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Morpho-phylogenetics and pathogenicity tests reveal a new species of *Fusarium* infecting *Cannabis sativa* L. (hemp) in Northern Thailand

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Abstract

Cannabis sativa L., widely known as hemp or cannabis, has been cultivated for centuries and also legalized in several countries around the world. In Thailand, permits to use cannabis only for medical and licensed purposes are allowed, and hemp (tetrahydrocannabinol [THC] < 0.2%), is legal for general use. The rapid expansion of hemp cultivation has increased the crop's susceptibility to fungal diseases. Species of the genus *Fusarium* are major phytopathogens, although they may also exist as endophytes. During a survey of pathogenic fungi in Northern Thailand, diseased stems of *C. sativa* were collected, from which a novel *Fusarium* species, *F. flavens* sp. nov., was isolated. Identification was performed using both morphological features and analyses of DNA sequence data. Phylogenetic trees were constructed based on analyses of combined dataset of the translation elongation factor 1-α (*tef1-*α), RNA polymerase II second largest subunit (*rpb2*), and calmodulin (*cal*) gene regions. A combined taxonomic approach of morphological comparisons and multi-locus phylogenetic analyses confirmed the distinctiveness of the species. Pathogenicity was assessed by inoculating spore suspensions and mycelium plugs on hemp leaves, stems, and seedlings. Typical symptoms were reproduced, including leaf and stem wilting and root rot. Re-isolation and re-identification of the same fungus confirmed Koch's postulates. Based on this comprehensive approach, *F. flavens* was identified as the causal agent of stem and root rot in hemp. This study highlights the significance of *F. flavens* as a novel pathogen of hemp and emphasizes the importance of integrating morphology, molecular phylogenetics, and pathogenicity tests to clarify its role in hemp diseases.

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Introduction

Cannabis sativa L. (cannabis and hemp) is an important medicinal and industrial crop of the family Cannabaceae[1]. Originating from Central Asia, this annual herb has been widely cultivated for food, fiber, and medicinal and narcotic uses[2]. Cannabis or marijuana is grown primarily for psychoactive cannabinoids produced in glandular trichomes on female flowers[3], whereas hemp is a sustainable multipurpose crop valued for its efficient resource use and high-quality biomass for fiber and construction materials[4]. These two forms differ mainly in cannabinoid composition, particularly delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), with THC being psychoactive and CBD non-psychoactive, but medically beneficial[5]. Cannabis and industrial hemp are cultivated in more than 40 countries under diverse legal frameworks, with industrial hemp defined by low THC content (generally ≤ 0.2%–0.3%)[6,7]. Despite its growing economic value, *C. sativa* production is constrained by pests and diseases, particularly fungal pathogens that affect yield, quality, and product safety during both pre- and post-harvest stages[8]. Favorable environmental conditions, genotype susceptibility, and improper post-harvest handling can promote fungal contamination. Among these pathogens, *Fusarium* is a large and diverse genus of ubiquitous filamentous fungi, distributed worldwide in soil, air, water, and plant tissues. Species of *Fusarium* are among the most economically important fungi, acting as major plant pathogens that cause significant yield losses in agricultural crops; producing mycotoxins that contaminate food and feed which pose serious health risks to humans and livestock, and are opportunistic pathogens of humans and animals, particularly in immunocompromised individuals. Control of *Fusarium* diseases remains challenging due to their broad distribution, long-term survival in soil and plant debris, and their ability to overcome host resistance[9,6].

Plant diseases, particularly root and crown rots and vascular wilts, are caused by members of the *Fusarium solani* species complex (FSSC), and affect a variety of plants such as soybeans, potatoes, cucurbits, peas, sweet potatoes, Chinese roses, and other legumes[10–13]. Moreover, *Fusarium* species are reported to be some of the most destructive pathogens affecting *C. sativa*, especially

during the root and vegetative growth phases, leading to decreased crop quality and potential total yield loss[8]. For instance, brown rot of flower buds, foliar blight (which is characterized by mycelium covering inflorescences and leaves), and foliar and flower blight (which affects both flowers and leaves) are among the symptoms caused by several *Fusarium* species. These symptoms underscore the possibility of serious impacts on the plant's reproductive systems and overall health[14–16]. Furthermore, symptoms of *F. oxysporum* infection include internal stem decay, crown rot, and yellowing. In addition, it may cause pith browning, brown rot of the roots, root necrosis, chlorosis, and irregular wilt. Notably, it also causes damping-off in cuttings, which negatively affects seedling survival[16–19]. Similarly, *F. solani* and *F. proliferatum* are known to cause pith necrosis, crown infections, and tissue decay, all of which reduce plant health and productivity. At present, *C. sativa* productivity and health are seriously threatened by these *Fusarium* species, especially during crucial stages of growth[8,14,20].

Our ongoing work focuses on fungal pathogens associated with *C. sativa* (hemp) cultivated in Northern Thailand. During our investigation, we have found more than 10 pathogenic fungi, including one species that was found to be different from extant ones, and deemed to be a novel species. The objectves of this study were: (1) to characterize the novel isolate through detailed morphological descriptions; (2) to determine its phylogenetic position within the genus *Fusarium* by analysing DNA sequences from regions of the *tef-1α*, *rpb2*, and *cal* genes; and (3) to access its pathogenicity. The resulting data provides a framework for accurate quarantine screening and field diagnostics, supporting improved management of *Fusarium* in hemp production.

Materials and methods

Sample collection and fungal isolation

Symptomatic parts of *C. sativa* (hemp) stems were collected from an outdoor plantation of Mae Sa Noi Village, Mae Rim District, Chiang Mai Province, Northern Thailand (18°51'50.06" N, 98°50'45.57" E; elevation 1,285 m/4,216 ft). Ten symptomatic stems were randomly collected from the site. The samples were individually kept at 4 °C in sterile polyethene bags and processed in the laboratory within 24 h of collection. The single-spore isolation method on water agar (WA) was used to obtain pure cultures for subsequent molecular work, following Senanayake et al.[21]. All pure cultures were maintained at the Sustainable Development of Biological Resources Laboratory, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand (SDBR-CMU), and the Kunming Institute of Botany Culture Collection, Kunming, China (KUNCC). One-month-old cultures on potato dextrose agar (PDA) were used to prepare dried fungal cultures, which were deposited as herbarium specimens in the Herbarium of the Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand (CMUB).

