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Occurrence of *Aspergillus chevalieri* and *A. niger* on Herbal Tea and Their Potential to Produce Ochratoxin A (OTA)

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Abstract: Herbal teas, including *Camellia sinensis* (black and green teas), are popular beverages with health benefits for consumers worldwide. These products are prepared from natural materials of different plant parts containing antioxidant properties and vitamins. The aim of this study was to investigate fungal contaminants and their ability to produce ochratoxin A (OTA) in herbal tea samples. Seven herbal teas were obtained from local markets in Chiang Rai, northern Thailand. Samples were incubated on potato dextrose agar (PDA), and the growing mycelia were isolated into a pure culture. The cultures were identified via both morphology and molecular analysis to confirm species identification. The identified species were subjected to OTA analysis using high-performance liquid chromatography (HPLC) with a fluorescence detector. Ochratoxin A was produced by *Aspergillus chevalieri* and *A. niger*, isolated from seven herbal tea samples (black tea, green tea, bael fruit, goji berry, jasmine, lavender, and rose). This finding raises concerns about the safety of herbal tea and should be investigated further for potential health implications.

Keywords: *Aspergillus*; herbal teas; molecular phylogeny; ochratoxin A; taxonomy

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1. Introduction

Phytotherapeutic sources have become important for healthy drink and food consumption and healthcare. Herbal teas have become popular beverages [1,2], with *Camellia sinensis* L. tea being the most consumed drink in the world [3]. In this paper, herbal tea refers to the aromatic brewing of diverse parts of plants known as herbs (such as leaves, flowers, seeds, bark, stems, and roots) [4–6].

The naturally occurring bioactive substances or phytochemicals in herbal teas are released through infusion [7–9]. These bioactive compounds include antioxidants and other therapeutic properties, while tea contains rather high amounts of caffeine [1,4,10]. Some of the most popular herbal teas include bael fruit, chamomile, chrysanthemum, jasmine, lavender, marigold, pomegranate, safflower, and rose [11–13]. In Thailand, there are several popular flower teas, such as butterfly-pea, chrysanthemum, jasmine flower, rose, roselle, or safflower. They contain color, flavor, taste, fragrance, aesthetic qualities, and antioxidant activities [14,15]. However, most of the herbal teas are produced by local farmers and do not undergo quality inspections, unlike tea products processed by the industry. Some of these herbal plants have been shown to be contaminated by toxigenic fungi in Asian countries such as China, India, Sri Lanka, and Thailand [16].

Herbal tea products include several parts of dried plants, which are suitable substrates for the growth of microorganisms, especially toxicogenic fungi [16–19]. In the natural environment, fungi habitually grow on organic and inorganic substrates [20–22]. Their

presence can negatively impact human health, cause infectious diseases, and contaminate food or food ingredients [23,24]. They can also deteriorate agricultural food crops and products under poor post-harvest facilities [25]. A large number of foodborne fungi, also known as storage fungi, are able to produce one or more toxic secondary metabolites (mycotoxins) that cause a wide array of negative effects and other complications in animals and humans [26–28].

In Thailand, climatic conditions characterized by high temperatures and high humidity promote the growth of fungi that can produce mycotoxins [29–32]. Therefore, several agricultural commodities are subjected to mycotoxin contamination such as animal feed, beans, cereal grains, spices, leguminous plants, dried fruits, mushrooms, herbs, and teas [32–37]. Several fungal genera can produce mycotoxins, with the most prevalent species being, e.g., aflatoxins (*Aspergillus flavus*, *A. parasiticus*), fumonisins (*Fusarium verticillioides*), ochratoxins (*A. ochraceus*, *Penicillium verrucosum*), sterigmatocystin (*A. versicolor*), trichothecenes (*F. graminearum*), and zearalenone (*F. graminearum*) [38–47].

Ochratoxin A, produced by species within *Aspergillus* and *Penicillium*, is one of the most significant toxins that affects agricultural products and human health worldwide [48,49]. Magan and Aldred [50] reported that *A. niger* within section *Nigri*, especially *A. carbonarius*, can produce OTA contamination in grapes, wine, and vine fruits. Han et al. [51] reported OTA contamination in the Chinese food industry from some strains of *A. niger*. On the other hand, *A. chevalieri* produces aflatoxins, citrinin, flavoglaucin, gliotoxin, and sterigmatocystin, but OTA production has not been reported in this species [52–55].

In this study, we investigated the potential of *Aspergillus* species isolated from herbal tea samples from local markets in Chiang Rai Province and their ability to produce OTA. *A. chevalieri* and *A. niger* isolated from seven herbal tea samples were found to produce OTA. The species were identified and are illustrated using both morphological and molecular data. The implications of detecting these species in such products are discussed.

2. Materials and Methods

2.1. Samples Collection and Fungal Isolation

Herbal teas were randomly purchased from five local markets in Chiang Rai Province (Doi Mae Salong, Fah Thai, Mae Sai, and Lan Muang). They included bael fruit, *Camellia sinensis* (black and green teas), jasmine, goji berry, lavender, and rose (Table 1). Isolation of fungi from the samples was performed under sterile conditions following the method described by Senanayake et al. [56]. One random piece of each tea sample was placed directly on potato dextrose agar (PDA) and incubated at 25 °C for 5 days. Mycelia growing from the herbal tea samples were individually transferred to fresh PDA plates to obtain pure cultures and for identification.

Table 1. Tea and herbal teas used in this study obtained from local markets in northern Thailand.

No.	Herbal Tea	Scientific Name and Family	Herbal Tea Substrate	Number of Samples (Package)	Local Markets
1	Black Tea	*Camellia sinensis*, *Theaceae*	leaves	6	Doi Mae Salong Fah Thai Mae Sai
2	Bael Fruit	*Aegle marmelos*, *Rutaceae*	fruits	4	Doi Mae Salong Fah Thai Mae Sai
3	Goji Berry	*Lycium* sp., *Solanaceae*	fruits	2	Doi Mae Salong Fah Thai
4	Green Tea	*Camellia sinensis*, *Theaceae*	leaves	6	Doi Mae Salong Mae Sai Lan Muang

Table 1. *Cont.*

No.	Herbal Tea	Scientific Name and Family	Herbal Tea Substrate	Number of Samples (Package)	Local Markets
5	Jasmine	*Jasminum* sp., *Oleaceae*	flowers	2	Doi Mae Salong
6	Lavender	*Lavandula* sp., *Lamiaceae*	flowers	4	Lan Muang Mae Sai
7	Rose	*Rosa* sp., *Rosaceae*	flowers	2	Doi Mae Salong

2.2. Macro- and Microscopic Identification of Fungi

Macroscopic and microscopic characteristics were examined by following the identification methods used in previous studies [57–59] and structures were measured following Senanayake et al. [56]. Macro- and micro-characteristics, such as conidiophores, conidiogenous cells, and conidia, were observed and photographed using the Nikon Eclipse Ni-U compound microscope connected to the Nikon DS-Ri2 digital camera. The photoplates were prepared with Adobe Photoshop CS3 Extended version 10.0 software (Adobe Systems, San Jose, CA, USA). Specimens were deposited at the Fungarium of Mae Fah Luang University (MFLU), and living cultures were deposited at Mae Fah Luang University Culture Collection (MFLUCC), Chiang Rai, Thailand.

