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Fungi as endophytes in Chinese *Artemisia* spp.: juxtaposed elements of phylogeny, diversity and bioactivity

Cosoveanu A[1*], Hernandez M[2], Iacomi-Vasilescu B[3], Zhang X[4], Shu S[4], Wang M[4] and Cabrera R[1]

[1] Departmento de Botánica, Ecología y Fisiología vegetal, Facultad de Ciencias, Universidad de La Laguna, Tenerife, Avda. Astrofisico Fco. Sanchez s/n, La Laguna, 38207, Islas Canarias, Spain
[2] Departamento de Bioquímica, Microbiología, Biología celular y Genética, Instituto Universitario de Enfermedades Tropicales y Salud Publica de Canaria, Tenerife, Avda. Astrofisico Fco. Sanchez s/n 38207, Islas Canarias, Spain
[3] Departamentul de Stiintele Plantelor, Facultatea de Agricultura, Universitatea de Stiinte Agronomice si Medicina Veterinara, Bd. Marasti 59, 11464, Bucuresti, Romania
4 College of Plant Science&Technology, Huazhong Agricultural University, 430070, Wuhan, China

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Abstract

Fungal endophytes were isolated from *Artemisia lavandulifolia*, *A. tangutica*, *A. brachyloba*, *A. subulata*, *A. argy* and *A. scoparia* in two Chinese localities, Qichun and Wuhan. 21 species were identified as belonging to one of the following: *Diaporthe*, *Colletotrichum*, *Nigrospora*, *Botryosphaeria*, *Aspergillus*, *Penicillium*, *Neofusicoccum*, *Cercospora*, *Rhizoctonia*, *Alternaria* and *Curvularia*. The evolutionary relationships were estimated through a phylogenetic tree using ITS1-5.8S-ITS2 region sequences. Members of the Diaporthaceae family were not clustered with Trichosphaeriaceae and Glomerellaceae though all are members of the Sordariomycetes class. Analysis of fungal diversity engaged various indices with results revealing contradictory aspects. Two new genera and two new species were reported as endophytes in *Artemisia* spp. (*Nigrospora*, *Curvularia*, *Neofusicoccum parvum* and *Penicillium chrysogenum*). Only two fungal species were found common in both localities. In dual culture assays with *Sclerotinia sclerotiorum*, *Alternaria alternata* and *Fusarium oxysporum*, *Nigrospora* endophytes provoked lysis, parasitism and had the highest values as antagonists against all pathogens. Fungal endophyte extracts were assayed against the mentioned pathogens. The three extracted fungi with the highest activity were: *Botryosphaeria dothidea* and *Curvularia geniculata* against *A. alternata* and *Curvularia spicifera* against *S. sclerotiorum*.

Key words – evolutionary relationships – fungal endophytes – medicinal plants – phytopathogens

Introduction

Endophytic fungi (EF) are estimated to be represented by at least one million species residing in plants (Idris et al. 2013). While some endophytic fungi appear to be ubiquitous (e.g. *Fusarium* spp., *Alternaria* spp., *Pestalotiopsis* spp., *Aspergillus* spp, *Botryosphaeria* spp.) others apparently present host specificity and/or host preference (Petrini 1996, Suryanarayanan et al. 2000, Schulz & Boyle 2005, Hu et al. 2007, Slippers & Wingfield 2007, Pang et al. 2008, Toju et al. 2013).

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Corresponding Author: Andreea Cosoveanu – e-mail – andreeacosoveanu@gmail.com

Many endophytes of the same species are often isolated from the same plant and only one or a few strains produce highly biologically active compounds in culture (Li et al. 1996). During the long period of co-evolution, endophytic fungi have gradually adapted themselves to their special microenvironments by genetic variation; including suggestions as uptake of plant DNA segments into their own genomes (Li et al. 1996, Long et al. 1998) as well as vice versa (Wink 2008) have arisen. This could have led to certain endophytes having the ability to biosynthesize various 'phytochemicals' originating from their host plants (Tan & Zou 2001, Strobel & Daisy 2003, Idris et al. 2013). One typical example was the production of gibberellins from both fungi and plants (Zhao et al. 2011).

Disease symptoms might express miscommunication with the host rather than active pathogenicity, leading to the hypothesis that plants participate in or initiate the disease processes (Rodriguez & Redman 2008, Aly et al. 2011). On the other hand, *B. dothidea* grows 'endophytically' in pedicels and spreads into the fruit a few weeks before they reach harvest maturity forming quiescent infections. On ripening, the fungus resumes growth and further invades the fruit (Plan et al. 2002).