Morphological study

The morphological analysis of *Fusarium* isolates was conducted as described by Wang et al.[22]. Cultural characteristics, including colony shape, colour, and odour, were noted during a 7-d incubation in the dark at 30 °C on PDA, oatmeal agar (OA), yeast extract agar (YEA), V8 juice agar (V8), and synthetic nutrient-poor agar (SNA). Colony characteristics were further observed after incubation on PDA at 30 °C for 15–30 d until sporulation. Phenotypic characteristics such as colony shape, size, colour, exudates, colony margins, spore colour and size were observed, recorded, and photographed using a Nikon ECLIPSE Ni-U compound microscope equipped with a Nikon DS-Ri2 camera[23]. For each anatomical structure (such as conidia, conidiophores, conidiogenous cells, and chlamydospores), at least 50 measurements were made using the Tarosoft (R) Image Framework program.

DNA extraction, PCR amplification, and sequencing

Genomic DNA from fungal mycelia was extracted using the Biospin Fungus Genomic DNA Extraction Kit (BioFlux, Hangzhou, China) based on manufacturer's protocols. The translation elongation factor-1 alpha (*tef1-α*), RNA polymerase second largest subunit (*rpb2*) and calmodulin (*cal*) were amplified by the polymerase chain reaction (PCR) using the primer pairs EF1/EF2 (EF1: 5'-A TGGGTAAG GAGGACAAGAC-3', EF2: 5'-GGAGGTACCAGTGATCATGTT-3')[24,25]; RPB2-5f/RPB2-7cR (RPB2-5f: 5'-GGGGWGAYCAGAAGAAGGC-3', RPB2-7cR: 5'-CCCATRGCTTGYTTRCCCAT-3')[26], and Cal-228F/Cal-2Rd (Cal-228F: 5'-GAGTTCAAGGAGGCCTTCTCCC-3', Cal-2Rd: 5'-TGRTCNG CCTCDCGGATCATCTC-3')[25]. PCR amplification was performed in a 25 μL reaction volume, containing 12.5 μL Master Mix (mixture of EasyTaqTM DNA Polymerase, dNTPs, and optimised buffer; Beijing TransGen Biotech Co., Ltd., Chaoyang District, Beijing, China), 8.5 μL of double-distilled water (ddH_2O), 2 μL DNA template, and 1 μL each forward and reverse primer (10 μM). The PCR thermal cycle program used for gene amplification is shown in Table 1. Purification of PCR products and DNA sequencing were conducted by TsingKe Company (Kunming City, Yunnan Province, China).

Phylogenetic analyses

The reverse and forward sequences were combined and examined using BioEdit v7.2.5[27]. The sequences were BLASTn searched in GenBank (https://blast.ncbi.nlm.nih.gov/) to identify taxa with the highest level of similarity (Table 2). The sequence alignment was completed using the MAFFT online server (https://mafft.cbrc.jp/alignment/server/index.html)[28] and minor modifications were performed in BioEdit v7.2.5[27] as needed. Maximum likelihood analysis (ML) was done on the CIPRES Science Gateway v3.3 (www.phylo.org/portal2)[29] using RAxML-HPC2 on XSEDE (8.2.12)[30] with the GTRGAMMA substitution model with 1,000 bootstrap iterations. The best-fit substitution models were evaluated using MrModeltest v2.3[31]. Bayesian Inference (BI) analysis was carried out using MrBayes on XSEDE v3.2.7a[32–34] using the same platform[29]. Six Markov chains were run continuously for 10 million generations and automatically terminated when the standard deviation of split frequency exceeded 0.01. The trees were sampled every 100th generation, and the first 25% of the sampled trees were eliminated

Table 1. The PCR thermal cycle programs for gene amplification used in this study.

Locus	Primer name	PCR procedures	Ref.
tef1-α	EF1/EF2	94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 50 s, elongation at 72 °C for 1 min, and final extension at 72 °C for 10 min	[24,25]
rpb2	RPB2-5f/ RPB2-7cR	95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 2 min, elongation at 72 °C for 1.5 min, and final extension at 72 °C for 10 min	[26]
cal	Cal-228F/ Cal-2Rd	94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30s, extension at 72 °C for 1 min and final step at 72 °C for 10 min	[25]

Table 2. GenBank accession numbers of the *Fusarium incarnatum-equiseti* species complex (FIESC) used in phylogenetic analysis.

