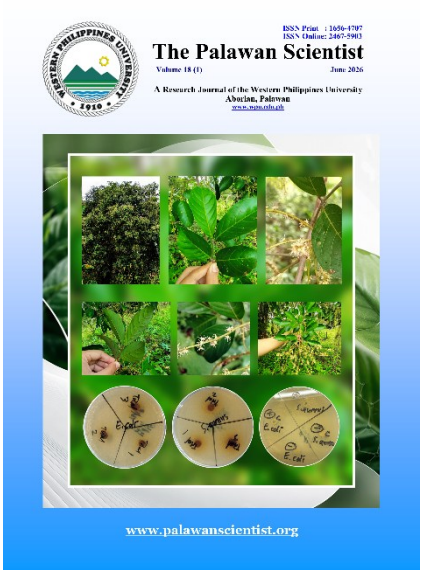

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Inhibition of ESKAPE bacterial pathogens by endolichenic fungi *Nemania bipapillata* and *Xylaria badia* associated with the fruticose lichen *Ramalina* spp.

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ABSTRACT

Endolichenic fungi (ELF), filamentous fungi that live asymptomatically within the lichen thalli, hold promise for combating ESKAPE bacteria — *Enterococcus faecalis* Schleifer and Kilpper-Bälz 1984, *Staphylococcus aureus* Rosenbach 1884, *Klebsiella pneumoniae* Trevisan 1887, *Acinetobacter baumannii* Bouvet and Grimont 1986, *Pseudomonas aeruginosa* Migula, and *Enterobacter aerogenes* Hormaeche and Edwards 1960 (current name: *Klebsiella aerogenes* Tindall et al. 2017). Twelve ELF from *Ramalina* were cultured in rice medium and potato dextrose broth (PDB), extracted with ethyl acetate, and assessed for antibacterial activity using a disc diffusion assay against ESKAPE bacterial pathogens. Specifically, 40 μL of 10 mg/mL crude extracts were impregnated onto each disc and placed on 15 mL Mueller-Hinton agar plates previously swabbed with 1 mL of bacterial suspension equivalent to a 0.5 McFarland standard. Crude culture extracts of *Xylaria badia* Pat. grown in PDB showed promising inhibitory activities against *E. faecalis* and *S. aureus*, with zones of inhibition (ZOI) of 18.17 mm and 21.70 mm, respectively. In addition, crude culture extracts of *Nemania bipapillata* (Berk. & M.A.Curtis) Pouzar grown in rice medium showed weak inhibition against *A. baumannii* (10.23 mm ZOI). These findings support the potential of endolichenic fungi, particularly *X. badia* and *N. bipapillata,* as sources of secondary metabolites active against ESKAPE bacterial pathogens.

Keywords: bioprospecting, disc diffusion assay, fruticose lichen, Philippines

The fruticose lichen *Ramalina* is widespread globally (Kirk et al. 2010), with extensive studies on its bioactivity, e.g., antimicrobial (Moreira et al. 2015), herbicidal (Gazo et al. 2019), cytotoxic (Koopaie et al. 2023), and insecticidal (da Silva et al. 2021). Moreover, endolichenic fungi associated with the thalli of *Ramalina* showed antioxidant activities (Galinato et al. 2021). In the study of Santiago et al. (2021a, 2022), endolichenic fungus (ELF) produced metabolites that inhibited Gram-negative bacteria, as opposed to the lichen acids produced by their lichen hosts, which target mainly Gram-positive bacteria.

The fungus *Nemania* has been isolated as an ELF in the fruticose lichen *Usnea* (Santiago et al. 2021a, 2022), as well as in the foliose *Parmotrema rampoddense* (Nyl.) Hale. (Tan et al. 2020), although it can also occur as an endophytic fungus in *Aquilaria sinensis* (Lour.) Spreng. (Kumarihamy et al. 2019; Tibpromma et al. 2021). On the other hand, *Xylaria* is known as an endophyte, e.g., of *Gingko biloba* L. (Suryelita et al. 2021), *Haematoxylum brasiletto* H. Karst (Sánchez-Ortiz et al. 2016), and *Geophila repens* (L.) I.M. Johnst. (Rajendran et al. 2023), and as an endolichenic fungus, e.g., of *Cladonia* (Cañón et al. 2019) and *Usnea* (Santiago et al. 2021a, 2022). Owing to the prolific production of secondary metabolites by ELF and the documented bioactivities of its lichen host, we assessed the antibacterial activities of selected *Xylaria* and *Nemania* species associated with the lichen *Ramalina* to contribute to the growing list of endolichenic fungi targeted for bioprospecting.