The secondary metabolites of many organisms are employed by the modern medicine into the creation of pharmaceutical products, including, but not limited to: penicillin which is derived from the fungal secondary metabolites of *Penicillium notatum*, bacitracin which is derived from the secondary metabolites of the hard working prokaryote *Bacillus subtilis* and the platinum awarded taxol which is synthesized by various endophytes in *Taxus* spp (Abdou 2013). As of 2005, approximately 22,000 bioactive secondary metabolites from microorganisms have been described; about 8,600 (38%) of these are of fungal origin, highlighting the biochemical richness of this diverse clade of eukaryotes (Xing & Guo 2011, Higginbotham et al. 2013). They produce a number of important secondary metabolites: growth hormones, anticancer, anti-Alzheimer, anti-fungal, antibacterial, anti-diabetic and immunosuppressant compounds (Giménez et al. 2007, Idris et al. 2013, Wang et al. 2014). Natural products from endophytic fungi were observed to inhibit many pathogenic organisms including bacteria, fungi, viruses and protozoans (Petrini 1986, Horn et al. 1995, Strobel et al. 1999, Moreno et al. 2011).

Stating that *Artemisia* spp. is a genus of plant evaluated for medicinal and bio pesticide traits (Bailen et al. 2013, Nageeb et al. 2013, Joshi 2013), this study displays the bioactivity of the endophytes residing in seven plant species collected in China. In addition, the evolutionary relationships, the fungal diversity and the plant-species specificity were taken into observation.

Materials & Methods

Sampling and isolation techniques

Samples of *Artemisia* spp.: *A.* t*angutica*, *A. brachyloba*, *A. subulata*, *A. argy*, *A. scoparia* and *A. lavandulifolia* were collected from Qichun and Wuhan South Lake (GPS coordinates at request) in October 2013. Three ecotypes of *A. brachyloba* were sampled: one from an ecologically cultivated field of aromatic plants (CH9) and the other two from wild areas (CH6 and CH7) inside Qichun. *A. lavandulifolia* was collected from 3 types of microenvironments: waste (CH1), lake shore (CH3) and agricultural land (CH2) in Wuhan. Only one ecotype was sampled for the rest of the species. Specimens were preserved in a herbarium (College of Plant Science & Technology, HAU, Wuhan; voucher numbers- codes in Table 1). From each plant species stems were cut, labelled and kept in paper bags at 4-5º C until transported to the laboratory and then processed within 24 hours.

Following Núñez-Trujillo et al. (2012), a surface sterilization method was used in order to suppress epiphytic microorganisms from the plant samples. The isolation procedure was performed according to Cosoveanu et al. (2014). In order to analyze the fungal diversity, each replicate of the distinct stem fragments was registered. When an endophyte was acquired in pure culture it was preserved (Czapek, T=5° and Glycerol 20% DI H2O, T= -30°), bioactively tested and identified.

Genomic DNA extraction, amplification and identification

The extraction procedure was carried out as described by Shu et al. (2014) with the following modifications: samples were centrifuged for 15 min at 12.000rpm ; after the chloroform procedure the supernatant was mixed with 10% Sodium acetate and 60% Isopropyl alcohol, incubated for 10 min at -30° C and centrifuged (10 min, 12.000rpm). Finally the pellet was washed twice with 75% ethanol (before maintained at -20°C) and centrifuged (10 min, 12.000rpm). The solvent was removed by evaporation, leaving the sample in the laminar flow cabinet. The purified DNA was resuspended in 20µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). RNase A was added and the sample was incubated for 1 hour at room temperature (long term storage at -20°C). For plants, stem fragments were sterilized superficially and sliced with only the laminae of medulla being availed of as material for the DNA extraction. These were carried out using Extract-N-Amp ™ Plant PCR Kit according to the manufacturer's indications (Sigma-Aldrich Co.).

The molecular identification of the fungal strains was performed using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primer pair to amplify the 5.8S rDNA and the two internal transcribed spacers ITS1 and ITS2 (White et al. 1990). PCRs were performed according to Shu et al. (2014). As for plants, the nuclear ribosomal sequence was amplified using ITS2F as the forward primer and ITS2R as the reverse primer (Yao et al. 2010). PCR protocol was as follows: 94°C for 3min; 30 cycles of 94°C for 30 s, 58° C for 30 s, and 72° C for 1 min and 45 sec; and a final extension at 72°C for 10 min. PCR products were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Co.) and sequenced by Sangon Biotech (Shanghai, China) and Sequencing Services SEGAI (La Laguna, Spain). All sequences were submitted to GenBank under accession numbers KU 360596-KU360638. The sequences were run through the BLASTN search page using Megablast program (National Center for Biotechnology Information) where the most identical hits and their accession numbers were obtained (Table 1).