Taxon	Strain	GenBank assession number		
		tef1-α	*rpb2*	*cal*
Fusarium aberrans	CBS 131385^T	MN170445	MN170378	MN170311
F. aberrans	CBS 131387	MN170446	MN170379	MN170312
F. arcuatisporum	LC12147	MK289584	MK289739	MK289697
F. brevicaudatum	NRRL 43638	GQ505665	GQ505843	GQ505576
F. bryceae	BRIP 74865c	PP209369	PP209368	—
F. caatingaense	URM 6779	LS398466	LS398495	—
F. camptoceras	CBS 193.65	MN170450	MN170383	MN170316
F. cateniforme	CBS 150.25^T = ATCC 11853	MN170451	MN170384	MN170317
F. caulendophyticum	GUCC 191050.1 = CGMCC3.25474^T	OR043881	OR043826	OR043731
F. caulendophyticum	GUCC 191050.2	OR043882	OR043827	OR043732
F. caulicola	GUCC 191051.1	OR043883	OR043828	OR043733
F. citri	CGMCC 3.19467^T	MK289617	MK289771	MK289668
F. citrullicola	SDBR-CMU422	OP020920	OP020928	OP020924
F. clavus	CBS 394.93 = BBA 64265 = NRRL 25795	GQ505597	GQ505775	GQ505509
F. clavus	CBS 126202^T	MN170456	MN170389	MN170322
F. clavus	CBS 130395 = NRRL 34032 = UTHSC 98- 2172	GQ505635	GQ505813	GQ505547
F. clavus	CBS 119881 = MRC 8412	MN170457	MN170390	MN170323
F. clavus	NRRL 32871 = FRC R-9561	GQ505619	GQ505797	GQ505531
F. coffeatum	CBS 635.76^T = BBA 62053 = NRRL 208	MN120755	MN120736	MN120696
F. coffeatum	CBS 430.81 = NRRL 28577	MN120756	MN120737	MN120697
F. compactum	NRRL 36323	GQ505648	GQ505826	GQ505560
F. concolor	NRRL 13459	GQ505674	GQ505852	—
F. croceum	CBS 131777	MN170463	MN170396	MN170329
F. curculicola	PPRI 20458^T = PREM 61345	MF787266	MN605062	—
F. duofalcatisporum	CBS 384.92^T = NRRL 36448	GQ505652	GQ505830	GQ505564
F. duofalcatisporum	CBS 264.50 = NRRL 36401	GQ505651	GQ505829	GQ505563
F. equiseti	CBS 245.61 = NRRL 20697	—	JX171595	—
F. equiseti	NRRL 26419 = CBS 307.94 = BBA 68556^T	GQ505599	GQ505777	GQ505511
F. equiseti	NRRL 36136 = CBS107.07, IMI 091982	GQ505644	GQ505822	GQ505556
F. equiseti	NRRL 36466 = CBS 414.86	GQ505653	GQ505831	GQ505565
F. extenuatum	LLC1501	OP487158	OP486728	OP486039
F. fasciculatum	CBS 131382^T	MN170473	MN170406	MN170339
F. fasciculatum	CBS 131383	MN170474	MN170407	MN170340
F. fasciculatum	CBS 131384	MN170475	MN170408	MN170341
F. fecundum	LC15875	OQ125250	OQ125544	OQ125281
F. flagelliforme	NRRL 36269	GQ505645	GQ505823	GQ505557
F. flavens	SDBR-CMU869 = KUNCC 25-20161^T	PX380329	PX380327	PX380330
F. goeppertmayerae	BRIP 64547d	OQ626868	—	—
F. gracilipes	NRRL 43635^T	GQ505662	GQ505840	GQ505573
F. guilinense	CBS 161.25	MN170448	MN170381	MN170314
F. guilinense	MFLUCC 24-0626	PV394837	PV394827	PV297811
F. hainanense	LC11638	MK289581	MK289735	MK289657
F. hainanense	MFLUCC 24-0630	PV394834	PV394828	PV297812
F. heslopiae	BRIP 74746a	PP209371	PP209370	—
F. humuli	CGMCC 3.19374 = CQ1039^T	MK289570	MK289724	MK289712
F. humuli	CQ1048	MK289571	MK289725	MK289713
F. incarnatum	CBS 132.73	MN170476	MN170409	MN170342
F. ipomoeae	CGMCC 3.19496 = LC12165 = M0111^T	MK289599	MK289752	MK289704
F. ipomoeae	LC12166	MK289600	MK289753	MK289706
F. irregulare	LC7188	MK289629	MK289783	MK289680
F. jinanense	LC15878	OQ125131	OQ125521	OQ125271
F. khuzestanicum	IRAN 4863C	PP858510	PP858506	PP858502
F. khuzestanicum	SCUA-Af-108–2	PP858512	PP858508	PP858504
F. kotabaruense	Indo172	LS479445	LS479859	LS479429
F. kotabaruense	MFLUCC 24-0633	PV394836	PV394831	PV297813
F. lacertarum	NRRL 20423 = ATCC 42771 = CBS 130185^T = IMI 300797	GQ505593	JX171581	GQ505505
F. lacertarum	NRRL 36123 = CBS102300, BBA 70843	GQ505643	GQ505821	GQ505555
F. longicaudatum	CBS 123.73^T = ATCC 24370 = IMI 160825 = NRRL 25477	MN170481	MN170414	MN170347
F. longifundum	NRRL 36372	GQ505649	GQ505827	GQ505561
F. luffae	LC12167	MK289601	MK289754	MK289698
F. makinsoniae	BRIP 64547a	OQ626867	—	—
F. melonis	SDBR-CMU424	OP020922	OP020930	OP020926

(to be continued)

Table 2. (continued)

Taxon	Strain	GenBank assession number		
		tef1-α	*rpb2*	*cal*
F. mianyangense	LC15879	OQ125232	OQ125510	OQ125335
F. mianyangense	MFLUCC 24-0625	PV394834	PV394829	PV297814
F. monophialidicum	NRRL 54973	MN170483	KC808362	MN170349
F. mucidum	CBS 102395	MN170485	MN170418	MN170351
F. multiceps	NRRL 43639	GQ505666	GQ505844	GQ505577
F. nanum	CGMCC 3.19498 = LC12168 = GXGL14-2^T	MK289602	MK289755	MK289651
F. nanum	LC1384	MK289611	MK289764	MK289661
F. nanum	LC1385	MK289612	MK289765	MK289662
F. nanum	LC1516	MK289613	MK289766	MK289663
F. neoscirpi	NRRL 26922	GQ505601	GQ505779	GQ505513
F. neosemitectum	CBS 189.60	MN170489	MN170422	MN170355
F. nothincarnatum	LC18436	OQ125147	OQ125509	OQ125290
F. oryzicola	IRAN 4864C	PP858509	PP858505	PP858501
F. oryzicola	SCUA-Af-125–2	PP858511	PP858507	PP858503
F. pernambucanum	MUM 1862^T = URM 7559	LS398489	LS398519	—
F. pernambucanum	URM 6810	LS398485	LS398515	—
F. pernambucanum	MUM 1864 = URM 7554	LS398486	LS398516	—
F. pernambucanum	SDBR-CMU744	PX233235	—	PX233226
F. pernambucanum	SDBR-CMU745	PX233236	—	PX233227
F. persicinum	CBS 479.83	MN170495	MN170428	MN170361
F. radicigenum	GUCC 197371.1	OR043907	OR043851	OR043752
F. radicigenum	GUCC 197425.1	OR043908	OR043852	OR043753
F. radicigenum	GUCC 197221.1 = CGMCC3.25478^T	OR043909	—	OR043754
F. rhinolophicola	KUMCC 21–0449	OR026001	OR025917	OR022061
F. ruthhalliae	BRIP 72406h	OP627085	OP627084	—
F. scirpi	NRRL 36478	GQ505654	GQ505832	GQ505566
F. serpentinum	CBS 119880	MN170499	MN170432	MN170365
F. sulawesiense	Indo138	LS479443	LS479855	LS479422
F. sulaweaiense	MFLUCC 24-0629	PV394835	PV394830	PV297816
F. sylviaearleae	BRIP 72816b	OR269444	OR269438	—
F. tanahbum-buense	Indo176	LS479448	LS479863	LS479432
F. tangerinum	JZG-2022b	OP487189	OP486758	OP486067
F. toxicum	CBS 406.86	MN170508	MN170441	MN170374
F. weifangense	LC18333	OQ125107	OQ125515	OQ125276
F. wereldwij-sianum	NL19-94.009	MZ921850	MZ921718	MZ921538