2.3. DNA Extraction, PCR Amplification, Sequencing, and Phylogenetic Analyses

Genomic DNA was extracted from fresh mycelium colonies grown on PDA using the manufacturer's protocol for Genomic DNA Extraction Kits (OMEGA Bio-Tek Inc., Norcross, GA, USA). Four gene regions were amplified using the corresponding pairs of primers: the internal transcribed spacer (ITS), ITS5/ITS4 [60,61]; β-tubulin (BenA), Bt2a/Bt2b [62,63]; calmodulin (CaM), CMD5/CMD6, CL1/CL2A [63,64]; and RNA polymerase II second largest subunit (RPB2), RPB2f-5f/RPB2f-7cr [65].

Polymerase chain reaction (PCR) was performed in a volume of 25 µL reaction process containing 12.5 µL of 2× Power Taq PCR Master Mix, 1 µL of each primer (20 µM), and 1 µL of 50 ng of DNA template in 9.5 µL of deionized water. PCR amplification conditions for each gene were performed following previous studies (Table 2). The PCR products were purified according to the company protocols and DNA sequencing was performed using Sanger sequencing at Solgent Co., Ltd., Daejeon, South Korea.

Phylogenetic analyses to identify fungal species were performed as described in Dissanayake et al. [69]. The fungal sequence data obtained from this study were deposited in GenBank (Tables 3 and 4).

Table 2. PCR amplification conditions used in the thermal cycler of each gene.

Gene	Primers (Forward/Reverse)	PCR Condition	References
β-tubulin (BenA)	Bt2a/Bt2b	1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s; 55 °C for 50 s; 72 °C for 1 min; and a final extension at 72 °C for 10 min	Frisvad et al. [58], Hubka et al. [66,67], Samson et al. [68]
Calmodulin (CaM)	CMD5/CMD6 CL1/CL2A	1 cycle at 95 °C for 5 min; 35 cycles of 94 °C for 45 s; 55 °C for 45 s; 72 °C for 1 min; and a final extension at 72 °C for 10 min	Frisvad et al. [58], Hubka et al. [66,67], Samson et al. [68]

Table 2. *Cont.*

Gene	Primers (Forward/Reverse)	PCR Condition	References
Internal transcribed spacer (ITS)	ITS5/ITS4	1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s; 55 °C for 50 s; 72 °C for 1 min; and a final extension at 72 °C for 10 min	Frisvad et al. [58], Hubka et al. [66,67], Samson et al. [68]
RNA polymerase II second largest subunit(RPB2)	RPB2f-5f/RPB2f-7cr	1 cycle at 95 °C for 5 min; 40 cycles of 95 °C for 1 min; 57 °C for 1 min; 72 °C for 1 min; and a final extension at 72 °C for 10 min	Frisvad et al. [58], Samson et al. [68]

Table 3. GenBank and culture collection numbers of *Aspergillus* section *Aspergillus* used in the phylogenetic analysis. The newly generated sequences are indicated in blue.

Taxa	Culture Collection No.	GenBank Accession No.			
		ITS	BenA	CAM	RPB2
A. aerius	CBS 141771	LT670916	LT670990	LT670991	LT670992
A. appendiculatus	CBS 101746	HE615133	HE801334	HE801319	HE801308
A. appendiculatus	CBS 374.75 ^{T}	HE615132	HE801333	HE801318	HE801307
A. aurantiacoflavus	CBS 141930 ^{T}	LT670917	LT670993	LT670994	LT670995
A. aurantiacoflavus	CCF 5391	LT670918	LT670996	LT670997	LT670998
A. brunneus	CBS 113.27	EF652056	EF651904	EF651997	EF651938
A. brunneus	CBS 112.26 ^{T}	EF652060	EF651907	EF651998	EF651939
A. caperatus	CBS 141774 ^{T}	LT670922	LT671008	LT671009	LT671010
A. chevalieri	DTO 092-D3	LT670929	LT671029	LT671030	LT671031
A. chevalieri	CBS 141769	LT670927	LT671023	LT671024	LT671025
A. chevalieri	CBS 522.65 ^{T}	EF652068	EF651911	EF652002	EF651954
A. chevalieri	MFLUCC 23-0094	OR478693	OR508967	N/A	N/A
A. chevalieri	MFLUCC 23-0095	OR478694	OR508966	OR508963	N/A
A. chevalieri	MFLUCC 23-0096	OR478695	OR508968	OR508964	N/A
A. chevalieri	MFLUCC 23-0097	OR478696	OR508969	OR508965	N/A
A. chevalieri	MFLUCC 23-0184	OR502380	OR573934	OR604627	OR604629
A. chevalieri	MFLUCC 23-0185	OR501403	OR573933	N/A	OR604628
A. cibarius	KACC 46346 ^{T}	JQ918177	JQ918180	JQ918183	JQ918186
A. cibarius	KAC 46764	JQ918178	JQ918184	JQ918181	JQ918187
A. costiformis	CBS 101749 ^{T}	HE615136	HE801338	HE801320	HE801309
A. cristatus	CBS 123.53 ^{T}	EF652078	EF651914	EF652001	EF651957
A. cumulatus	KACC 47316	KF928303	KF928297	KF928300	KF928294.
A. endophyticus	CBS 141766 ^{T}	LT670941	LT671067	LT671068	LT671069
A. equitis	NRRL 25823	EF652073	EF651895	EF652015	EF651961
A. glaucus	CBS 516.65 ^{T}	EF652052	EF651989	EF651887	EF651934
A. glaucus	NRRL 117	EF652053	EF651990	EF651888	EF651935
A. heterocaryoticus	NRRLA-13891 ^{T}	EU021619	EU021670	EU021687	EU021659
A. intermedius	CBS 377.75	HE974459	HE974432	HE974437	HE974425
A. intermedius	CBS 523.65 ^{T}	EF652074	EF651892	EF652012	EF651958
A. leucocarpus	CBS 353.68 ^{T}	EF652087	EF651925	EF652023	EF651972
A. levisporus	CBS 141767 ^{T}	LT670950	LT671094	LT671095	LT671096
A. mallochii	CBS 141928 ^{T}	KX450907	KX450889	KX450902	KX450894
A. mallochii	CBS 141776	KX450908	KX450890	KX450903	KX450895
A. megasporus	CBS 141772	KX450911	KX450893	KX450906	KX450898
A. megasporus	CBS 141929 ^{T}	KX450910	KX450892	KX450905	KX450897
A. microperforatus	CBS 142376 ^{T}	LT627271	LT627296	LT627321	LT627346
A. microperforatus	UTHSCSADI16-400	LT627270	LT627295	LT627320	LT627345
A. montevidensis	CBS 518.65	EF652076	EF651897	EF652017	EF651963
A. montevidensis	CBS 491.65 ^{T}	EF652077	EF651898	EF652020	EF651964
A. neocarnoyi	EXF-10029	LT670955	LT671109	LT671110	LT671111