Endolichenic fungi were isolated from two *Ramalina* species, i.e., *Ramalina* sp. and *Ramalina* cf. *farinacea*, following the protocol of Paranagama et al. (2007). Briefly, lichen thalli were surface sterilized by successive dipping in 96% ethanol (10 sec), 0.5% NaOCl (2 min), and 70% ethanol (2 min), and then plated after rinsing on Dichloran Rose Bengal Chloramphenicol agar plates. Colonies of six *Nemania* and six *Xylaria* isolates were selected and characterized morphologically on Malt Extract Agar (MEA) and Corn Meal Agar (CMA). Colonial growth was measured daily, and the mean colony extension rates (MCER) were computed following the method of dela Cruz et al. (2006). To confirm species identity, the isolated ELF were sent to Macrogen, South Korea, for sequencing using the primer pairs internal transcribed spacer 4 (ITS4) and ITS5 for the nuclear ribosomal (ITS) region. For phylogenetic analysis, phylogenetic trees were generated and constructed using the Randomized Axelerated Maximum Likelihood program with 1000 bootstrap values through the TrEase Web Service.

The twelve *Ramalina*-ELF were cultivated using solid-state and submerged liquid-state fermentation to produce secondary metabolites. For solid-state fermentation, 60 g of long-grain white rice in 90 mL distilled water were prepared in culture bottles and inoculated with ten agar blocks of seven-day-old ELF cultures. The culture bottles, in duplicate, were incubated for 30 days at room temperature. For submerged liquid-state fermentation, ten mycelial agar blocks of each ELF isolate were aseptically inoculated into 120 mL potato dextrose broth (PDB) (in duplicate) and cultured for 14 days under a rotary shaker (100 rpm) at room temperature. Secondary metabolites were extracted with 120 mL analytical-grade ethyl acetate and concentrated in vacuo using a rotary evaporator (80 rpm) at 45°C. Finally, the crude culture extracts were air-dried and then reconstituted with methanol at a concentration of 100 mg/mL.

The antibacterial activity was tested using the following bacteria: *Enterococcus faecalis* Schleifer and Kilpper-Bälz 1984 UST-CMS 10029, *Staphylococcus aureus* Rosenbach 1884 UST-CMS 1090, *Klebsiella pneumoniae* UST-CMS 1194, *Acinetobacter baumannii* Bouvet and Grimont 1986 UST-CMS 10005, *Pseudomonas aeruginosa* Migula UST-CMS 10013, and *Enterobacter aerogenes* Hormaeche and Edwards 1960 UST-CMS 1021 (current name: *Klebsiella aerogenes* Tindall et al. 2017), which were generously provided by the University of Santo Tomas Collection of Microbial Strains. Each bacterium was prepared in Mueller-Hinton Broth as an inoculum equivalent to a 0.5 McFarland standard. Then, 1 mL of the standardized bacterial suspension was swabbed onto 15 mL Mueller-Hinton Agar (MHA) plates in triplicate, onto which paper disks (6 mm) containing 40 μL of the crude culture extracts were placed. Briefly, 40 μL of the crude culture extracts were impregnated into blank discs in two aliquots of 20 μL followed by a 30-minute interval between applications to allow the solvent to evaporate and ensure uniform absorption. Disks containing erythromycin (15 μg, positive control), methanol (negative control), and extracts of uninoculated rice medium or PDB (negative control) were used as controls. All culture plates were incubated at 37°C for 24 hours, and the zones of inhibition (ZOI) for each disk were measured.

The six *Ramalina*-ELF isolates described as *Xylaria* generally exhibited white-gray, opaque colonies with cottony and velvety surface textures on MEA, with a usually white-cream underside (Figure 1). The colony diameter ranged from 18 to 30 mm on day four. When grown on CMA, these *Ramalina*-ELF had a general colonial morphology that was cottony and powdery, with a white-gray surface color and a white-gray to white-yellowish underside. The colony diameter ranged from 14 to 22 mm. On the other hand, the six *Ramalina*-ELF described as *Nemania* were cottony and velvety on MEA, with colony diameters of 19 to 24 mm after four days of incubation (Figure 1). When grown on CMA, the colony diameter ranged from 11 to 28 mm. When the mycelial colony extension rate was computed, most *Ramalina*-ELF isolates exhibited faster growth on CMA than on MEA (Figure 2). This finding is unexpected, given the limited nutrient content of CMA compared with MEA.