The new taxon is indicated in bold. The ex-type strains are indicated by ^{T}.

during the burn-in phase of the analysis, which was assessed using Tracer v1.7[35]. The phylograms were shown and changed using FigTree v1.4.0[36] and Adobe Illustrator version 24.3 (Adobe Systems, San Jose, CA, USA).

Pathogenicity tests

Fungal inoculum preparation

The pathogenicity test was performed using the *Fusarium flavens* (SDBR-CMU869 = KUNCC 25-20161) for Koch's postulates. The fungal isolate was grown on PDA plates and incubated at 25 ± 2 °C for one week. To prepare for the suspension, 15 mL of sterile distilled water (DW) was added to PDA plates containing *F. flavens*. The spores were gently dislodged using a sterile rod, the resulting liquid was filtered through four layers of sterile gauze, and collected in a 50 mL tube. The filtrate was then centrifuged at 900 rpm, 4 °C for 10 min. After discarding the supernatant, the pellet was resuspended in 20 mL of sterile distilled water, centrifuged again, and decanted. The final pellet was suspended in 15 mL of sterile distilled water, and the spore concentration was determined using a hemocytometer under the Olympus light microscope (Olympus CH30RF200, Japan), then adjusted to 10^6 spores·mL^{-1}.

Detached leaf and stem assay procedure

Before being tested, asymptomatic plant tissues (leaves and stems) were collected from a *C. sativa* (hemp) plantation (45-day-old plants), kept in sterile plastic bags, and carried to the laboratory. Leaves and stems were sterilized with a 0.2% sodium hypochlorite (NaOCl) solution (v/v) for 3 min, washed three times with DW, and air-dried. After that, a uniform wound (3 pores, 1 mm in width) was made at the equator of each leaf and stem using an aseptic needle. To fulfil Koch's postulates, inoculations were performed using a mycelial plug and a spore suspension. A 5-mm-diameter plug of *F. flavens* was taken from the actively growing edge of the fungal colonies and placed onto the surface of each healthy plant tissue. Sterile PDAC (potato dextrose agar supplemented with chloramphenicol) plugs served as negative controls. The inoculated tissues were positioned on a sterile plastic net inside sterile 90 mm petri dishes, which contained moistened sterile paper towels soaked with 1 mL of DW to maintain humidity. The 10 μL of spore suspension was evenly dropped into healthy plant tissues[14]. Three replications of each treatment were performed. The samples were incubated at 25 ± 2 °C for 3–7 d until disease symptoms appeared. After the appearance of symptoms, the plates were examined for fungal growth and symptom progression.

Detached seedlings assay procedure

To perform a pathogenicity test on hemp seedlings, spore suspensions were prepared from cultures of *F. flavens* as previously described. Hemp seeds were surface-sterilized with 2.5% NaOCl solution for 5 min, washed three times with DW, and then germinated on moist filter paper in sterile plastic boxes for 3–5 d at 25 ± 2 °C. The seedlings were transferred into pots (7 cm high × 8 cm wide) containing a sterilized mixture of vermiculite : perlite : peat moss (1:1:2) for 2 weeks under greenhouse conditions. Fifteen two-week-old hemp seedlings were gently washed with DW to remove debris and then soaked in the spore suspensions, while the control seedlings were soaked in DW for 30 min. After inoculation, each seedling was transplanted into a pot (14.5 cm high × 14 cm wide) containing 800 g of sterilized soil[14]. The plants were cultivated at ambient temperatures ranging from 25 to 34 °C and substrate moisture content within a range of 58% to 81% for 30 d. The pot experiment was conducted in a greenhouse located at the Faculty of Science, Chiang Mai University, Thailand. Throughout the experiment, plants were monitored for wilt symptoms, such as slow growth and yellowing. The level of disease was assessed based on plant growth rates and categorized into four classes: mild (1%–25%), moderate (26%–50%), severe (51%–75%), and very severe (76%–100%). Disease incidence (DI) was calculated as the percentage of diseased plants using the formula: DI (%) = (Number of diseased plants/Total number of plants) × 100. Each isolate was tested in triplicate to ensure consistency and reliability.

To fulfill Koch's postulate, fungi associated with disease symptoms were re-isolated from infected detached tissues and seedlings. The re-isolated fungi were identified based on morphological characteristics and DNA sequence analysis, thus fulfilling Koch's postulates.

