 30˚C. Pure cultures were maintained on PDA slants at -20˚C in culture collection. Cultures were grown in 500-ml Erlenmeyer flasks containing 200 ml of culture media (PDA). Radial growth rate of the isolates were measured in petri plates containing PDA after 15, 20, 25 and 30 days at 30˚C.

Each specimen was examined for morphological characteristics of asci, ascospores, paraphyses and other structures of taxonomic value. Spore dimensions were determined for 50 spores. Lactophenol cotton blue and distilled water were used as mounting media for microscopy. Dried materials were rehydrated in 3% aqueous KOH. Photography was carried out with a light microscope and binocular microscope (COSLAP) with computer attached.

DNA extraction, amplification and sequencing

Fungal isolates were incubated 2–3 weeks at 30˚C on PDA. The mycelium was harvested and transferred into 2 ml plastic tubes using a spatula and lyophilized for DNA isolation. Total genomic DNA was extracted using the method of Doyle & Doyle **(**1987**).** The ITS1 5.8s and ITS2 regions of nrDNA were amplified using the primers ITS1 and ITS2 **(**White et al. 1990**)** and the 18S region was amplified using the (5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3') primers (White et al. 1990). Polymerase chain reactions (PCR) were performed using a Perkin Elmer 480 with 35 cycles of 94°C denaturation for 1 min, 50°C annealing for 30 s, and 72°C extension for 1 min with an additional 7 min extension at 72°C after cycling. The PCR amplicons were purified using Qiaquick spin-columns according to manufacturer protocols. All the PCR products were sequenced at Applied Biosystems, Foster City, California, USA and an additional internal 18S primer NS 1.5, NS 2, NS 4 (White et al. 1990) and BMB-BR (Lane et al. 1985) were used to improve sequencing results. Isolates of 18s rRNA fungal sequences obtained were submitted to GenBank (NCBI, USA) (accession numbers: JF795289 and JF795290). All the studies of DNA extraction and isolation were done by Synergy Scientific Services, Chennai.

Phylogenetic analysis

Phylogenetic analysis was conducted in MEGA 5 software (Tamura et al. 2007). Sequenced ITS1-5.8S-ITS2 regions were aligned initially using the alignment algorithm Clustal W (Thompson et al. 1997) with the gap

Fig. 1 – Macro and microscopic features of *Xylaria curta*. 1, 2. Natural habitat of fruiting bodies. 3. Cultural morphology on PDA plates (diameter 9 cm) after 2 weeks of incubation. 4. Ascal apical rings and ascospores in water (×200). 5. Cross section showing prethecia and ascospores 6. A partial rosette of maturing asci.

open penalty 7.0 and gap extension penalty 4.0. Due to some variation in areas of ITS1 and ITS2 regions, an alignment was then improved manually. The evolutionary history was inferred using the neighbor- joining method (Saitou & Nei 1987). All positions containing gaps with missing data were eliminated from the dataset. Strengths of internal branches of resulting trees were statistically tested by the bootstrap analysis of 1000 replications **(**Felsenstein 1985). Additional sequences were retrieved from GenBank (Table 1).

Results

Identification of fungal strain

The two fungal strains were identified as *Xylaria curta* and *Xylaria* sp. based on the nuclear ribosomal ITS1-5.8S-ITS2 sequence analysis. The ITS sequence analysis revealed that the fungal strains had more than 90% sequence similarity with those strains obtained from GenBank.

Morphology and cultural characteristics

Xylaria curta

Fruiting bodies 6–15 cm in length, 0.5–1 cm in broad (Fig. 1), growing either singly or in groups which are typically seen emerging from soil, dark brown, becoming darker at maturity. Stromata with fertile part is cylindrical-clavate, with rounded, fertile apices, unbranched, single or clustered, short-stipitate, stipe smooth and black. External surface is blackish with golden brown scales, roughened with small wrinkles, internally white and occasionally becoming hollow. Perithecia completely immersed, up to 0.5 mm diam., ostioles black, papillate. Asci 8-spored, uniseriate, cylindrical, stipitate, Ascospores are 17.3–17.8 µm broad, 33.1–33.8 µm in length. ellipsoid-inequilateral to broad ellipsoid-inequilateral, dark brown, unicellular, smooth, germ slit conspicuous, straight, running full-length of spore (Fig. 1).

Substrate is undetermined decaying wood. Growth rate is high, 5.8–7.5 cm/week, covering petri dish in 6–8 days. Mycelial mat is white at early stage, later it is brown to black coloured. Hyphae are thin-walled and branched. The fruiting bodies arise in the spring. They may persist for several months or even years and can release spores continuously during this time.