Of the twelve *Ramalina*-ELF, only ten were successfully sequenced and submitted to National Center for Biotechnology Information for accession numbers. Phylogenetic analysis identified these as: *Xylaria badia* Pat. [D1A.2, PV413362], *Xylaria grammica* (Mont.) Mont. [D2A.5.2, PV425956], *Nemania* sp.1 [D2C.3.2, PV425957], *Xylaria laevis* Lloyd [D3D.4.1, PV425959], *Nemania primolutea* Y.M. Ju, H.M. Hsieh & J.D. Rogers [D4A.4, PV425958;

D6B.2, PV425978], *Nemania bipapillata* (Berk. & M.A. Curtis) Pouzar [D4D.1, PV425960; D5D.5.2, PV425962], *Nemania* sp.2 [D5C.2.2, PV425961], and *Xylaria feejeensis* (Berk.) Fr. [D6D.1.1, PV425977]. The *Nemania* isolates formed two clades (Figure 3). Two ELF clustered with *N. bipapillata* with high bootstrap support, while another *Nemania* formed a distant group. Two ELF also clustered with *N. primolutea*. In previous reports, the separation of *Nemania* from *Annulohypoxylon*, *Daldinia*, and *Hypoxylon,* and its close relationship with *Xylaria,* was established only by combined data of ITS and *rpb2* genes (Tang et al. 2007), but may require detailed morphological and multi-gene phylogenetic analyses using ITS, α-actin, *rpb2,* and β-tubulin genes (Pi et al. 2021); hence, the use of additional reference genes may shed light on the correct placement of our *Nemania* isolates.