Phylogeny analysis

Sequences were aligned with the multiple alignment program ClustalW (Thompson et al. 1994) as implemented in Mega 6.0 (Tamura et al. 2013) and indels corrected manually to minimize alignment gaps (Foronda et al. 2011). Designated outgroup was *Glomus* sp. (GenBank Accession no. FJ164242.1). After the exclusion of non-overlapping leading/trailing gaps the length of the alignment was of 645 bps. Because of the high number of indels, the fragments were recoded as a binary matrix by means of the simple indel coding algorithm (Simmons & Ochoterena 2000) , appending the fragments to the nucleotide data as additional characters [as implemented in FastGap 1.21 (Borchsenius 2009)]. This "indel matrix" was used in all Bayesian analyses. Formerly, Gblocks program was used to eliminate poorly aligned positions and divergent regions (Dereeper et al. 2008). Best-fit models were compared in jModel test according to Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) (Posada 2008). Best fit model for this ITS dataset was K80+G+I. Bayesian Inference analysis was conducted with MrBayes (Huelsenbeck & Ronquist 2001) and run for 1×10^7 generations with a sampling frequency of 10^2 generations. Of the resulting trees, the first 25.000 were discarded as burn-in and the following 75,001 trees were used to estimate topology and tree parameters. The percentage number of times a node occurred within these 75,001 was interpreted as the posterior probability of the node (Ramírez-Bahena et al. 2012). Convergence of the runs was indicated by an average standard deviation of split frequencies between duplicate runs of less than 0.01; starting value was 0.11, values less than 0.01 were attained within the first 2.78×10^5 generations and final values were ~ 0.002. The consensus tree was visualized and edited using Treegraph software (Stöver & Müller 2010).

Dual culture assays

Dual culture technique was the screening method employed to find endophytic fungi that produce metabolites which inhibit *S. sclerotiorum, F. oxysporum* and *A. alternata* growth *in vitro*.

PDA plates were incubated at 25ºC in darkness for 7 days and observed daily; plates were left for a further week to check the stability of the interaction. The following criteria were used to read the results:

0- No apparent interaction

1- Mycelia grow until making contact with each other and in the area where the contact is produced; morphological changes occur / slight growth inhibition of both fungi with narrow demarcation line (1-2 mm)

2- Pathogen growth is detained at a certain distance from the endophyte (> 2 mm)

RDP- Rapid development and parasitism of the endophyte

RD- Rapid development of the endophyte

RDL- Rapid development of the endophyte and lysed mycelia of the pathogen

L- Opponent fungus presents lysed mycelia

P- Endophyte displays parasitism on pathogen

Bioactivity assays of endophytic extracts

Fungal isolates (endophytic and pathogenic strains) were maintained on PDA, T=25° in darkness while bacteria, *Pectobacterium carotovorum* and *Klebsiella oxytoca*, were maintained on LBA, T=28° and T=32° (respectively) in darkness. Pathogens were selected due to their different interactions with the host and their high economic importance: *Alternaria alternata*- seed borne, *Fusarium oxysporum*- vascular attack, *Sclerotinia sclerotiorum*- soil borne, *Pectobacterium carotovorum*- Gram negative degrading pectin and *Klebsiella oxytoca*- Gram negative, opportunistic pathogen of mammals and insects. Fungal and plant extract procedures and screening of potential antibacterial compounds (4mg/disc) have been performed according to Cosoveanu et al. (2012, 2013). Antifungal activity was checked using biometric agar dilution for *S. sclerotiorum* assays and spectrophotometry microplate and cuvette readings for *F. oxysporum* and *A. alternata*. Concentrations used were 1mg/ml, 0.5mg/ml, 0.1mg/ml, 0.05mg/ml, 0.01mg/ml and 0.005mg/ml. The absorbance was read at 570nm. Each sample was replicated 8 times. Absorbance corrections were performed (cultivation media and extracts concentrations).

Growth of the target organisms in screening [1mg/ml] was compared with the control using Mann Whitney test and Wilcoxon matched pairs test ($p < 0.05$). The Log-dose Probit regression model was used to obtain EC_{50} where the dose-effect response was observed (Mondino et al. 2015). All analysis were made using IBM SPSS Statistics 21.0.

Fungal diversity

The colonization rate was calculated as the total number of stem fragments in a sample (plant) yielding at least one isolate divided by the total number of stem fragments in that sample. The isolation rate was expressed as the total number of isolates yielded by a given sample (plant) divided by the total number of stem fragments in that sample. Frequency (%) was calculated as the total number of fragments in a sample (plant/location) colonized by a species divided by the total number of fragments plated. The Shannon diversity index was calculated according to the formula $H = \sum_{i=1}^{S} pi \ln pi$, where s is the total number of species and pi is the relative proportion of each species. The species evenness was estimated with Shannon's equitability index according to the formula: EH = H / Hmax, where H max is the maximum value of H, equal to lnS. Margalef's index of diversity was calculated using the formula (S-1) / lnN, where S is the number of species and N is the total number of individuals in the sample. The dominance of Simpson was calculated according to the formula $D = \Sigma ni (ni-1) / N (N-1)$, where ni is the number of individuals belonging to i species and N is the total number of individuals. The diversity of Simpson was calculated as 1 – D and the evenness of Simpson was calculated as D / S, where S is the total number of different species. Finally the dominance of Berger Parker was calculated as DBP = Nmax / N, where Nmax is the number of the most abundant species and N is the total number of species. The reciprocal form of Berger Parker index was also applied, 1 / DBP.

Table1 Endophytic fungi's host plants (species and codes); endophytic fungi's codes, species, GenBank accession numbers, most similar sequences accession numbers and identity between query and hits.