Xylaria sp.R005

Fruiting bodies 2–8 cm in length, 1–3 cm in diameter (Fig. 2), often growing in groups of three clustered into "finger-like or hand-shaped" forms which emerge around stumps or decaying trees, dark grey to brown, becoming dark black at maturity, subcylindric at first, becoming flattened; upper branches appear powdered white, finally tipped black when mature, stalk black and hairy. Stromata cylindrical, dichotomously branched several times or infrequently unbranched, surface with conspicuous perithecial mounds, wrinkled. Asci 8-spored, uniseriate, cylindrical, Ascospores are 15.1–16.5 µm broad, 30.1–33.5 µm long, brown to dark brown, unicellular, short with narrowly rounded ends, smooth.

Growth rate is slow, colonies not reaching the edge of petri dish (9 cm diameter) in 1 month. The mycelial mat white at first, later black, mostly submerged, with irregular margins.

Phylogenetic analysis

Phylogenetic relationships inferred from ITS1-5.8S-ITS2 region sequences of *Xylaria* species are shown in Fig. 3. The tree is divided into two main clusters (A and B), each divided into two sub-clusters (A1 and B1). In sub-cluster A1 xylariaceae sp, *X. longipes* and *Xylaria* sp. were grouped together. *X. curta*, *Xylaria* sp. and *X. longipes* were grouped together in sub-cluster A2. In sub-cluster B1 *Xylaria* sp., *X. juruensis* and *X. polymorpha* were grouped together at a bootstrap value of 55%. *X. levis*, *X. plebeja*, *X. polymorpha*, *X. curta* and *Xylaria* sp. were grouped together at a bootstrap value of 72% in sub-cluster B2. Based on the sequence data the *X. curta* and *Xylaria* sp. were connected to each other in the same group and nested in a cluster consisting of *X. longipes*. The tree showed no phylogenetically close relationship between the two fungi and certain genera of Xylariaceae.

Discussion

This study provides molecular evidence for identification of *Xylaria curta* and *Xylaria*

Table 1 ITS sequence data used in this study.

Species	Geographic origin	GenBank No
Xylaria polymorpha	China	AB274817
Xylaria sp.SOF11	China	JF703668
*Xylaria curta**	India	JF795289
Xylaria sp.XF10	India	HQ435666
Xylaria sp.CH2*	India	JF795290
Xylaria polymorpha	Japan	AB512310
Xylaria longipes	Netherlands	AF163038
Xylaria longipes	Spain	AY909016
Xylaria curta	Taiwan	GU322443
Xylaria curta	Taiwan	GU322444
Xylaria juruensis	Taiwan	GU322439
Xylaria laevis	Taiwan	GU324747
Xylaria plebeja	Taiwan	GU324740
Xylaria curta	Thailand	DQ322144
Xylaria sp.E10500C	USA	JN572047
Xylariaceae sp.vega244	USA	EU010004
Xylariaceae sp.vega348	USA	EU009959
Xylariaceae sp.vega457	USA	EU010003

Asterisks indicate the sequences obtained from the present study

sp. in southern Tamil Nadu, India. Kshirasagar et al. **(**2009**)** reported that ten species of *Xylaria* from Western Ghats of Maharashtra could not be distinguished phylogenetically. Similarly, *X. escharoidea* and *X. nigripes* have been characterized by Rogers et al. (2005).

The genus *Xylaria* shows great variation in morphology, but few phylogentic studies have been conducted to infer the relationships of taxa within the genus. Nuclear small subunit ribosomal RNA gene regions are usually used as a molecular tool to analyze fungal taxa at a family or order level and ITS regions are commonly used to examine phylogenetic positions or relationship at a species or intraspecies level. Morphological and anatomical data clearly differentiated these two species from each other. *X. curta* was found in soil whereas *Xylaria* sp.R005 was found in decaying wood. The morphological characters of these two *Xylaria* species are identical with *X. angulosa* (AB274814) found from soil (Rogers et al. 1987). On the other hand, many species of *Xylaria* are actively found in decaying wood of angiosperms and are known to be saprobic (Rogers 1979).

In this study *X. curta* and *Xylaria* sp.R005 were differentiated from *X. polymorpha* and other species of *Xylaria* on the basis of variation in internal transcribed spacers ITS1 and ITS2. Our fungal strains formed a segregated clade with *X. curta* and *Xylaria* sp., supported by low bootstrap values of 68 and 72%, respectively (Fig. 3). Phylogenetic relationship of some *Xylaria* species was also studied by Lee et al. (2000) in which *Xylaria* species were classified into three groups based on morphological and molecular similarity, viz. *X. apiculata*, *X. arbuscula*, *X. muli* in group A, *X. acuta*, *X. castorea*, *X. cornu-damae*, *X. enteroleuca*, *X. fioriana* and *X. longipes* in group B, *X. hypoxylon* and *X. polymorpha* in group C. A few characters of ascospores, perithecia and stromata support the grouping of *Xylaria* inferred from molecular data, but there seems to be no character of universal significance that can justify the phylogenetic results. It may indicate that convergent evolution of characters occurred many times within *Xylaria*. Such possible changes in convergent evolution, along with variations associated with developmental stages of fruit bodies might have caused confusions in identifying and classifying *Xylaria* species. Phylogenetic analyses based on molecular data such as ITS