Results

Phylogenetic analyses

The BLAST results revealed that our isolate had the highest similarity to members of the *Fusarium incarnatum-equiseti* species complex. Therefore, this complex was selected for detailed phylogenetic analysis. The combined sequence dataset of *tef1-α*, *rpb2,* and *cal* comprised 96 *Fusarium* strains in *F. incarnatum-equiseti* species complex (FIESC), and one outgroup taxon, *F. concolor* (NRRL 13459) (Fig. 1). The aligned dataset consisted of a total 2,954 characters with gaps (*tef1-α*: 1–623 bp, *rpb2*: 624–2,356 bp, *cal*: 2,357–2,954 bp). The best-scoring maximum likelihood (RAxML) tree was selected to represent the relationships among taxa, with a final likelihood value of –15,909.427308. The matrix contained 963 distinct alignment patterns, with 24.92% undetermined characters or gaps. Estimated base frequencies were as follows: A = 0.260395, C = 0.258686, G = 0.244464, and T = 0.236456; substitution rates were AC = 1.412250, AG = 4.053456, AT = 1.124038, CG = 0.659120, CT = 8.715739, and GT = 1.000000; gamma distribution shape parameter α = 0.305373, Tree Length = 1.140524. For Bayesian inference (BI) analysis, the best-fitting model of each locus and the final average standard deviation of split frequencies were determined as follows: the average standard deviation of split frequencies was 0.009952, while the best-fit substitution models were GTR + I + G for *tef1-α*, GTR + I + G for *rpb2*, and SYM + G for *cal*. Similar tree topologies were acquired from ML and BI analyses. In the phylogenetic tree (Fig. 1), the new species, *F. flavens* (SDBR-CMU869 = KUNCC 25-20161), formed an independent branch basal to *F. caulendophyticum* (CGMCC 3.25474 and GUCC 191050), and *F. weifangense* LC 18333 with 72% ML and 1.00 BYPP support (Fig. 1).

Taxonomy

Fusarium flavens. Ei, Phookamsak, Monkai & S. Lumyong, *sp. nov*. (Fig. 2)

Index Fungorum number: IF 904383.

Etymology—name refers to the yellowish colour of cultures on the PDA and V8 juice agar.

Holotype—(CMUB 40081) isolated from *Cannabis sativa* (hemp) stems, Chiang Mai, Thailand.

Pathogenic fungus occurring on the stems of *Cannabis sativa* (hemp) in Northern Thailand. Sexual morph: not observed. Asexual morph: *Sporodochia* not observed. *Conidiophores* macronematous and developed on the aerial mycelium. *Aerial phialides* subulate to subcylindrical, smooth- and thin-walled, 6–33 × 2–5 μm ($\overline{x}$ = 20.9 × 3.4 μm, n = 15). *Microconidia* not observed. *Macroconidia* formed by phialides on aerial mycelium, falcate, curved dorsiventrally with almost parallel sides tapering slightly towards both ends, 2–5-septate, hyaline, smooth- and thin-walled; 21–47 × 2–5 μm ($\overline{x}$ = 34.1 × 4.2 μm, n = 30). Chlamydospores not observed.

Culture characteristics: *Fusarium flavens* (SDBR-CMU869 = KUNCC 25-20161) grew in darkness at 30 °C after 7 d on PDA, SNA, YEA, V8, and OA. Colonies on PDA reached 80 mm in diam. after 7 d of incubation, with abundant mycelial growth, the colony surface turned to creamy white, reverse pale yellow. Colonies on SNA reached 82 mm in diam. after 7 d of incubation, colonies are membranous to woolly, white, with abundant sporulation on the surface, giving a powdery appearance; reverse colourless. Colonies on YEA reached 82 mm in diam. after 7 d of incubation, white, with sparse to moderate aerial mycelium, slightly raised center, and the same reverse colour. Colonies on V8 juice agar medium reached 80 mm in diam. after 7 d of incubation, pale orange with floccose mycelium, reverse saffron yellow. Colonies on OA reached 83 mm in diam. after 7 d of incubation, white to orangish-white, dense and woolly, resembling a cotton-like mass, reverse creamy white colour (Fig. 2).

Material examined—Thailand, Chiang Mai Province, Mae Rim District, Mae Sa Noi Village, unhealthy stems of hemp (*Cannabis sativa*, Cannabaceae), 18°51'50.06" N, 98°50'45.57" E; elevation 1,285 m/4,216 ft, 12 December 2023, Toe Swe Zin Ei, PS4 (CMUB 40081, holotype), ex-type, SDBR-CMU869 = KUNCC 25-20161.

Notes: According to nucleotide BLAST search, the *tef1-α*, *rpb2,* and *cal* sequences of the new isolate *Fusarium flavens* (SDBR-CMU869 = KUNCC 25-20161) showed the highest similarity to *Fusarium* sequences available in GenBank. Details of the closest BLAST matches and percentage identities are presented in Table 3. Our phylogeny depicts a close association of *F. flavens* with *F. humuli,* as well as *F. weifangense* and *F. caulendophyticum* (Fig. 1). The nucleotide comparison among our new isolate (SDBR-CMU869 = KUNCC 25-20161), and the ex-type strains of *F. caulendophyticum*, *F. weifangense*, and *F. humuli* indicated reasonable nucleotide differences to justify the establishment of a novel species as recommended by Jeewon & Hyde[37] (Table 4).

Fusarium caulendophyticum, described from healthy stems of *Rosa roxburghii* in Guizhou Province, China[38], possesses irregularly branched conidiophores with lateral and terminal mono or polyphialides. In contrast, *F. flavens* develops simpler, subulate to subcylindrical aerial phialides, with larger conidiogenous cells (6–33 × 2–5 vs 10–24 × 2–3 μm) and larger macroconidia (21–47 × 2–5 μm vs 16–27 × 2.5–4 μm),[38] compared with those in *F. caulendophyticum*.