Plant species	Plant code	EF codes	EF species	EF Accession No.	Hit Accession No.	Identity
A. argy	CH10	HCH280	*Alternaria alternata*	KU360605	KJ526175.1	100%
		HCH288	*Nigrospora oryzae*	KU360608	KF516962.1	99%
		HCH285	*Nigrospora sphaerica*	KU360607	KC505176.1	99%
		HCH284	*Curvularia geniculata*	KU360606	HE861840.1	99%
A. brachyloba	CH6	HCH330	*Diaporthe longicolla*	KU360629	JQ753971.1	99%
		HCH328	*Colletotrichum capsici*	KU360628	JX867217.1	96%
		HCH326	*Nigrospora sphaerica*	KU360627	KC505176.1	99%
		HCH323	*Cercospora capsici*	KU360625	HQ700353.1	99%
		HCH332	*Rhizoctonia solani*	KU360630	KJ152163.1	100%
		HCH325	*Curvularia geniculata*	KU360626	HE861840.1	99%
	CH7	HCH256	*Alternaria alternata*	KU360596	JX406532.1	100%
	CH9	HCH320	*Nigrospora oryzae*	KU360622	JQ863242.1	99%
		HCH322	*Nigrospora sphaerica*	KU360624	JN198501.1	98%
		HCH321	*Alternaria alternata*	KU360623	KJ526175.1	100%
A. lavandulifolia	CH1	HCH260	*Diaporthe ceratozamiae*	KU360597	JQ044420.1	99%
		HCH266	*Colletotrichum gloeosporioides*	KU360599	GU066673.1	99%
		HCH271	*Colletotrichum gloeosporioides*	KU360602	JN887346.1	99%
		HCH334	*Colletotrichum gloeosporioides*	KU360631	JN887346.1	99%
		HCH267	*Nigrospora sp.*	KU360600	JF817271.1	99%
		HCH263	*Botryosphaeria dothidea*	KU360598	JN809914.1	99%
		HCH269	*Botryosphaeria dothidea*	KU360601	KF293883.1	99%
		HCH335	*Botryosphaeria dothidea*	KU360632	KC197764.1	97%
	CH2	HCH306	*Diaporthe hordei*	KU360617	KC343120.1	98%
		HCH304	*Penicillium chrysogenum*	KU360615	JN851002.1	99%
		HCH310	*Neofusicoccum parvum*	KU360618	KJ381071.1	99%
		HCH305	*Alternaria alternata*	KU360616	JX985742.1	99%
	CH3	HCH317	*Nigrospora sphaerica*	KU360620	KJ767121.1	99%
		HCH311	*Botryosphaeria dothidea*	KU360619	KC197789.1	99%
		HCH314	*Aspergillus flavus*	KU360621	LN482585.1	99%
A. scoparia	CH11	HCH337	*Diaporthe sp.*	KU360633	DQ145734.1	99%
		HCH345	*Nigrospora oryzae*	KU360637	JQ863242.1	99%
		HCH343	*Nigrospora sphaerica*	KU360636	KC505176.1	99%
		HCH339	*Alternaria alternata*	KU360634	KJ526175.1	100%
		HCH341	*Alternaria alternata*	KU360635	JX406532.1	100%
		HCH346	*Alternaria alternata*	KU360638	KJ526175.1	99%
A. subulata	CH8	HCH295	*Alternaria alternata*	KU360612	KJ008698.1	99%
		HCH297	*Alternaria alternata*	KU360613	KF293964.1	99%
		HCH300	*Curvularia spicifera*	KU360614	KC315931.1	99%
A. tangutica	CH4	HCH279	*Alternaria alternata*	KU360604	KJ526175.1	99%
		HCH274	*Curvularia intermedia*	KU360603	HE861855.1	98%
Artemisia sp.	CH5	HCH289	*Nigrospora sphaerica*	KU360609	KJ767121.1	99%
		HCH293	*Nigrospora sphaerica*	KU360610	KJ767121.1	99%
		HCH294	*Aspergillus aculeatus*	KU360611	KJ653817.1	97%

The statistical significance between the ranks of the isolates from both localities was questioned using paired wise Wilcoxon Signed Ranks Test (IBM SPSS 21.0) as the data could not fill the assumption for normal distribution, nor could the samples be regarded as independent.

Results & Discussion

Isolation and identification of endophytic fungi in *Artemisia* spp. (Table1)

Of all the fungi recovered from a single host plant only the morphologically different

endophytes were selected for further preservation and analysis (97 isolates). The similar non purified isolated fungi were recorded and used for frequency and biodiversity indices. Morphological identification of the pure isolates was carried out based on macroscopic and microscopic observations using taxonomic keys (Arx 1981) so only the different isolates were subjected to DNA analysis. 21 species were identified as belonging to one of the following genera: *Diaporthe*, *Colletotrichum*, *Nigrospora*, *Botryosphaeria*, *Aspergillus*, *Penicillium*, *Neofusicoccum*, *Cercospora*, *Rhizoctonia*, *Alternaria* and *Curvularia*. Results from macroscopic and microscopic characteristics were congruent with results of ITS sequence blasting analysis of these isolates, less in the case of the species of Cochliobolus (HE861840.1, HE861840.1, KC315931.1 and HE861855.1) where only asexual forms of *Curvularia* were isolated. Furthermore, to obtain an even higher probability of matching, Bayesian tree was used to select the most similar sequence according to GenBank.