Table 3. *Cont.*

Taxa	Culture Collection No.	GenBank Accession No.			
		ITS	BenA	CAM	RPB2
A. neocarnoyi	CBS 471.65 ^{T}	EF652057	EF651903	EF651985	EF651942
A. niveoglaucus	CBS 101750	HE615135	HE801331	HE801323	HE801312
A. niveoglaucus	CBS 114.27 ^{T}	EF652058	EF651905	EF651993	EF651943
A. osmophilus	CBS 134258 ^{T}	KC473921	KC473924	KC473918	KX512310
A. porosus	CBS 375.75	LT670963	LT671136	LT671137	LT671138
A. porosus	CBS 141770 ^{T}	LT670961	LT671130	LT671131	LT671132
A. proliferans	CBS 121.45 ^{T}	EF652064	EF651891	EF651988	EF651941
A. proliferans	NRRL 114	EF652051	EF651886	EF651987	EF651933
A. pseudoglaucus	CBS 379.75	HE615131	HE801336	HE801322	HE801311
A. pseudoglaucus	CBS 123.28 ^{T}	EF652050	EF651917	EF652007	EF651952
A. ruber	CBS 530.65 ^{T}	EF652066	EF651920	EF652009	EF651947
A. ruber	CBS 101748	HE615134	HE801337	HE801325	HE801315
A. sloanii	CBS 138178	KJ775542	KJ775076	KJ775313	KX450900
A. sloanii	CBS 138177 ^{T}	KJ775540	KJ775074	KJ775309	KX463365
A. tamarindosoli	CBS 141775 ^{T}	LT670981	LT671191	LT671192	LT671193
A. teporis	CBS 141768 ^{T}	LT670982	LT671194	LT671195	LT671196
A. tonophilus	CBS 405.65 ^{T}	EF652081	EF651919	EF652000	EF651969
A. umbrosus	NRRL120	EF652054	EF651889	EF651991	EF651936
A. xerophilus	CBS 938.73 ^{T}	EF652085	EF651923	EF651983	EF651970
A. xerophilus	NRRL 6132	EF652086	EF651924	EF651984	EF651971
A. zutongqii	CGMCC 3.06103	LT670989	LT671215	LT671216	LT671217
A. zutongqii	CBS 141773 ^{T}	LT670986	LT671206	LT671207	LT671208

Note: The ex-type cultures are marked as "^{T}", and "N/A" indicates sequence is unavailable.

Table 4. GenBank and culture collection numbers of *Aspergillus* section *Nigri* used in the phylogenetic analysis. The newly generated sequences are indicated in blue.

Taxa	Culture Collection No.	Gene Bank Accession No.			
		ITS	BenA	CAM	RPB2
A. aculeatinus	CBS 121060 ^{T}	EU159211	EU159220	EU159241	HF559233
A. aculeatus	CBS 172.66 ^{T}	EF661221	HE577806	EF661148	EF661046
A. awamori	ITEM 4509 ^{T}	AM087614	AY820001	AJ964874	HE984360
A. brasiliensis	CBS 101740 ^{T}	FJ629321	FJ629272	FN594543	KY006765
A. brunneoviolaceus	CBS 621.78 ^{T}	AJ280003	EF661105	EF661147	EF661045
A. carbonarius	CBS 111.26 ^{T}	EF661204	EF661099	EF661167	EF661068
A. chiangmaiensis	SDBR-CMUI4	MW588209	MW602898	MK457199	MW602899
A. costaricaensis	CBS 115574 ^{T}	DQ900602	FJ629277	FN594545	HE984361
A. ellipticus	CBS 482.65 ^{T}	EF661194	EF661122	EF661170	EF661051
A. eucalypticola	CBS 122712 ^{T}	EU482439	EU482435	EU482433	MN969070
A. flavus	CBS 100927 ^{T}	AF027863	EF661485	EF661508	EF661440
A. flavus	NRRL 447 ^{T}	EF661560	EF661483	EF661506	EF661438
A. fijiensis	CBS 313.89 ^{T}	FJ491680	FJ491688	FJ491695	N/A
A. floridensis	NRRL 62478 ^{T}	N/A	HE984412	HE984429	HE984376
A. foetidus	CBS 121 28 ^{T}	FJ491683	FJ491690	FJ491694	N/A
A. homomorphus	CBS 101899 ^{T}	EF166063	AY820015	FN594549	N/A
A. heteromorphus	CBS 11755 ^{T}	EU821305	EF661103	EF661169	EF661050
A. hydei	KUMCC 18-0196	MT152332	MT161679	MT178247	MT384370
A. ibericus	ITEM 4776 ^{T}	NR 119514	AM419748	AJ971805	N/A
A. ibericus	NRRL 35645 ^{T}	EF661201	EF661101	EF661164	N/A
A. indologenus	CBS 114.80 ^{T}	AJ280005	AY585539	AM419750	HE984366
A. japonicus	CBS 114.51 ^{T}	AJ279985	HE577804	FN594551	N/A
A. labruscus	CCT 7800 ^{T}	KU708544	KT986014	KT986008	N/A
A. lacticoffeatus	CBS 101883 ^{T}	FJ629336	AY819998	EU163270	HE984367

Table 4. *Cont.*

Taxa	Culture Collection No.	Gene Bank Accession No.			
		ITS	BenA	CAM	RPB2
A. luchuensis	CBS 205.80 ^{T}	JX500081	JX500062	JX500071	LC179910
A. niger	CBS 554 65 ^{T}	EF661186	EF661089	EF661154	EF661058
A. niger	IHEM 2312	MH613218	MH614521	MH645010	OP082127
A. niger	IHEM 5296	MH613217	MH614519	MH645009	OP082167
A. niger	MFLUCC 23-0192	OR502379	OR594234	OR502379	OR604630
A. niger	MFLUCC 23-0193	OR501408	OR594235	OR501408	OR604631
A. niger	MFLUCC 23-0194	OR501405	OR594237	OR501405	OR604634
A. niger	MFLUCC 23-0195	OR500483	OR594236	OR500483	OR604632
A. niger	MFLUCC 23-0200	OR501402	OR573928	OR501402	OR604633
A. neoniger	CBS 115656 ^{T}	FJ491682	FJ491691	FJ491700	KC796429
A. piperis	CBS 112811 ^{T}	EU821316	FJ629303	EU163267	KC796427
A. piperis	CMV011A9	N/A	MK451187	MK451493	MK450798
A. pseudopiperis	SDBR-CMUI7 ^{T}	MK457204	MK457206	MK457205	MK457208
A. pseudopiperis	SDBR-CMUI1 ^{T}	MW588212	MW602913	MW602912	MW602914
A. pseudotubingensis	SDBR CMUO2 ^{T}	MK457204	MK457206	MK457205	MK457208
A. pseudotubingensis	SDBR CMU20 ^{T}	MW588212	MW602913	MW602912	MW602914
A. sclerotiicarbonarius	CBS 121057 ^{T}	EU159216	EU159229	EU159235	N/A
A. saccharolyticus	CBS 127449 ^{T}	HM853552	HM853553	HM853554	HF559235
A. sclerotioniger	CBS 115572 ^{T}	DQ900606	AY819996	FN594557	HE984369
A. trinidadensis	NRRL 62479 ^{T}	N/A	HE984420	HE984434	HE984379
A. tubingensis	PW3161	AB987902	LC000547	LC000560	LC000573
A. tubingensis	NRRL 4875 ^{T}	EF661193	EF661086	EF661151	EF661055
A. uvarum	CBS 121591 ^{T}	AM745757	AM745751	AM745755	HE984370
A. uvarum	ITEM 14819	N/A	HE984421	HE984435	HE984380
A. vadensis	CBS 113365 ^{T}	AY585549	AY585531	FN594560	HE984371
A. violaceofuscus	CBS 102.03 ^{T}	FJ491677	FJ491686	FJ491697	HF559234
A. welwitschiae	CBS 139.54 ^{T}	MH857271	FJ629291	KC480196	MN969100

Note: The ex-type cultures are marked as "^{T}", and "N/A" indicates sequence is unavailable.