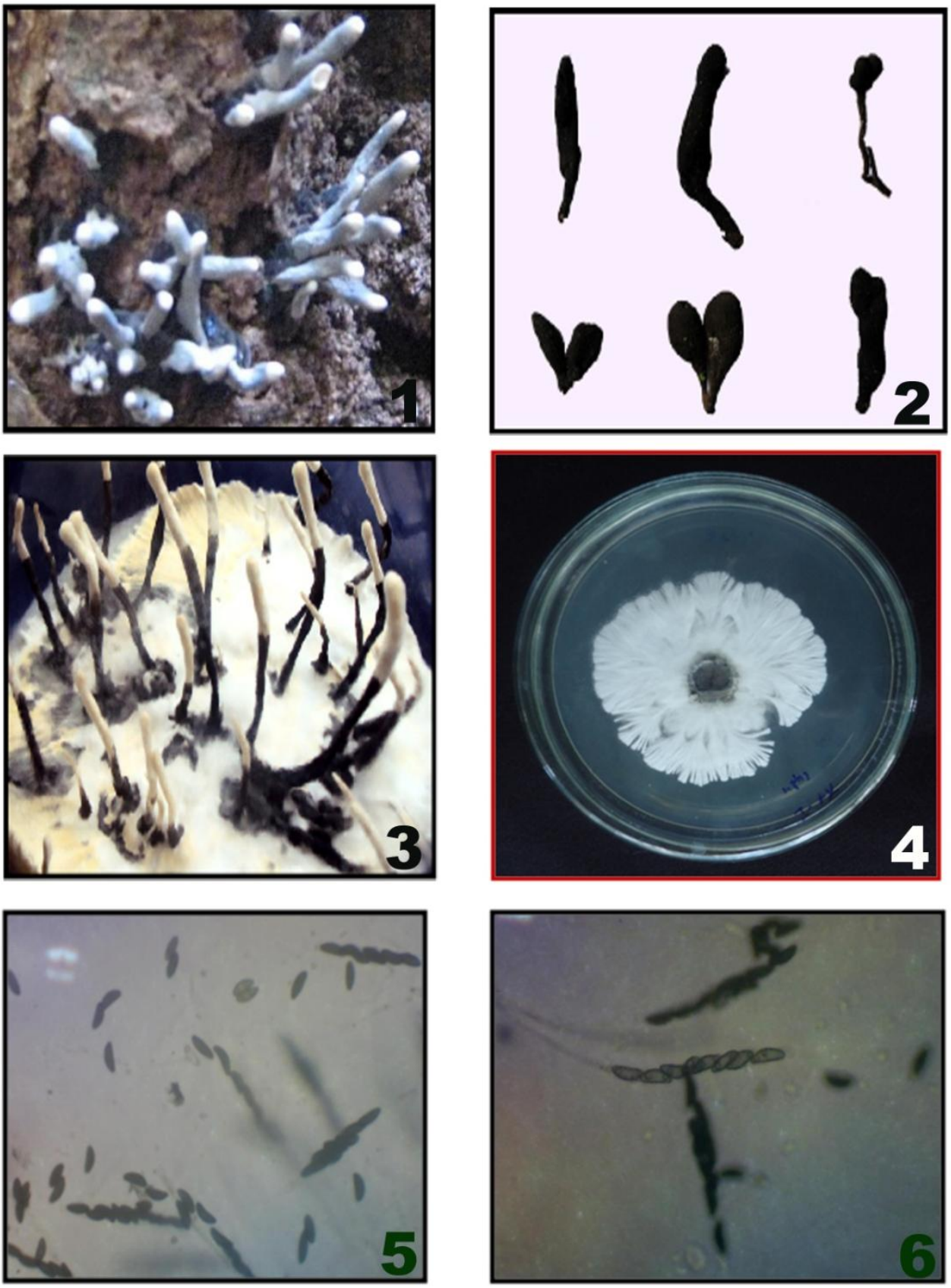

Fig. 2 – Macro and microscopic features of *Xylaria* sp. 1. Natural habitat of fruiting body. 2. Morphological variability of fruiting body. 3. Cultural morphology on liquid medium (PDB) in 500 ml conical flask. 4, Cultural morphology on agar plates (diameter 9 cm) after 4 weeks of incubation. 5, 6. Ascal apical rings and ascospores in water (×200).