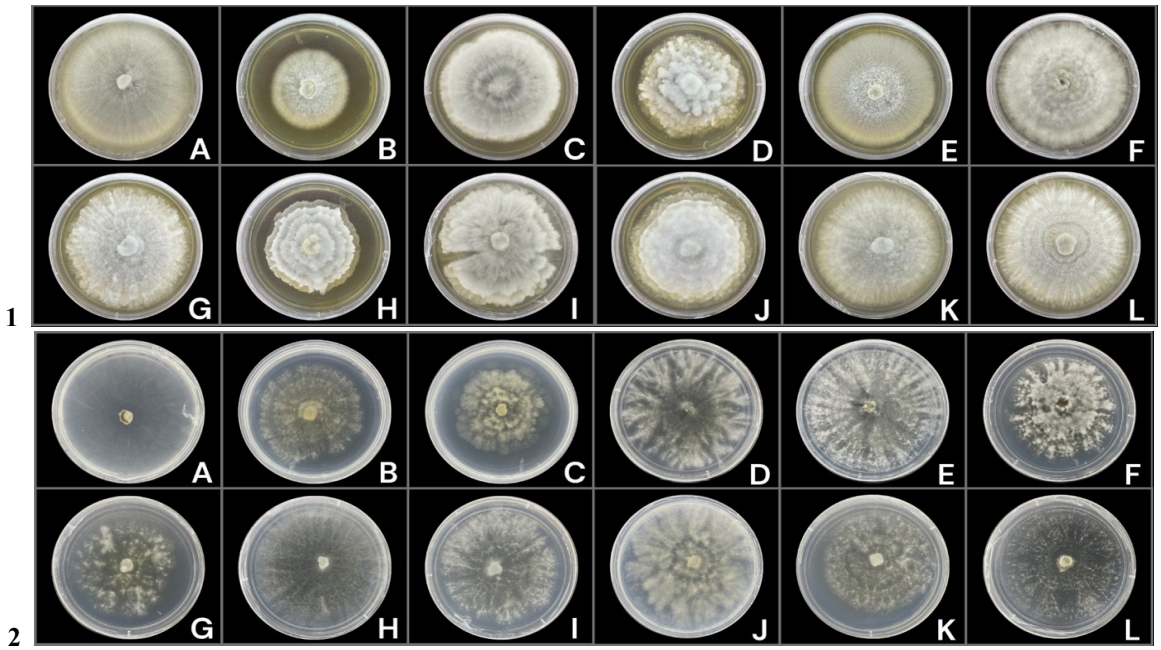

Figure 1. Colonial growth of the endolichenic fungi grown on (1) malt extract agar and (2) corn meal agar. A. *Xylaria badia* (D1A.2) B. *Xylaria* sp. 1 (D1B.3) C. *Xylaria grammica* (D2A.5.2) D. *Xylaria* sp. 2 (D2C.3.2) E. *Nemania* sp. 1 (D3C.5.1) F. *Xylaria laevis* (D3D.4.1) G. *Nemania primolutea* 1 (D4A.4) H. *Nemania bipapillata* 1 (D4D.1) I. *Nemania* sp. 2 (D5C.2.2) J. *Nemania bipapillata* 2 (D5D.5.2) K. *Nemania primolutea* 2 (D6B.2) L. *Xylaria feejeensis* (D6D.1.1)

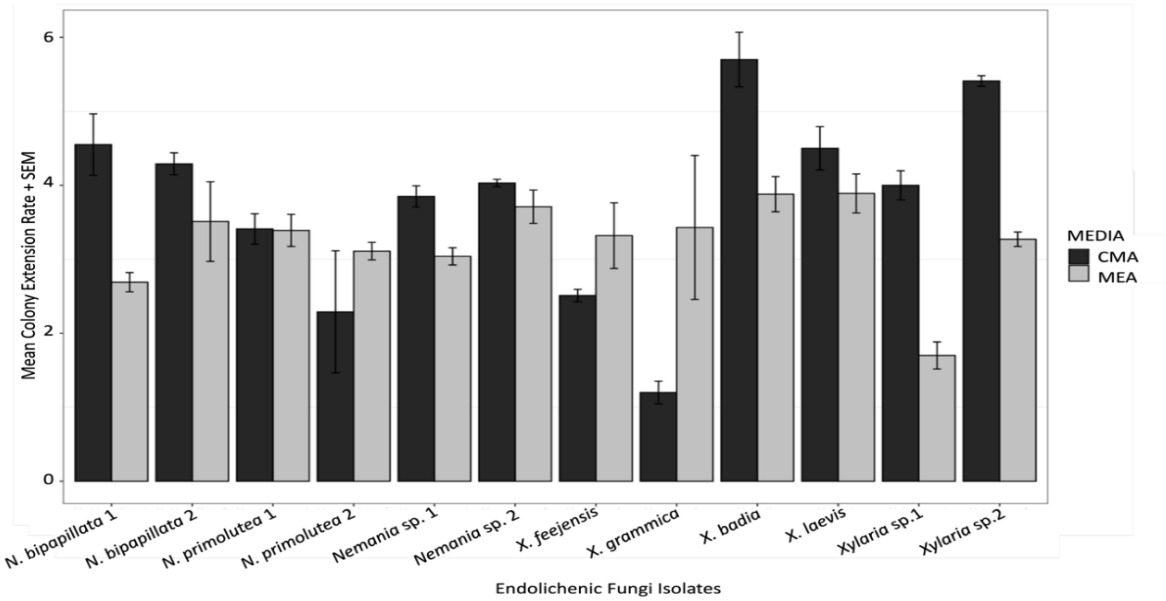

Figure 2. Growth rates of ELF isolates in corn meal agar (CMA) and malt extract agar (MEA) expressed as mean colony extension rates.