2.4. OTA Extraction and Quantification

Isolates of *A. chevalieri* and *A. niger* were grown on yeast extract sucrose agar (YES) [68,70,71] and incubated at 25 °C in darkness for 14 days for OTA production [70]. Small pieces of culture agar plugs (6 mm diameter) of each isolate were transferred to 50 mL centrifuge tubes, and 16 mL methanol (HPLC grade) was added, followed by orbital shake at 230 rpm for 60 min, vortexing at every 20 min, and centrifugation at 2683× *g* (5000 rpm) for 15 min [48,72]. Five microliters of the solution were collected and evaporated to dryness under a nitrogen stream at 50 °C [48,73,74]. The dried extracts were dissolved in 1 mL of methanol, filtered through a 0.22 µm Polyvinylidene difluoride (PVDF) membrane filter into 2 mL amber vials, and sent to the Scientific and Technological Instruments Center (STIC), Mae Fah Luang University, for HPLC analysis. The analyses were performed on a Waters HPLC System with a 2998 PDA detector, following the manufacturer's instructions.

3. Results

3.1. Phylogenetic Analyses

Phylogenetic analyses were performed using two separate datasets, one for *Aspergillus* section *Aspergillus* and the other for *Aspergillus* section *Nigri*. Maximum likelihood and Bayesian phylogenetic trees from combined DNA sequences of BenA, CaM, ITS, and RPB2 gene regions had the same topology. The sequence dataset of *Aspergillus* section *Aspergillus* consists of 62 taxa (Table 3). Our six isolates (MFLUCC 23-0094, MFLUCC 23-0095, MFLUCC 23-0096, MFLUCC 23-0097, MFLUCC 23-0184, and MFLUCC 23-0185) are clustered with *A. chevalieri* with 100% MLBS/1.00 BYPP support (Figure 1). We, therefore, identify these strains as *A. chevalieri* (Table 5).

The sequence dataset of *Aspergillus* section *Nigri* consists of 51 taxa (Table 4). Our five isolates (MFLUCC 23-0192, MFLUCC 23-0193, MFLUCC 23-0194, MFLUCC 23-0195, and MFLUCC 23-0200) clustered with *A. niger* with 100% MLBS/1.00 BYPP support (Figure 2). We, therefore, identify these strains as *A. niger* (Table 5).

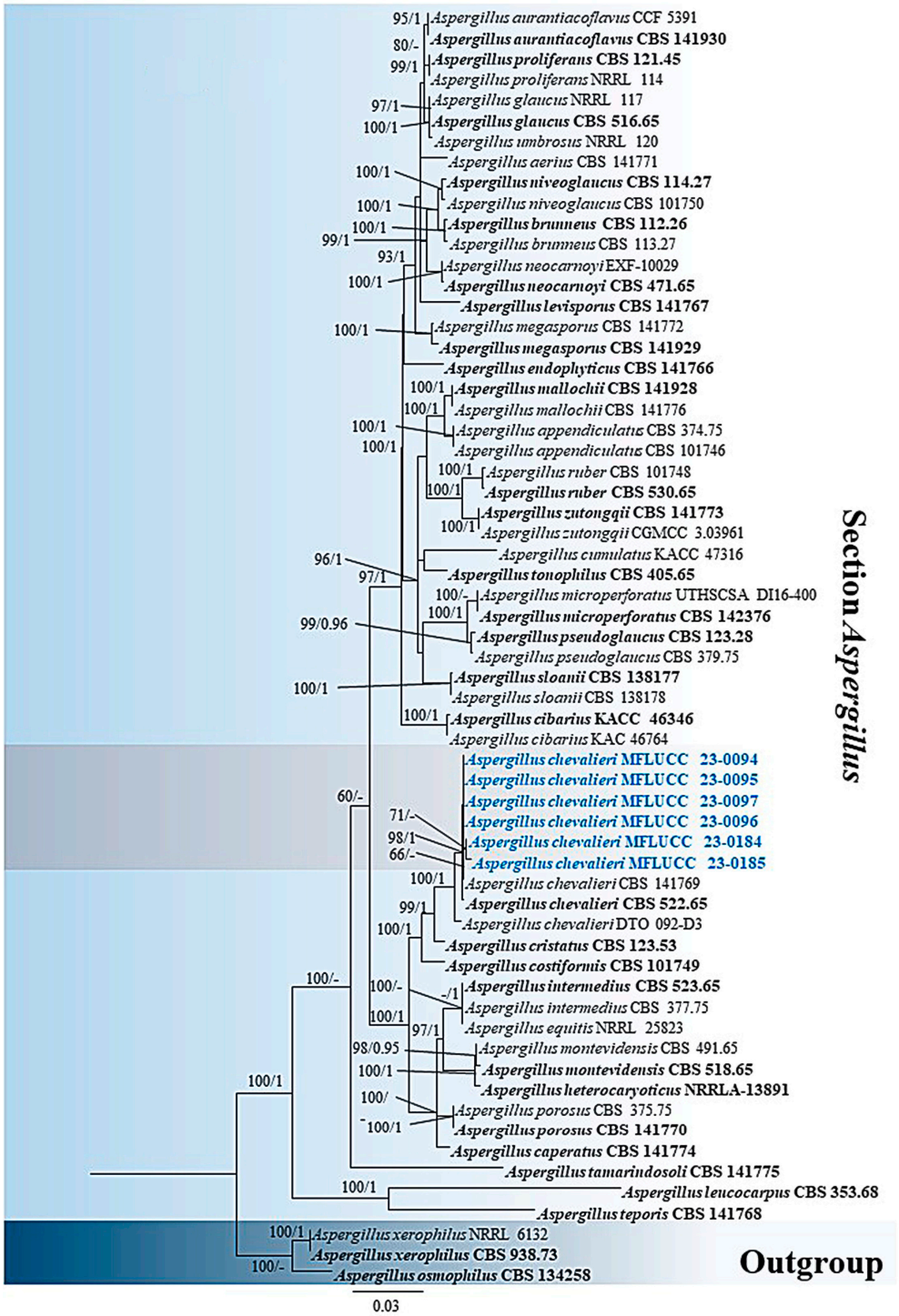

Figure 1. Phylogram generated from RAxML analysis based on combined BenA, CaM, ITS, and RPB2 sequence data of *Aspergillus* section *Aspergillus* taxa. *A. osmophilus* (CBS 134258) and *A. xerophilus* (CBS 938.73, NRRL 6132) are selected as the outgroup taxa. Bootstrap support values for ML values equal to or >60% and BYPP values equal to or >0.90 are shown as MLBS/BYPP above the nodes. Newly generated sequences in this study are in blue. Type strains are indicated in bold.