Fusarium weifangense, isolated from symptomatic tissues of *Triticum aestivum* in Shandong Province, China[39], forms densely

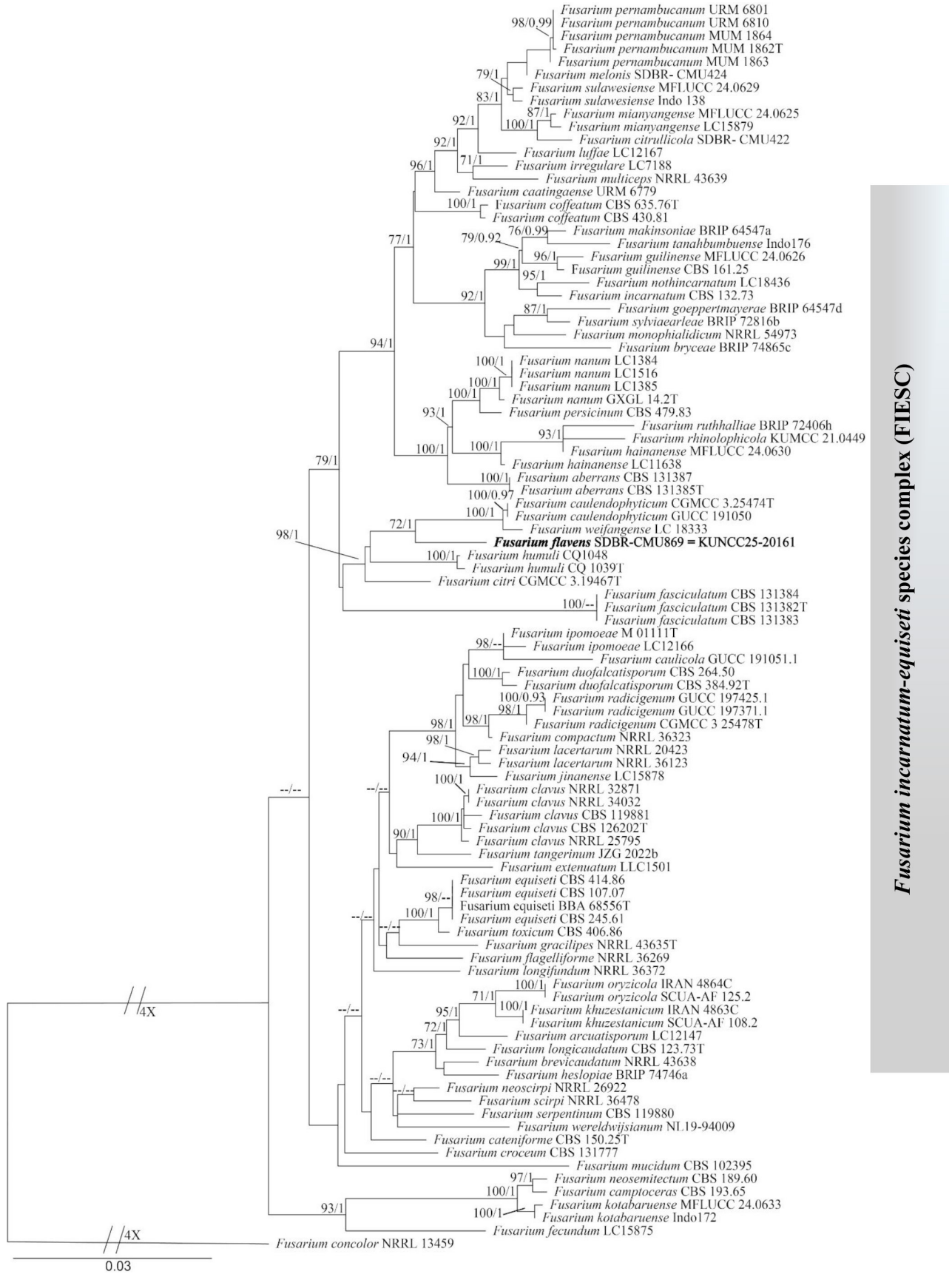

Fig. 1 Phylogram constructed by maximum likelihood (ML) analyses of the combined *tef1-α*, *rpb2*, and *cal* sequence dataset of *Fusarium*. Bootstrap support valued for ML and Bayesian posterior probabilities (BYPP) equal to or greater than 70%, and 0.90, are shown above the nodes as ML/BYPP. The tree is rooted to *F. concolor* (NRRL 13459). The newly generated strain is indicated by black bold. T indicates the ex-type strains.