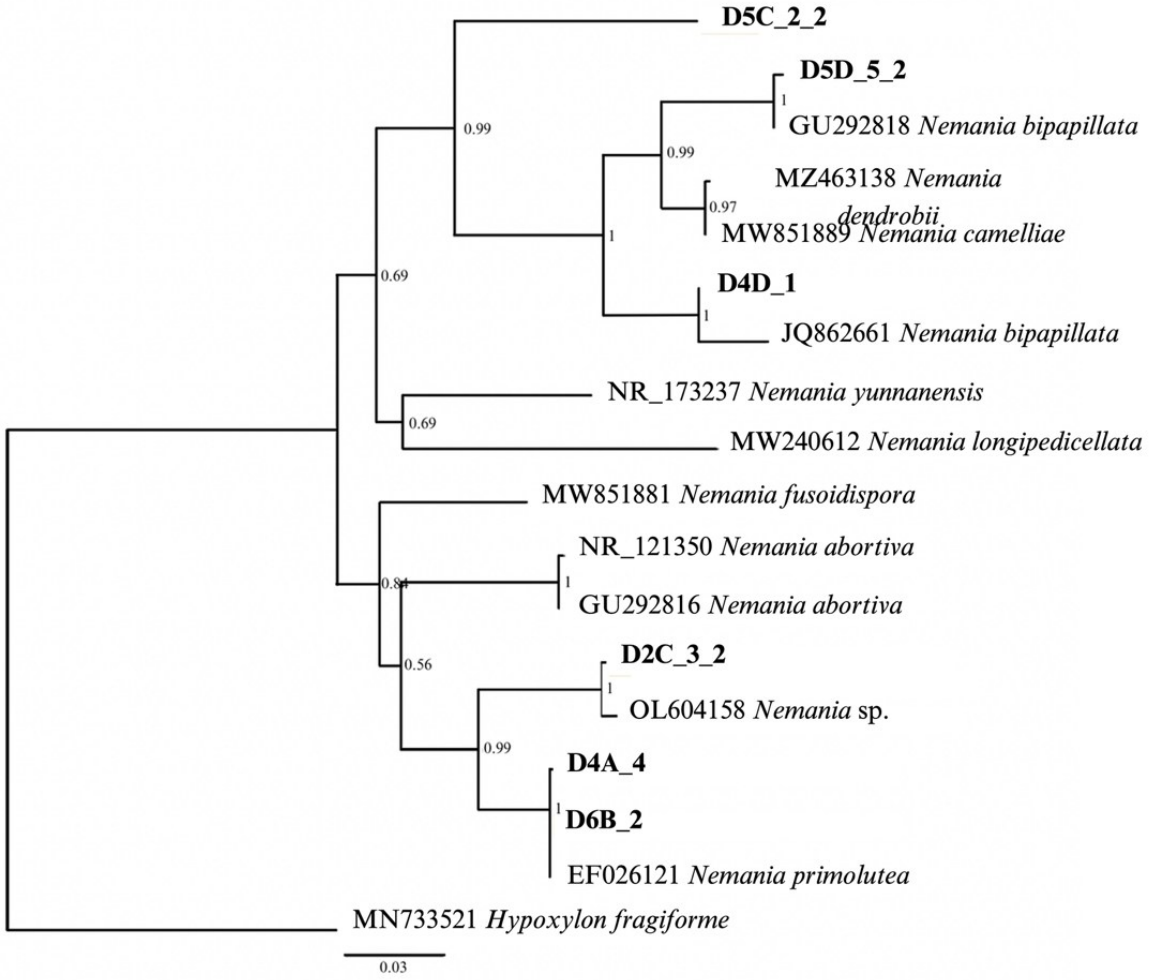

Figure 3. Phylogenetic tree of our isolated *Ramalina* endolichenic fungi within the genus *Nemania*. Accession codes are as follows: D4A.4 (PV425958), D6B.2 (PV425978), D2C.3.2 (PV425957), D4D.1 (PV425960), D5C.2.2 (PV425961), D5D.5.2 (PV425962).

Only four *Xylaria* isolates were successfully sequenced and were placed as closely related to *X. feejeensis* with moderately supported low posterior probability, to *X. grammica*, also with low support, and to *X. laevis* and *X. badia*, both with relatively good support (Figure 4). Similarly, ITS coupled with *rpb2* genes were used to resolve the identities of *Xylaria* (Lee et al. 2000; Pan et al. 2022).