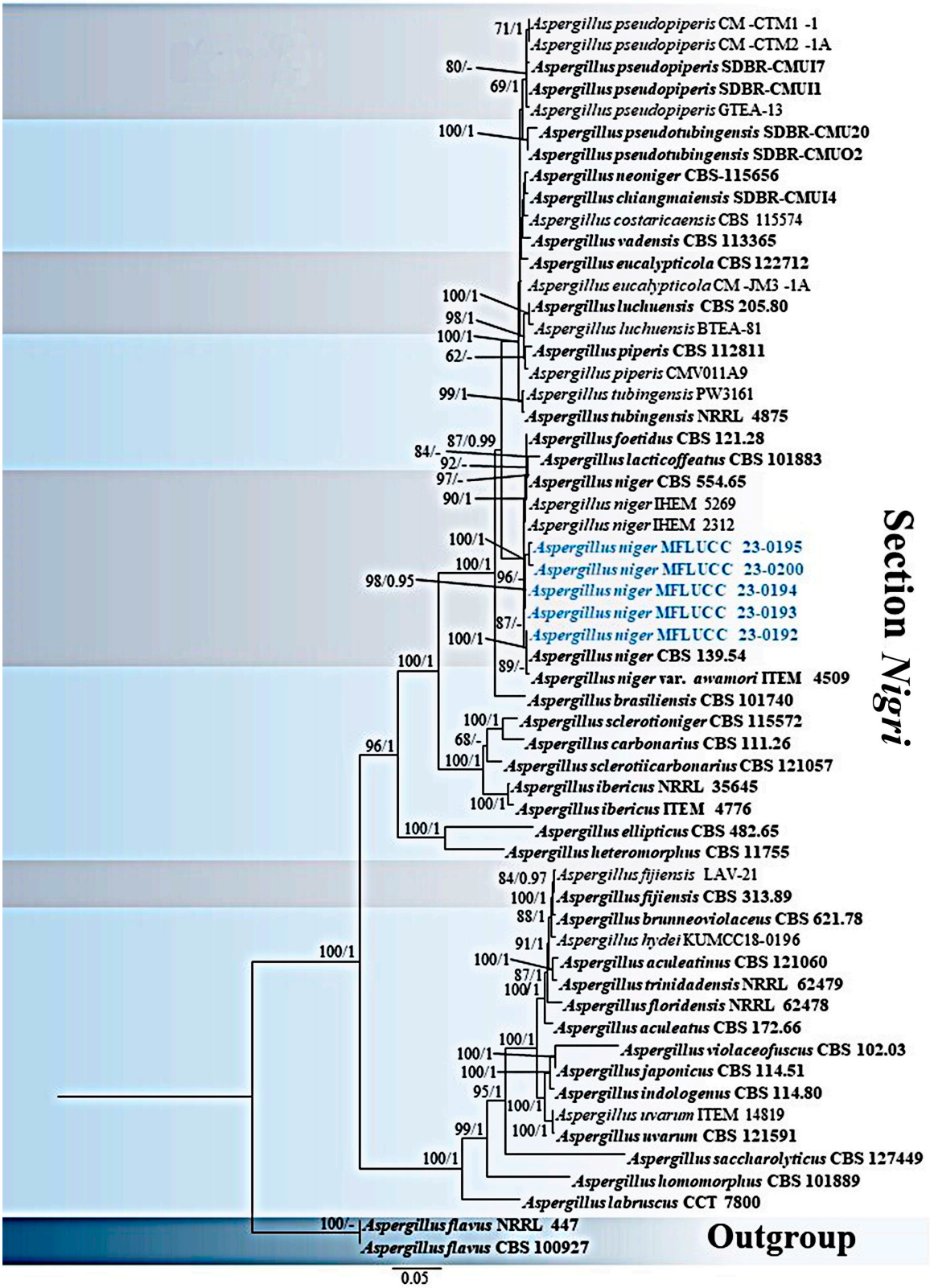

Figure 2. Phylogram generated from RAxML analysis based on combined BenA, CaM, ITS, and RPB2 sequence data of *Aspergillus* section *Nigri* taxa. *A. flavus* isolates (CBS 100927 and NRRL 447) are selected as the outgroup taxa. Bootstrap support values for ML values equal to or >60% and BYPP values equal to or >0.90 are shown as MLBS/BYPP above the nodes. Newly generated sequences in this study are in blue. Type strains are indicated in bold.

Table 5. Fungal identification of 11 isolates from seven herbal tea samples.

No.	Isolate No.	Taxa	Herbal Tea/Market
1	MFLUCC 23-0094	*A. chevalieri*	Jasmine/Doi Mae Salong
2	MFLUCC 23-0095	*A. chevalieri*	Jasmine/Doi Mae Salong
3	MFLUCC 23-0096	*A. chevalieri*	Jasmine/Doi Mae Salong
4	MFLUCC 23-0097	*A. chevalieri*	Rose/Doi Mae Salong
5	MFLUCC 23-0184	*A. chevalieri*	Bael fruit/Doi Mae Salong
6	MFLUCC 23-0185	*A. chevalieri*	Black tea/Doi Mae Salong
7	MFLUCC 23-0192	*A. niger*	Goji berry/Doi Mae Salong
8	MFLUCC 23-0193	*A. niger*	Bael fruit/Mae Sai
9	MFLUCC 23-0194	*A. niger*	Lavender/Mae Sai
10	MFLUCC 23-0195	*A. niger*	Green tea/Fah Thai
11	MFLUCC 23-0200	*A. niger*	Green tea/Fah Thai

3.2. Taxonomy

3.2.1. *Aspergillus chevalieri* (L. Mangin) Thom & Church, The Genus *Aspergillus*: 111 (1926)

Index Fungorum: IF292839; Facesoffungi number: FoF 14734; Figure 3.

Colonies on PDA 16.5–26 mm diameter in 7 days at 25 °C, initially white, gradually becoming light yellow from the center outwards, granulose due to the presence of ascomata, sporulation abundant, with conidial masse olive green, margin entire.

Conidiophores up to 247 × 4–6 µm, uniseriate with radiating conidial heads, stipes hyaline to subhyaline, smooth-walled. *Vesicles* 24–46 µm diameter, subglobose to pyriform, hyaline, smooth-walled. *Phialides* variable in shape and size, ampulliform to cylindrical. *Conidia* 3–6 × 2–5 µm, globose to subglobose, sometimes pyriform, hyaline, rough-walled. *Ascomata* 118–128 µm diameter, cleistothecium, globose to subglobose, light yellow to yellow, surrounded by hyaline to light brown hyphae. *Peridium* consisting of one layer of *textura angularis* to *textura globulosa*, light yellow, smooth-walled. *Asci* 8–10 µm diameter, globose to subglobose, thin-walled. *Ascospores* 3–4 × 4–5 µm, globose to subglobose, lenticular, aseptate, hyaline, with a slight furrow in the equatorial region, convex surface smooth-walled to finely roughened.