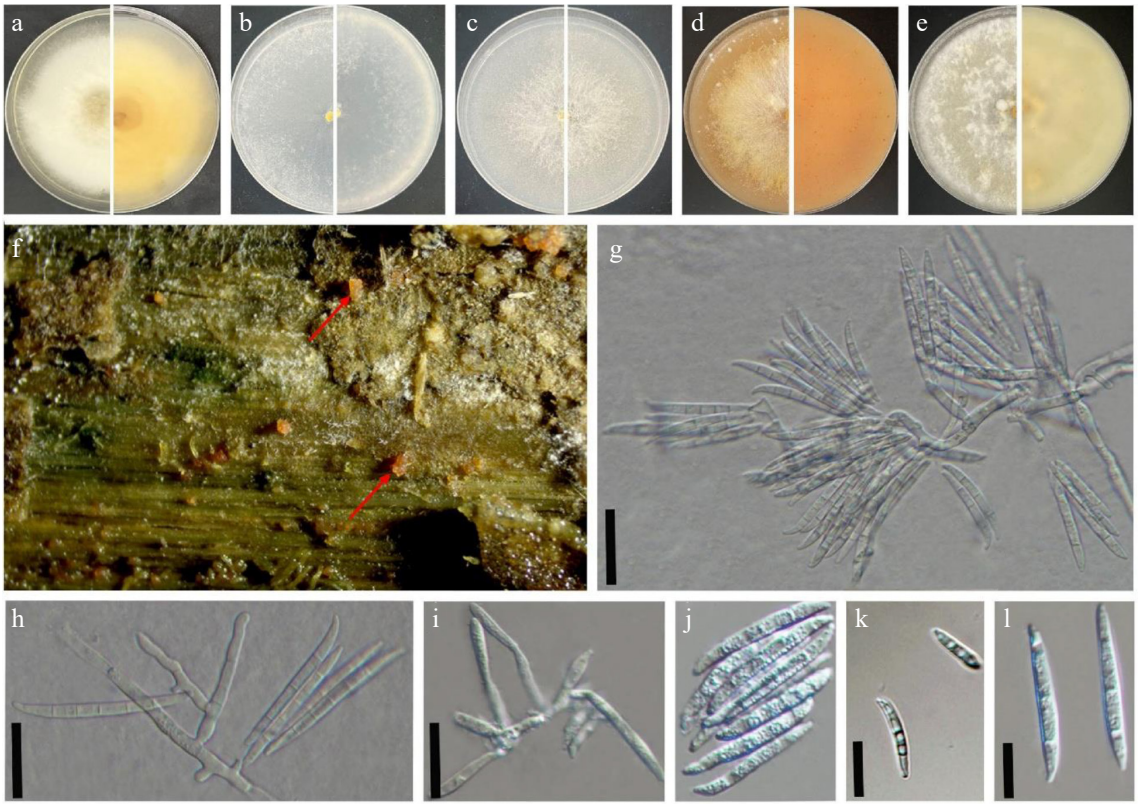

Fig. 2 *Fusarium flavens* (SDBR-CMU869 = KUNCC 25-20161, ex-type). (a)–(e) Seven-day-old culture on PDA, SNA, YEA, V8 juice agar, and OA incubated at 30 °C (top and reverse). (f) Natural symptoms of stem disease on *Cannabis sativa* (red arrow indicates the symptoms). (g), (h) Sporodochial conidiophores. (i) Phialides. (j)–(l) Macroconidia found on PDA media incubated at 30 °C. Scale bars: (g)–(i) = 100 μm; (k), (l) = 10 μm.

Table 3. Nucleotide BLAST similarity results among several similar strains of *Fusarium flavens*.

Genes	Percentage similarity across similar species		
tef1-α	*F. citri* NRRL 52765 (98.24%) JF740839	*Fusarium* sp. FS5 (98.10%) JQ244848	*Fusarium* sp. CMW55738 (97.94%) MZ966178
rpb2	*F. humuli* hnxryc2 (98.82%) OR257587	*F. humuli* JXRC 11 (98.71%) MZ824673	*F. humuli* GL 17-3 (98.71%) OQ512810
cal	*F. humuli* LC18763 (99.27%) OQ125277	*F. humuli* LC18553 (99.27%) OQ125278	*F. humuli* CMML20-13 (98.99%) OL331025

Table 4. Nucleotide differences among closely related strains of *Fusarium flavens*.

Compared strains	Gene region/locus		
	tef1-α	*rpb2*	*cal*
F. humuli (CQ 1039, ex-type)	2.8% (18/629 bp) MK289570	1.1% (10/874 bp) MK289724	0.5% (3/531 bp) MK289712
F. weifangense (LC18333, ex-type)	3.4% (19/551 bp) OQ125107	2.1% (15/751 bp) OQ125515	1.4% (8/548 bp) OQ125276
F. caulendophyticum (CGMCC3.25474, ex-type)	2.9% (10/650 bp) OR043881	2.0% (19/932 bp) OR043826	1.6% (10/595 bp) OR043731

branched sporodochial conidiophores with apical whorls of 3 to 4 phialides. In contrast, *F. flavens* produces macronematous aerial conidiophores with subulate to subcylindrical phialides and smaller conidia, compared with those in *F. weifangense* (21–47 × 2–5 μm vs 26.5–49.4 × 4.1–7.1 μm)[39].

Fusarium humuli, described from leaves of *Humulus scandens* in Jiangsu Province, China[22], produces pale orange sporodochia with densely packed, verticillately branched conidiophores bearing small subulate to subcylindrical phialides (6.3–11.9 × 2–3.4 μm). In contrast, *F. flavens* forms only aerial conidiophores with larger phialides (6–33 × 2–5 μm) and larger conidia (21–47 × 2–5 μm vs 21–35 × 2–3 μm)[22]. Based on these morphological differences and phylogenetic evidence, *F. flavens* (SDBR-CMU869 = KUNCC 25-20161) is introduced here as a novel species.

Pathogenicity test on detached tissues

The pathogenicity test of *F. flavens* (SDBR-CMU869 = KUNCC 25-20161) on hemp was carried out using a detached leaves and stems assay (Fig. 3a–h). After inoculation with the fungal mycelium plug, the symptoms of brown lesions developed rapidly and spread onto the leaves and stem surface after 3 d of incubation (Fig. 3b, d). In contrast, control leaves and stems receiving treatment with sterile PDAC plugs remained healthy and symptom-free, as shown in (Fig. 3a, c). In the spore suspension inoculation method, brown lesions and wilting developed and spread across the surface of the leaves and stems (Fig. 3f, h). Conversely, the control leaves and stems receiving DW remained healthy and showed no disease symptoms (Fig. 3e, g).

Pathogenicity test on detached seedlings

In the hemp seedling test, *Fusarium flavens* was also evaluated for its ability to cause disease (Fig. 3i–l). Four weeks after inoculation, seedlings inoculated with *F. flavens* exhibited slower growth compared to uninoculated controls. Slow development of symptoms

Fig. 3 Pathogenicity tests on detached hemp leaves, stems, and seedlings. (a) Control leaves, and (c) stems, inoculated with a PDAC plug. (b), (d) Symptoms after being inoculated with *Fusarium flavens* (SDBR-CMU869 = KUNCC 25-20161) mycelium plug for 7 d. (e) Control leaves, and (g) stems, inoculated with distilled water (DW). (f), (h) Symptoms after being inoculated with *F. flavens* (SDBR-CMU869 = KUNCC 25-20161) spore suspension for 7 d. Inoculation of hemp seedling control with (i) DW, compared with (j) that inoculated with *F. flavens* (SDBR-CMU869 = KUNCC 25-20161) spore suspension. (k) The roots of a plant inoculated with DW (control) shows no symptoms. (l) The roots of a plant inoculated with *F. flavens* (SDBR-CMU869 = KUNCC 25-20161) spore suspension shows root rot symptoms (30 d).