Phylogenetic analysis

The 5.8S rDNA and the ITS1 and ITS2 juxtaposed regions molecular phylogenetic reconstruction of *Artemisia* spp. fungal endophytes (Fig. 1) shows 2 distinct clades having a long edge length, distinguishing the Ascomycota taxa from the Basidiomycota taxon, *Rhizoctonia solani*. Inside the Ascomycota clade, Diaporthaceae forms a separate tree from the other families (Pleosporaceae, Botryosphaeriaceae, Trichocomaceae, Mycosphaerellaceae, Glomerellaceae and Trichosphaeriaceae). The tree of the remaining families is divided into two subtrees with Pleosporaceae, Botryosphaeriaceae, Mycosphaerellaceae (Dothideomycetes) and Trichocomaceae (Eurotiomycetes) on one side and Glomerellaceae and Trichosphaeriaceae (Sordariomycetes) on the other. One interesting aspect, given that Diaporthaceae is a member of Sordariomycetes, is the lack of a monophyletic clade (Hyde et al. 2011, Jaklitsch et al. 2016). Additionally, *Neofusiccocum parvum* though a member of Botryosphaeriaceae, shares only one common ancestor with each of Botryosphaeriaceae, Mycosphaerellaceae and Pleosporaceae families. This does not suggest that *N. parvum* is any close to Botryosphaeriaceae family than the other two families afore mentioned. Despite these 2 interferences the tree reflects the affiliation of genera to their families and classes. Multigene phylogenies strongly support the acceptance of the genera *Alternaria* and *Curvularia* (Jumpponen & Trappe 1998, García et al. 2012, Ariyawansa et al. 2015). Here both genera cluster in a well-supported clade within the family Pleosporaceae (PPs = 1).

From another point of view inside *Nigrospora* genus, no correlation could be made related to plant-endophyte species specificity. Two nested species specific clades are formed: one clade for *N. oryzae*, excepting undefined *Nigrospora* sp. isolate HCH267, and a second one for *N. sphaerica* (though with low PPs of 0.86 and 0.73). The isolates inside each clade are dwelling in different plant species. The rest of the *N. sphaerica* isolates were not forming a species specific clade with the correspondent ones and neither were they isolated from the same host species as the ones which do form a species specific clade. Moreover, HCH293, HCH289 and HCH317 had the same branch length but only the first two isolates were obtained from the same plant species.

The latter observation is sustained by *Botryospaheria dothidea* clade in which isolate HCH335 is presented as a younger event in the evolution of the tribe than the other two isolates (HCH269 and HCH263) all isolated from the same plant, CH1. Furthermore, though the same host plant species, CH1 and CH3 have different locations; HCH263 and HCH269 belonging to CH1 are closer to HCH311 dwelling in CH3 than to HCH335 hosted by CH1. The relationship between the two species of *Aspergillus* found in this study is similar to that obtained by Perrone et al. (2007). Despite the species' variability with the same identity and similar query covers from GenBank, according to Bayesian tree, the same species of *A. alternata* was selected for all of the isolates clustered in the same clade (PPs = 1). Note that for *Curvularia* isolates the most similar sequences found had been identified as *Cochliobolus* spp. Neither the articles cited inside sequences assignments nor do our microscopic observations ensure a sexual state of the isolates. All accession numbers of the selected hits, identities and host plants are shown in Table 1.