Material examined: Thailand, Chiang Rai Province, Mae Fah Luang District, Doi Mae Salong market, jasmine flower tea, 13 January 2022, Saranyaphat Boonmee, JM1–1A(CR), MFLU MFLU23-0273, living culture MFLUCC 23-0095, JM1–2A(CR), MFLU 23-0272, living culture MFLUCC 23-0094, JM3–2A(CR), MFLU 23-0274, living culture MFLUCC 23-0096; rose flower tea, 13 January 2022, Saranyaphat Boonmee RF3-1A(CR), MFLU 23-0275, living culture MFLUCC 23-0097; beal fruit, 10 June 2022, Maryam Tavakol Noorabadi, INB-043, MFLU 23-0365, living culture MFLUCC 23-0184; black tea, 10 June 2022, Maryam Tavakol Noorabadi, Btea-47, MFLU 23-0366, living culture MFLUCC 23-0185.

Notes: Six isolates obtained from this study clustered with the clade *A. chevalieri* (DTO 092-D3, CBS 522.65, and CBS141769) based on the phylogenetic analysis of BenA, Cam, ITS, and RPB2 sequence data (Figure 1). The isolates were morphologically identical to *A. chevalieri* strains (Figure 3). *A. chevalieri* is a member of section *Aspergillus* (formerly the genus *Eurotium*), which was described by Thom and Church [75]. They are generally characterized by yellow cleistothecia, lenticular, hyaline ascospores, and globose, subglobose, or ellipsoidal conidia [76,77]. This species produces some mycotoxins, such as aflatoxins, citrinin, gliotoxin, and sterigmatocystin, but it has not previously been shown to produce OTA [53–55,76,78–80]. In this study, we found that *A. chevalieri* isolated from herbal teas (bael fruit, black tea, jasmine flower, and rose flower) produced OTA.

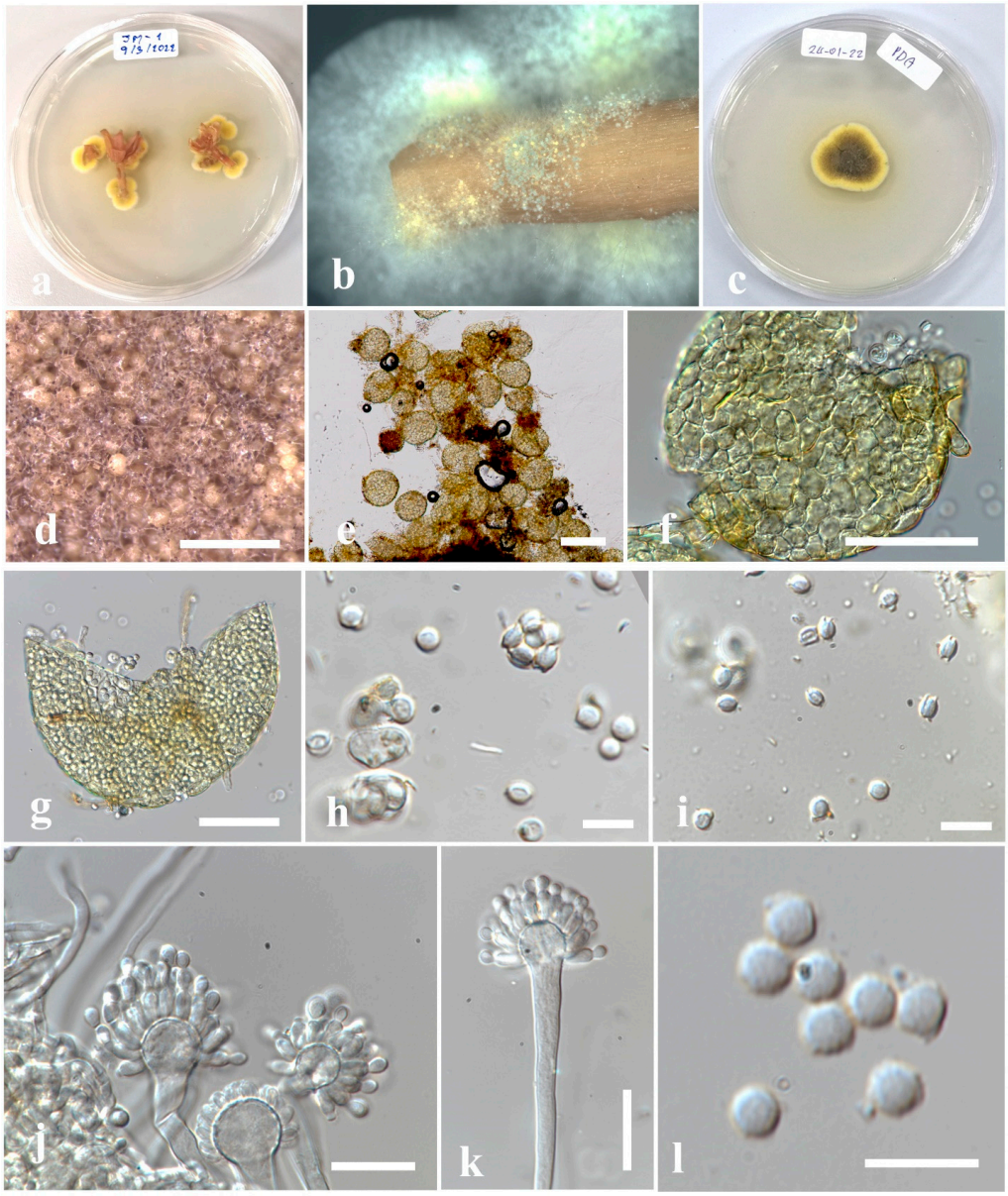

Figure 3. *Aspergillus chevalieri* (MFLUCC 23-0094). (**a**,**b**) Fungal isolation from jasmine flower samples on PDA. (**c**) Pure culture colony on PDA at 25 °C for 7 days from surface. (**d**,**e**) Ascomata. (**f**,**g**) Cleistothecial ascomata and peridium. (**h**,**i**) Asci and ascospores. (**j**,**k**) Phialides bearing apical uniseriate conidia. (**l**) Conidia. Scale bars: (**d**) = 500 μm, (**e**) = 100 μm, (**f**,**g**) = 50 μm, (**h**,**i**,**l**) = 10 μm, (**j**,**k**) = 20 μm.

3.2.2. *Aspergillus niger* Tiegh., Annls Sci. Nat., Bot., sér. 5 8: 240 (1867)

Index Fungorum: IF284309; Facesoffungi number: FoF 10087; Figure 4.

Colonies on PDA 57–63 mm diameter in 7 days at 25 °C, irregular, protuberant, margins narrow, entire. Mycelia white and then cream to light yellow, texture velutinous, soluble pigments yellow, exudates tiny, hyaline, and clear, reverse buff, yellow to orange, and with black sectors, from the center outwards, sporulation abundant, with conidial masse dark brown to black.

Conidiophores up to 730 × 12.5–16.5 μm, with biseriate, rarely uniseriate, radiating conidial heads, regularly splitting into columns, stipes smooth-walled to finely roughened, hyaline to light brown. *Vesicles* 55–78 μm, globose to subglobose, light brow to brown. *Metulae* 5.5–8.5 × 3–5 μm. *Phialides* 7.5–10.5 × 3.5–4.8 μm, ampulliform to cylindrical,

smooth-walled. *Conidia* 3.5–5.5 µm, globose, brown to dark brown, coarsely rough to echinulate-walled.