Table 5. Comparison of plant height between control and inoculated plants.

Treatment	Number of plants (*n*)	Plant height ($\bar{x}$ ± SD) (cm)	Disease incidence (%)
Control plant	15	83.82 ± 5.67	no symptoms
Inoculated plant	15	56.13 ± 9.03	73%

Disease severity was evaluated based on plant growth performance. Growth reduction (%) relative to the control was calculated and reported in the Results section.

was observed in approximately 73% of the seedling inoculated with *F. flavens*, respectively. The average plant height of control and inoculated plants is presented in Table 5. The remaining plants showed reduced and stunted development (Fig. 3i, j [right], and l), while the control, non-inoculated, saline-watered seedlings continued to grow healthily (Fig. 3i, j [left] and k). The results of isolated pathogenic strains of *F. flavens* caused varying symptoms in infected leaves, stems, and seedlings. The fungi were re-isolated from infected plant parts for Koch's postulate assays, and were confirmed based on their morphological characteristics that this fungus was the disease-causative agent.

Discussion

The identification of *Fusarium* species, particularly within the *Fusarium incarnatum–equiseti* species complex (FIESC), requires a combined taxonomic approach integrating both morphological and molecular phylogenetic analyses. Morphological traits, such as colony growth, spore type, size, and septation are important but often insufficient due to overlapping characteristics and cryptic variation among closely related species[22]. Ribosomal DNA markers (ITS and LSU) are widely used, but generally lack the resolution to distinguish among cryptic taxa[40–43]. Therefore, protein-coding

genes, including *tef1-α*, *rpb2*, and *cal*, are regarded as more informative and are increasingly adopted as standard markers for species delimitation in *Fusarium*[38,44].

In practice, accurate identification involves combining morphological examination with multigene phylogenetic analysis, which provides robust species boundaries and resolves hidden diversity. This approach is particularly important in FIESC, which contains numerous cryptic species and newly described taxa[38,44]. In the present study, *Fusarium* isolates were recovered from hemp stem rot in Northern Thailand. Based on both morphology and multigene phylogeny (*tef1-α*, *rpb2,* and *cal*), one isolate was determined to represent a novel species, *F. flavens* (SDBR-CMU869 = KUNCC 25-20161), in accordance with established taxonomic practices[38,44]. This study aimed to isolate and identify fungal pathogens associated with *C. sativa,* and to confirm their pathogenicity by fulfilling Koch's postulates using detached plant tissues and attached seedlings. The causal agent of stem rot, *F. flavens*, was found on hemp stems. Several *Fusarium* species are significant pathogens of *C. sativa* in North America. For instance, *F. falciforme* has been identified as the causal agent of root rot in the United States[45]. In Israel, soil-borne species such as *F. oxysporum*, *F. proliferatum*, and *F. solani* have been associated with disease development in cannabis[14]. Additionally, *F. proliferatum* has been documented as the cause of stem rot in Canada[46]. During our taxonomic investigation of pathogenic fungi, several *Fusarium* species were identified based on morphological characteristics, nucleotide pairwise comparisons, and phylogenetic evidence. One of them, *F. flavens*, a novel species, is reported here for the first time on *C. sativa*. The novel species, *F. flavens* was found to be phylogenetically related to *F. caulendophyticum* and *F. weifangense* (Fig. 1). However, the phylogenetic placement of *F. flavens* received relatively moderate bootstrap support (Fig. 1), indicating the need for further investigation. However, the independent lineage and the nucleotide differences support the establishment of a new species. In this study, pathogenicity was confirmed using both detached tissues and seedling tests, demonstrating that the *F. flavens* isolate obtained from hemp stems can also cause both stem and root rot symptoms. In pathogenicity tests, brown lesions and wilting developed in detached tissues, while seedling inoculations resulted in root rot symptoms. The severity varied among test conditions, indicating that the pathogenic potential of this isolate may be influenced by environmental factors.

In Thailand, *Fusarium* species have been documented to affect a variety of crops. For example, *F. proliferatum*, *F. thapsinum*, and *F. verticillioides* have been found on sorghum[47]. Additionally, *F. oxysporum* has been reported in banana farms in Chiang Rai, causing significant crop losses and posing a serious threat to banana production[48]. Moreover, two species of *Fusarium*, *F. citrullicola* and *F. melonis*, have been identified from rot lesions on watermelon and muskmelon fruits[49]. Recent studies have also discovered three novel causal agents of muskmelon fruit rot: *F. compactum*, *F. jinanense*, and *F. mianya*[50]. A recent study by Samarakoon et al.[51] described *F. pernambucanum* as a causal agent of bud rot of hemp in northern Thailand. There is a need to do further research on this genus in Thailand, given its potential to cause diseases in many economically important crops.

Conclusions

In conclusion, these findings highlight the presence and potential impact of *Fusarium* species in *C. sativa* cultivation in Chiang Mai Province, Northern Thailand. The confirmation of their pathogenicity provides a valuable basis for future research for effective disease management, and the development of resistant cultivars. Overall, this study expands our understanding of the fungal diversity and pathogenic threats affecting *C. sativa* in Thailand and underscores the need for ongoing monitoring and integrated management strategies to reduce potential economic losses in hemp production.

Author contributions

The authors confirm contributions to the paper as follows: study conception and design: Ei TSZ, Phookamsak R, Monkai J, Jatuwong K, Aiduang W, Lumyong S; data collection: Ei TSZ, Phookamsak R; Monkai J, Jatuwong K, Aiduang W; analysis and interpretation of results: Ei TSZ, Phookamsak R; Monkai J, Jatuwong K, Aiduang, Hongsanan S, Khuna S; draft manuscript preparation Ei TSZ, Phookamsak R, Jeewon R, Monkai J, Jatuwong K, Aiduang, Hongsanan S, Khuna S, Lumyong S. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All sequences generated in this study were submitted to GenBank.

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Conflict of interest

The authors declare that they have no conflict of interest.

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