Contrary to our expectation, only the crude culture extract of *X. badia* (D1A.2) grown in PDB showed inhibitory activities against *S. aureus* (ZOI = 21.7 ± 0.61 mm) and *E. faecalis* (ZOI = 18.17 ± 1.73 mm) (Figure 5). The crude culture extracts of *Nemania* sp. (D5C.2.2) grown in rice medium also showed minimal inhibitory activity against *A. baumanii* (ZOI = 10.23 ± 0.85 mm). These extracts were compared with the negative control, the solvent methanol, which showed no zone of inhibition as outlined in Guevara (2005). Nevertheless, our study confirmed the antibacterial activities of endolichenic fungi from *Ramalina,* specifically the crude culture extracts of *N. bipapillata* and *X. badia,* against ESKAPE bacterial pathogens, as similarly observed with those isolated from other lichen hosts, e.g., *Parmotrema* and *Usnea* (Tan et al. 2020; Santiago et al. 2021a, 2022). While inhibition was observed, the study does not report any detailed quantitative measures, e.g., MIC values or bacterial counts before and after treatment. Of particular interest are species of *Xylaria* that produce bioactive metabolites that inhibit pathogenic bacteria and are distinct from those of their lichen hosts (Santiago et al. 2021b). Endophytic *Xylaria* also showed varied bioactivities (Orachaipunlap et al. 2016). The ELF has also been shown to have other pharmacological potentials, e.g. antioxidant activities (Galinato et al. 2021). However, the varying degrees of bioactivity reported in our ELF culture extracts could be due to the media used for fermentation. For example, our *Ramalina*-ELF identified as *N. primolutea* did not exhibit any

antibacterial activity, whereas the same species from the lichen *P. rampoddense* showed antibacterial activity against *E. faecalis* and *S. aureus* (Tan et al. 2020). This indicates that the observed inhibitory activity was strain-dependent. In that study, malt extract broth was also used as a fermentation medium, as opposed to PDB and rice medium. Frisvad (2012) and Vandermolen et al. (2013) noted that growth media and incubation conditions greatly influence secondary metabolite production. Thus, it is recommended to characterize the bioactive metabolites in the crude extract to identify compounds responsible for the antibacterial activity. It is also recommended to study the possible mechanism of action for each of the bioactive crude culture extracts. Although there is no consensus on which medium is optimal for bioactive metabolite production, one should note that the use of varying media and conditions may lead to variations in secondary metabolite production and should therefore be considered in any bioprospecting research.