Material examined: Thailand, Chiang Rai Province, Mae Fah Luang District, Doi Mae Salong, goji berry, 15 June 2022, Maryam Tavakol Noorabadi, Goji-41, MFLU 23-0373, living culture MFLUCC 23-0192; beal fruit, 5 June 2022, Maryam Tavakol Noorabadi, INB-50, MFLU 23-0374, living culture MFLUCC 23-0193; Chiang Rai Province, Mae Sai market, lavender flower tea, 5 June 2022, Maryam Tavakol Noorabadi, LAV-85, MFLU 23-0375, living culture MFLUCC 23-0194; Chiang Rai Province, Fah Thai market, green tea, 5 June 2022, Maryam Tavakol Noorabadi, GTEA-39, MFLU 23-0376, living culture MFLUCC 23-0195, and GTEA-78, MFLU 23-0377, living culture MFLUCC 23-0200.

Notes: Five isolates obtained from this study are phylogenetically (Figure 2) and morphologically similar to *A. niger* strains (Figure 4). This species belongs to the *Aspergillus* section *Nigri*. Bian et al. [81] revised the section *Nigri* based on BenA, CaM, and RPB2 sequence data and new whole-genome sequences for six species that comprise *A. brasiliensis*, *A. eucalypticola*, *A. luchuensis*, *A. niger*, *A. tubingensis*, and *A. vadensis*. Furthermore, *A. niger* in the section of *Nigri* can be mainly distinguished by significant differences in colony colors, vesicle, conidiophores, conidia, and sclerotia, i.e., micro- and macro-morphology [82–85]. *A. vinaceus* and *A. welwitschiae* are considered synonyms of *A. niger* [81]. Morphological comparisons with the ex-type of *A. niger* (CBS 139.54) indicated no differences between the type strain and our five isolates.

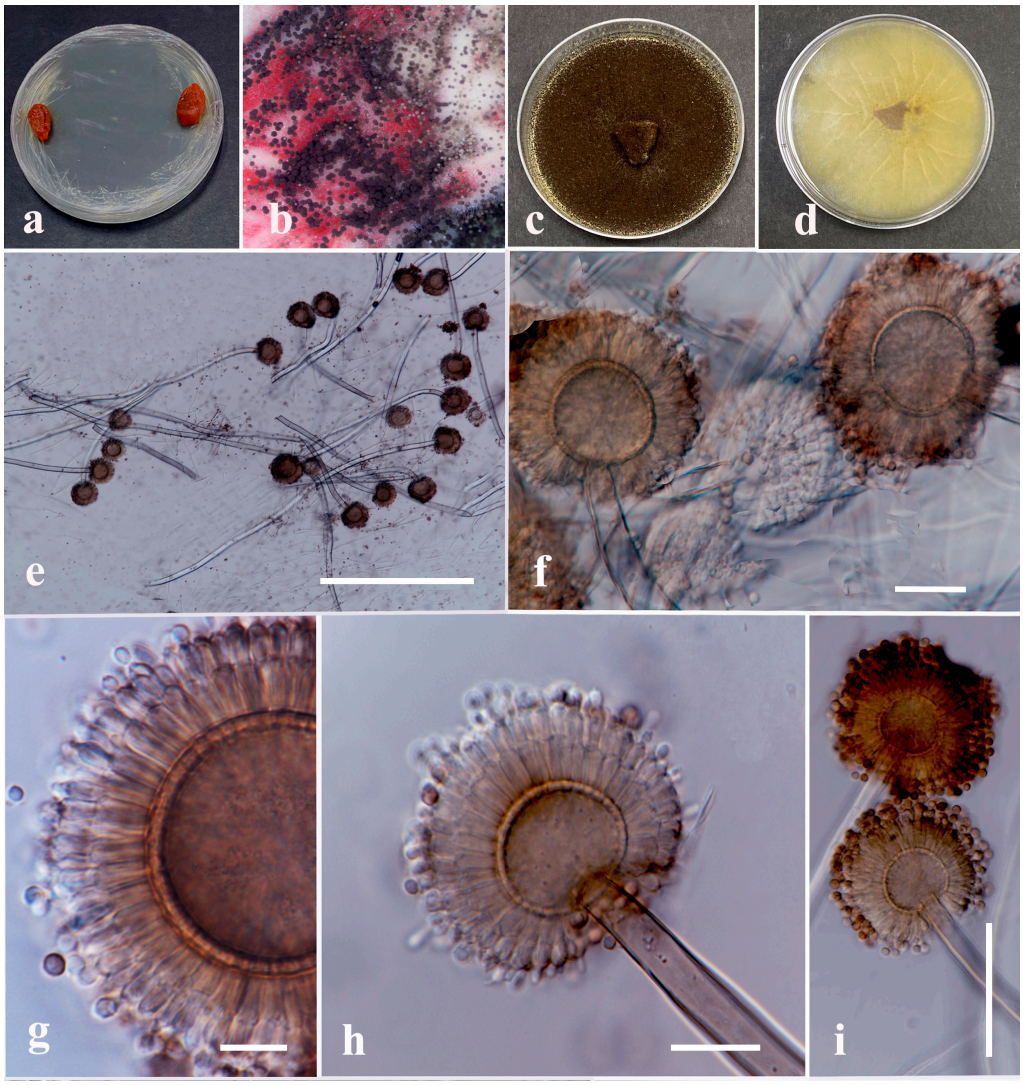

Figure 4. *Cont.*

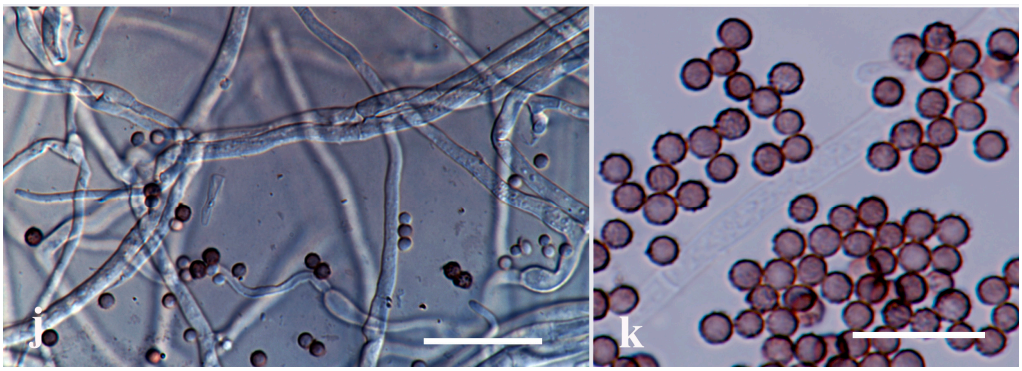

Figure 4. *Aspergillus niger* (MFLUCC 23-0192). (**a**,**b**) Fungal isolation from goji berry samples. (**c**,**d**) Pure colonies on PDA, at 25 °C, after 7 days from surface and reverse, respectively. (**e**,**f**) Conidiophores and apical dark conidia. (**g**–**i**) Phialide with apical radiating biseriate conidia. (**j**) Vegetative mycelium. (**k**) Conidia. Scale bars: (**e**,**g**) = 100 µm, (**f**,**h**,**i**) = 50 µm, (**j**,**k**) = 10 µm.