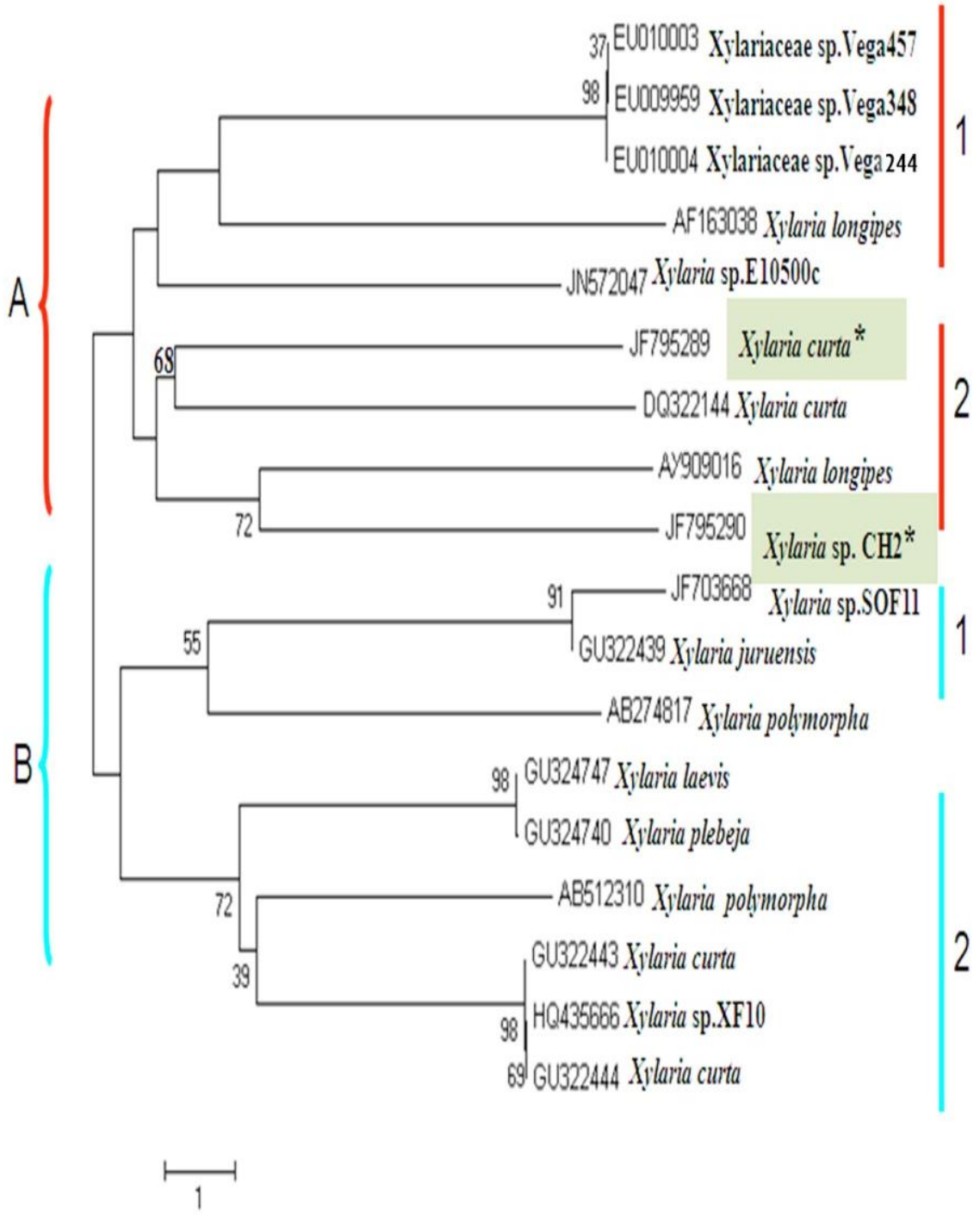

Fig. 3 – Phylogenetic relationship between *Xylaria* species, inferred from ITS nucleotide sequence data. Bootstrap values are shown for those branches that had >30% support in a bootstrap analysis of 1000 replicates. The numbers of nucleotide changes among taxa are represented by branch length and scale bar equals the number of nucleotide substitutions per site. Asterisks indicate the sequence obtained from the present study. A and B indicates major clusters and 1 and 2 indicates sub-clusters referred to in the text.

sequences of the present study proved to be very practical for taxonomic investigations at specific or generic levels in identification or classification of fungi with highly variable morphology like *Xylaria*.

Increased taxon sampling from other parts of India and other continents are needed to elucidate the genetic diversity of *Xylaria* species complex. In future the phylogenetic structure will be increased through additional gene sequences.

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