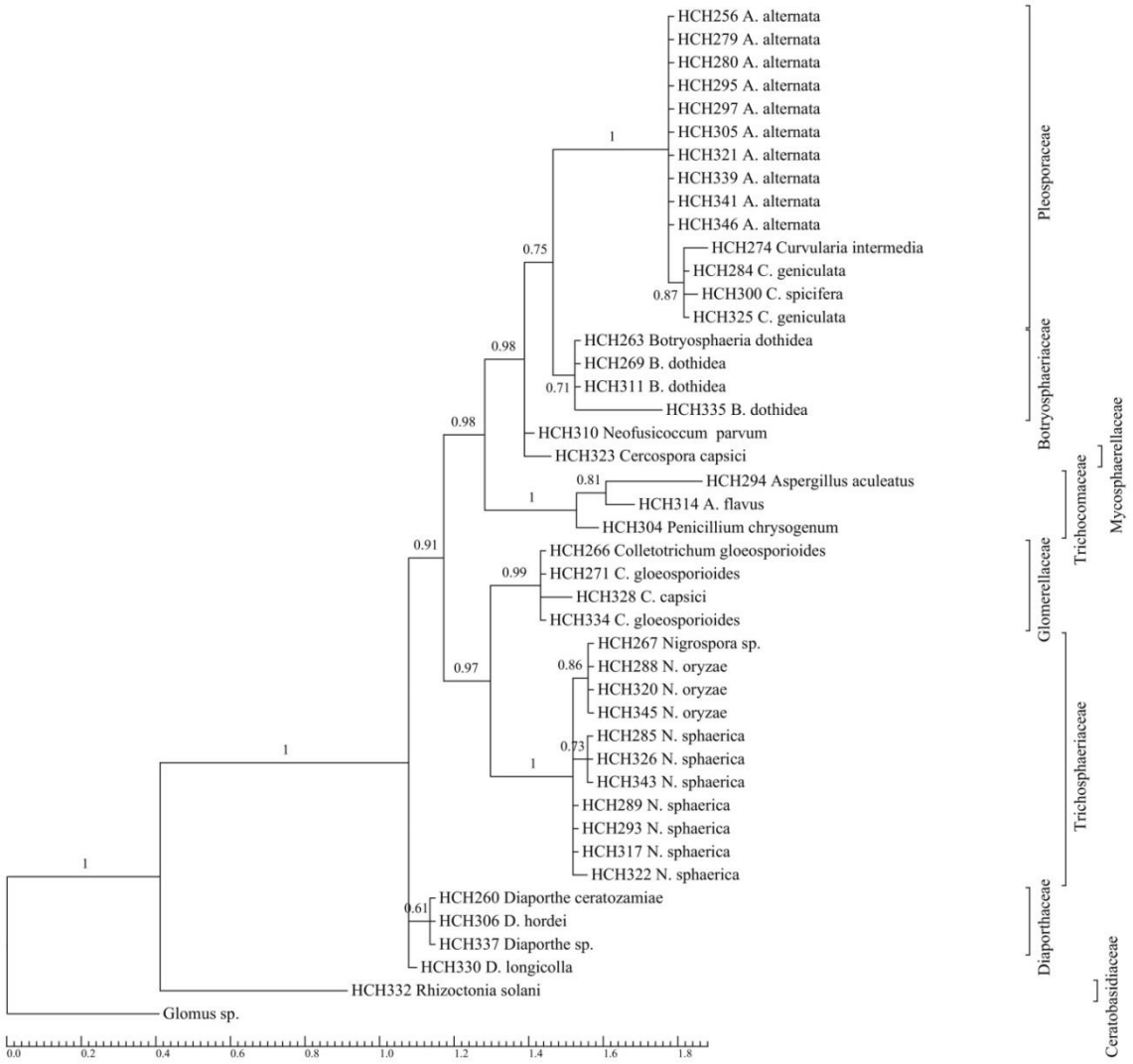

Fig. 1 – Phylogram of Bayesian inference phylogenetic analysis of ITS1-5.8S-ITS2 region sequences for endophytic fungi (EF) and the outgroup *Glomus* sp. The Bayesian clade-credibility values (posterior probabilities) are shown above the nodes. Host plants are presented in parentheses. Taxonomic families of the EF are shown in the right side of the tree.

Fungal diversity

Colonization rate and Isolation rate

With respect to colonization rate and isolation rate, no relations could be established between plant species and/or their localities (Table 2). Yet, it is to be mentioned that the lowest value in colonization rate was found in CH9 which was sampled from an ecologically cultivated area with medicinal plants in Qichun; the rest of the sampling being performed in a wild area.

Colonization frequency per plant

Only the highest incidences have been underlined: *Nigrospora sphaerica* in *Artemisia* sp. (CH5), *Nigrospora oryzae* in *A. argy*, *Alternaria alternata* in *A. subulata* and *A. tangutica* and *B. dothidea* in *A. lavandulifolia* (Table 2). Various reports upon *Alternaria* spp. and *Botryospheria* spp. in *Artemisia* spp. confirm the high incidence of these endophytes (Huang et al. 2007). On the other hand, to our knowledge, species of *Nigrospora* have not been reported to populate *Artemisia* species. During different studies of the authors, Chinese and Romanian ecotypes of *Artemisia* were observed as hosts but not Indian and Canarias ecotypes.

Table 2 Endophytic fungi: colonization frequency per plant and per sampled site; colonization rate and isolation rate per plant.