3.3. Mycotoxin Detection

The set of isolates (Figure 5), representing the different locations from where the various teas were bought, were tested for potential toxigenicity (Table 5). *A. niger* strains were shown to produce OTA with values ranging between 0.328 and 1.660 ng/L, while *A. chevalieri* strains produced OTA in the range between 0.663 and 39.182 ng/L (Figure 5).

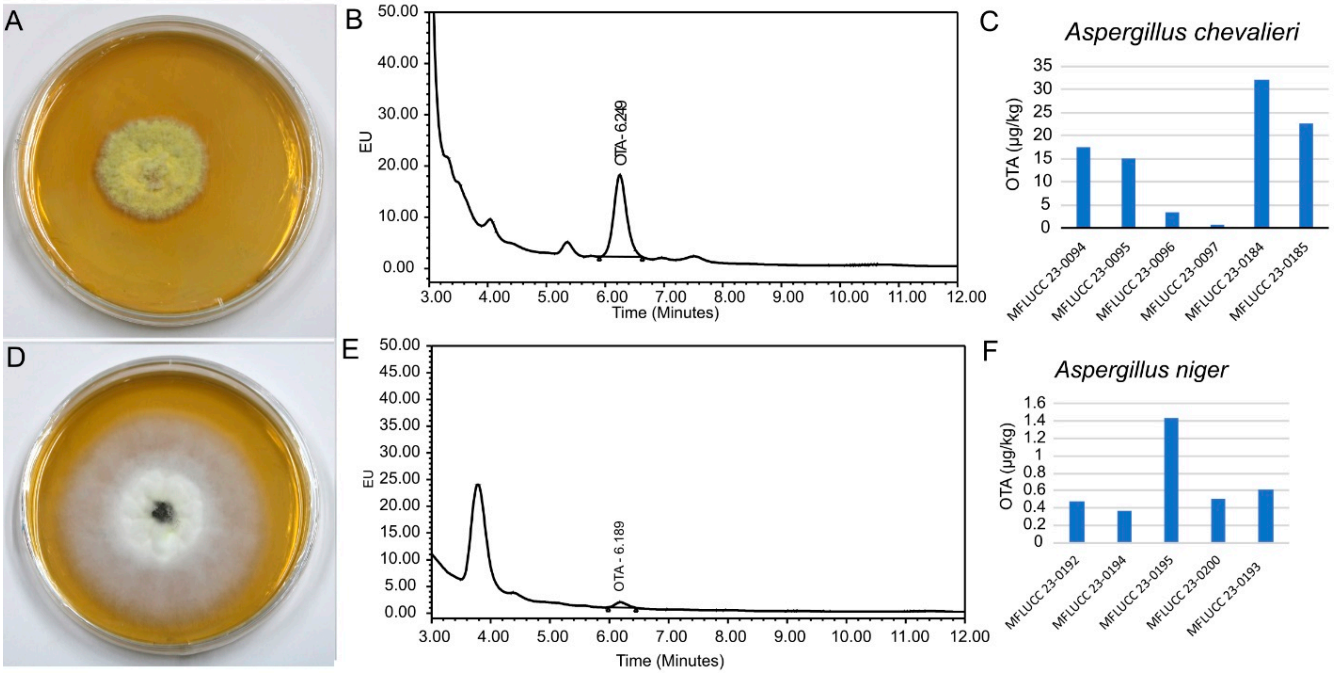

Figure 5. Analysis of OTA production by isolates of *A. chevalieri* and *A. niger* on YES at 25 °C for 14 days. (**A**,**D**) *A. chevalieri* (MFLUCC 23-0184) isolated from bael fruit and *A. niger* isolates from green tea (MFLUCC 23-0195), respectively. (**B**,**E**) UV absorbance traces of extracts from MFLUCC 23-0184 and MFLUCC 23-0195. (**C**,**F**) Amount of OTA production by different isolates of *A. chevalieri* and *A. niger*.

4. Discussion

In this study, 137 isolates were obtained from herbal teas and teas from local markets in northern Thailand. Eleven isolates, of which six isolates belonged to *A. chevalieri* and five isolates belonged to *A. niger,* produced OTA. Phylogenetic trees obtained from the analysis of combined BanA, Cam, ITS, and RPB2 sequence data provided good resolution

for identifying the *Aspergillus* isolates into two sections: (i) section *Aspergillus* = *A. chevalieri* (MFLUCC 23-0184, MFLUCC 23-0185, MFLUCC 23-0094, MFLUCC 23-0095, MFLUCC 23-0096, and MFLUCC 23-0097) and (ii) section *Nigri* = *A. niger* (MFLUCC 23-0192, MFLUCC 23-0194, MFLUCC 23-0195, MFLUCC 23-0200, and MFLUCC 23-0195). *Aspergillus* species are commonly isolated from tea samples. *A. tubingensis*, *A. fumigatus*, and *A. marvanovae* were isolated from China's Pu-erh tea [86], while *A. niger* was obtained from black and green teas [87]. *A. acidus*, *A. awamori*, and *A. tubingensis* were isolated from herbal teas [88]. In this study, the contamination of herbal teas by *Aspergillus* species includes five new substrate records: *A. chevalieri* on bael fruit, black tea, jasmine, and rose flowers; and *A. niger* on bael fruit, goji berries, green tea, and lavender flowers (Table 5).

Environmental factors such as temperature, air wetness, and water activity play a significant role in influencing mycotoxin production and contamination levels in pre- and post-harvest products. Various studies have highlighted the impact of these factors [89–94]. Mycotoxin production is influenced not only by the genetic makeup but also by environmental conditions, such as those from northern Thailand, and potential host effects. In our study, all isolates of *A. niger* produced low amounts of OTA, which was probably due to environmental factors. The European Union specifies the maximum limits for ochratoxin A (OTA) in dried herbs of 10 µg/kg [95]. OTA production from *A. chevalieri* has not previously been reported [53,89,96]. OTA production was detected in six isolates of *A. chevalieri* in this study (Figure 5). This finding raises concerns about OTA contamination in herbal teas and highlights the need for further research and monitoring to ensure consumer safety.

In conclusion, the increasing concern over *Aspergillus* contaminants and mycotoxin production in teas necessitates further research and analysis of OTA to ensure consumer health and safety. Analyzing and monitoring fungal contamination in teas is essential to meet consumer expectations and demands. The discovery of OTA production by *A. chevalieri* underscores the importance of continued vigilance in this area.

Author Contributions: Conceptualization, M.T.N. and S.B.; data curation, M.T.N. and S.B.; methodology, M.T.N. and S.B.; resources, M.T.N. and S.B.; formal analysis, M.T.N., S.B., and A.R.G.d.F.; investigation, M.T.N., A.R.G.d.F. and S.B.; writing—original draft, M.T.N., A.R.G.d.F., A.M. and S.B.; writing—review and editing, M.T.N., A.R.G.d.F., A.M., K.D.H. and S.B.; supervision, M.T.N., A.R.G.d.F., A.M., K.D.H. and S.B.; project administration, S.B.; funding acquisition, M.T.N., A.R.G.d.F., A.M., K.D.H. and S.B. All authors have read and agreed to the published version of the manuscript.

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