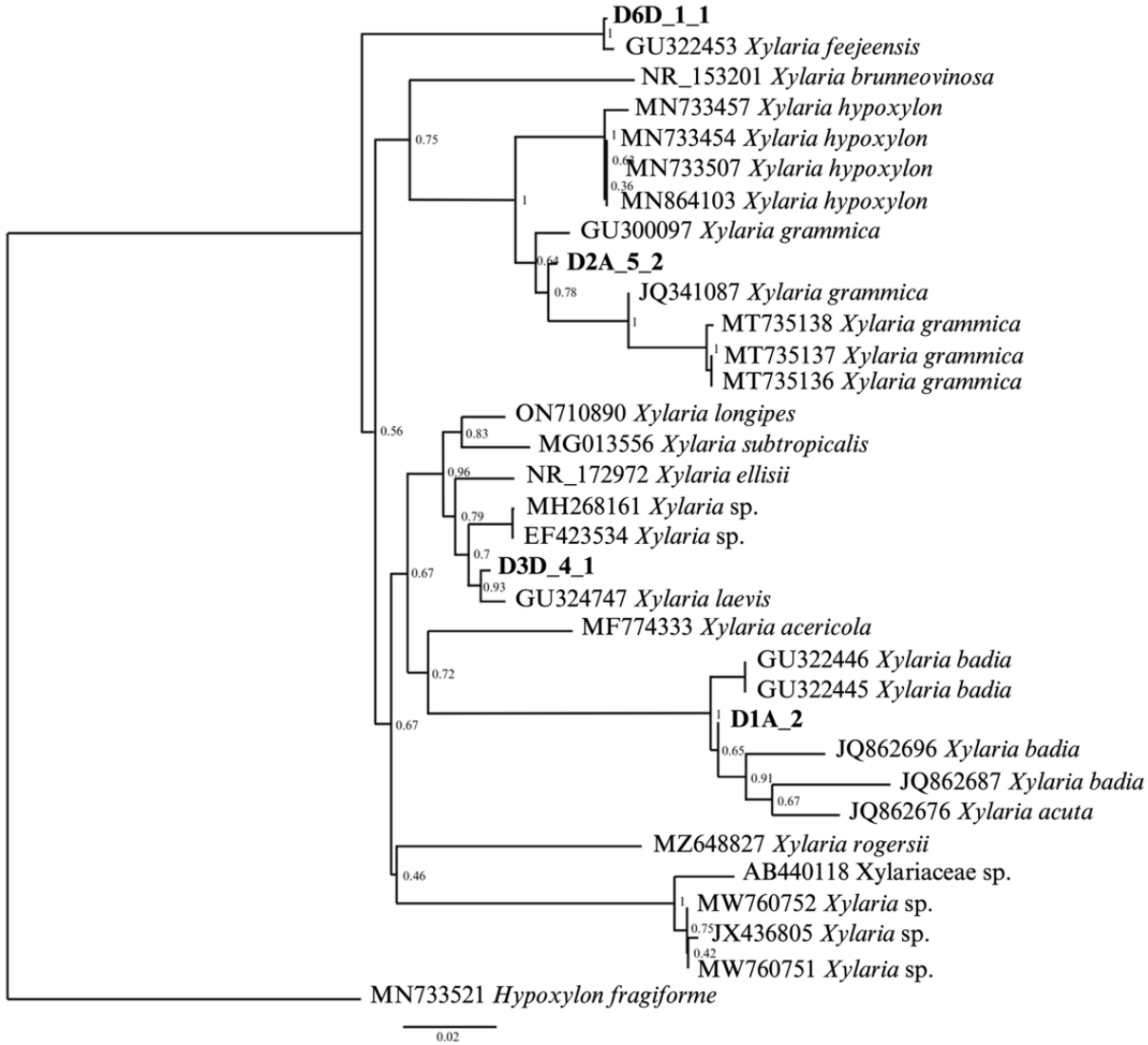

Figure 4. Phylogenetic tree of our isolated *Ramalina* endolichenic fungi within the genus *Xylaria*. Accession codes are as follows: D1A.2 (PV413362), D2A.5.2 (PV425956), D3D.4.1 (PV425959), D6D.1.1 (PV425977).

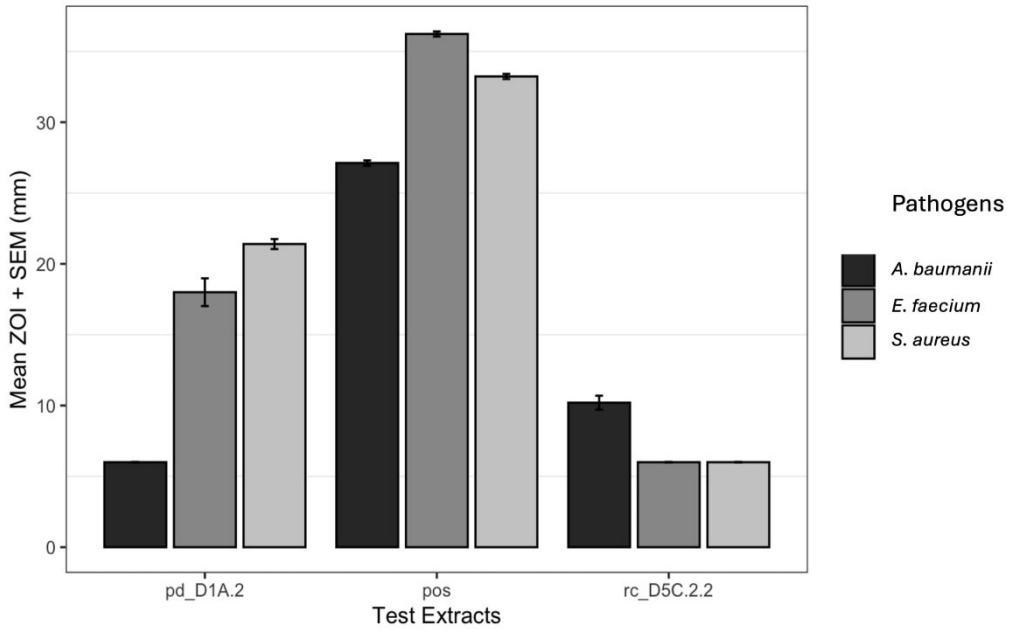

Figure 5. Mean zone of inhibition (ZOI), expressed in mm ± standard error of the mean (SEM), of the crude culture extracts of *Nemania bipapillata* (D5C.2.2) grown in rice medium (rc) and *Xylaria badia* (D1A.2) grown in PDA (pd), with erythromycin as the positive control (pos).

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GENERATIVE AI STATEMENT

We declare that this manuscript was prepared without the assistance of artificial intelligence. Hence, the content of this paper is original.

ETHICAL CONSIDERATIONS

No ethical clearance is required for this study.

DECLARATION OF COMPETING INTEREST

We declare no conflict of interest to disclose.

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