	CF (%)													
EF species	**CH1**	**CH2**	**CH3**	**CH4**	**CH5**	**CH6**	**CH7**	**CH9**	**CH8**	**CH10**	**CH11**	**WU**	**QI**	**Total**
A. alternata		41.60		83.30			16.60	8.30	58.30	33.30	33.30	12.80	31.00	25.50
A. aculeatus					8.30							0.00	1.07	0.70
A. flavus			25.00									8.30	0.00	2.32
B. dothidea	41.60		41.60									27.70	0.00	7.75
C. capsici						25.00						0.00	3.20	2.32
C. geniculatus						16.60				8.30		0.00	3.20	2.32
C. intermedius				16.60								0.00	2.10	1.50
C. spicifer									16.60			0.00	2.10	1.50
C. capsici						25.00						0.00	3.20	2.32
C. gloeosporioides	41.60											13.80	0.00	3.87
D. ceratozamiae	8.30											2.70	0.00	0.70
D. hordei		8.30										2.70	0.00	0.70
D. longicolla						16.60						0.00	2.10	1.50
Diaporthe sp.											8.30	0.00	1.07	0.70
N. parvum		8.30	2.70									2.70	0.00	0.70
N. oryzae								8.30		50.00	16.60	0.00	3.20	2.32
Nigrospora sp.	8.30											2.70	0.00	0.70
N. sphaerica			8.30		100.00	16.60		8.30		25.00	25.00	2.70	20.43	15.5
P. chrysogenum		8.30										2.70	0.00	0.70
R. solani						16.60						0.00	2.10	1.50
CR	1.00	0.80	0.90	0.90	1.00	1.00	0.70	0.40	0.90	0.90	0.90			
IR	1.75	0.92	0.92	1.00	1.08	1.33	0.67	0.42	1.75	1.33	1.50			

EF- endophytic fungi; CF- colonization frequency; CR- colonization rate; IR- isolation rate; WU- Wuhan; QI- Qichun

Lowest values of *D. ceratozamiae* and *D. hordei* inside *A. lavandulifolia* and their absence from the other species, in terms of species specificity, differ from the values presented in a previous study in which *Diaporthe* was found in all of the three Chinese *Artemisia* species (Huang et al. 2007). To the best of our knowledge *P. chrysogenum* and *N. parvum* were isolated for the first time from *Artemisia* spp. *Colletotrichum gloeosporoides* had a specific incidence in *A. lavandulifolia* (41.6%) as its relative *C. capsici* in *A. brachyloba* (25%). *A lavandulifolia* and *A. brachyloba* were the only two plants with the highest variety in endophytic species (10 and 9 species, respectively). *B. dothidea* (41.6%) and *C. gloeosporoides* (41.6%) were the dominant endophytes in *A. lavandulifolia*. *A. tangutica*, *A. subulata*, and *A. scoparia* are dominated by *A. alternata* (83.3%, 58.3% and 33.3%, respectively). *A. argy* and *Artemisia* sp. (CH5) resulted in a high incidence of *N. oryzae* and *N. sphaerica*, respectively.

As for plant species specificity, only *N. spaherica*, *N. oryzae* and *A. alternata* were present in various plants; except for *C. geniculatus* isolated from *A. brachyloba* and *A. argy*. Specificity of some fungus/plant interactions has been widely assumed at least at the genetic level, and it has been claimed that endophyte communities (or at least community profiles) are usually specific at the host species level (Fisher et al. 1992). Studies in the tropics (Arnold & Lutzoni 2007) have identified distinct host-related communities in tropical tree leaves, but on a quantitative rather than qualitative basis. Thus, few endophytic fungi were found to be entirely restricted to particular plant species, but significant differences were found in the frequency of infection of individual morphotaxa. This phenomenon has been termed as host preference, following similar observations of decomposer fungal communities by Lodge (1997). As expected, the *Alternaria* genus was the dominant one, being by far one of the most cosmopolite endophytes reported. Surprisingly *Nigrospora* received displayed the second highest rate of occurrence in our analysis, followed by *Botryosphaeria* whose incidence has been described more in woody plants.

Colonization frequency distribution by localities

Only two species were isolated from both locations: *A. alternata* and *N. sphaerica* (Table 2). This indicates a true distinction in the diversity of fungal species found in Qichun and Wuhan. Moreover the same degree of occurrence is maintained as in the colonization frequency per plant species. The distribution showed statistical significance ($p= 0.027$).

Fungal diversity indices

The isolated biodiversity is likely to be much lower than the real one as the isolated endophytic communities will be biased towards faster growing fungi that are capable of rapid development on sugar high media like *Colletotrichum*, *Phomopsis*, *Phyllosticta*, and *Xylaria* species (Hyde & Soytong 2008). Two of the selected indices (Margalef and Shannon) are mainly influenced by range, two (Simpson and Berger-Parker) are defined as dominance measures while two (Shannon Evenness and Simpson's evenness) are considered measures of evenness (Lexerod & Eid 2006).

The Shannon diversity index (H) for Qichun is 1.56 and for Wuhan 1.0 (Table 3). Shannon's evenness states a higher equitability in Qichun, differing from the one resulted from Simpson's index of evenness. Simpson's index has been less used as it emphasizes disproportionately the most common species in the sample, making them insensitive to changes of diversity that affect only the non-dominant species (Jost 2006).

Margalef's index results show a higher species richness in Wuhan than Qichun (3.11 vs. 2.51) and Berger-Parker index results show a positive correlation of a higher dominance in Qichun (0.35) than in Wuhan (0.28). Consequently the reciprocal of Berger-Parker concluded a higher diversity in Wuhan (3.57) than in Qichun (2.86). On the other hand, Simpson's and Shannon's indices of diversity are giving contradictory results. Opposite results generated by Shannon's species richness and Simpson's diversity were debated through various studies (Peet 1974, Abou-Moustafa et al. 2013). Further, Simpson's dominance is higher in Qichun (0.19) than in Wuhan (0.15), Simpson's diversity is congruent with the dominance, Qichun-0.85 and Wuhan-0.85 and finally the evenness is higher in Wuhan than in Qichun (0.08 vs. 0.076). As a counter argument comes Shannon's richness index with a higher value for Qichun (1.56) than Wuhan (1.00), maintaining the uniformity of distribution higher in Qichun (0.61) than in Wuhan (0.43). The species richness number which is higher in Qichun (12 vs. 10) is underlined by the presence of 6 different host plant species including 8 different plant populations versus 1 species and 3 different populations in Wuhan, respectively. More specifically, if one starts from the hypothesis of having a linear higher endophytic diversity in a higher number of plant samples altogether with a higher number of plant species then the present results showed that the outcome is not the predictable one. Having these preliminary data and results we may conclude intuitively that Wuhan's biodiversity is actually higher than Qichun's though Shannon's index values say the exact opposite. This may raise issues about not just the variation of diversity of species specificity of fungal endophytes but also about the distinct micro environments that promote different endophytic populations. The three Wuhan's ecotypes of *A. lavandulifolia* were collected from three types of microenvironments: waste, lake shore and agricultural land. The three ecotypes of *A. brachyloba* are distinct in that the CH9 samples were obtained from an ecologically cultivated field of aromatic plants while the rest were obtained from a wild area in Qichun. Of the 21 fungal species samples, only two (*N. sphaerica* and *A. alternata*) were found at both sites. Of the seven *Artemsia* species sampled, two species had three ecotypes. *N. sphaerica* was isolated only from CH3 (CH1, CH2 and CH3 are the same species). *A. alternata* was isolated only once from both CH2 and CH7 (CH6, CH7 and CH9 being the same species). Also, these two fungal species have been isolated from other *Artemisia* species in Qichun. This may lead to a lack of endophyte-plant species specificity and rather a host preference issue. Besides, four different species of endophytic fungi revealed the highest values in bioactivity assays. *C geniculatus* (HCH325) and *A. alternata* (HCH321) were isolated from the same plant species, but different ecotypes: *A. brachyloba* in Wuhan. On the other hand *C. spicifer* (HCH300) and *B. dothidea* (HCH311) were isolated from *A. subulata* and *A. lavandulifolia*, respectively, in Qichun. These results can neither establish a connection between plant species known activity and fungal isolates, nor provide proof with regard to which plant gathers more active fungi (apart from the fungal species belonging to *A. brachyloba*, however, this remains inconclusive as it was the only case). Finally, Berger-Parker Dominance is congruent with $D_{Simpson}$ index stating that Qichun has a higher number of dominant species. The inversed formula of Berger-Parker shows that Wuhan has a higher diversity agreeing with Simpson's Diversity value.

Table 3 Fungal endophytes: diversity indices for both sampled localities.

Diversity indices	WUHAN	QICHUN
Margalef Species Richness	3.11	2.51
Berger Parker Dominance (D)	0.28	0.35
Berger Parker Dominance (1/D)	3.57	2.86
Shannon Wiener Species Richness	1.00	1.56
Shannon's Evenness (EH)	0.43	0.61
Simpson's Diversity	0.85	0.81
Simpson's Evenness	0.08	0.07
Simpson Dominance	0.15	0.19

Table 4 Endophytic fungi (EF): dual culture activity against phytopathogenic fungi.

EF CODE	EF IDENTITY	Evaluation of the dual culture		
		S.sclerotiorum	***A.alternata***	***F.oxysporum***
HCH263	*Botryosphaeria dothidea*	1	0	0
HCH273	*Alternaria alternata*	1	1	0
HCH274	*Curvularia intermedia*	0	0	1
HCH284	*Curvularia geniculata*	L	0	1
HCH285	*Nigrospora sphaerica*	2	0	0
HCH288	*Nigrospora oryzae*	0	0	1
HCH289	*Nigrospora sphaerica*	0	P	P
HCH293	*Nigrospora sphaerica*	L	2	0
HCH294	*Aspergillus aculeatus*	RDP	RD	RDL
HCH300	*Curvularia spicifera*	2	1	0
HCH304	*Penicillium chrysogenum*	RDP;2	2;L	0
HCH310	*Neofusicoccum parvum*	1	0	0
HCH314	*Aspergillus flavus*	0	RDP	RDP
HCH317	*Nigrospora sphaerica*	0	2	P
HCH321	*Alternaria alternata*	2	0	0
HCH322	*Nigrospora sphaerica*	0	0	1
HCH325	*Curvularia geniculata*	2	0	0
HCH326	*Nigrospora sphaerica*	0	0	1
HCH328	*Colletotrichum capsici*	2	0	0
HCH330	*Diaporthe longicolla*	0	1	P
HCH332	*Rhizoctonia solani*	0	P	P
HCH335	*Botryosphaeria dothidea*	0	1	0
HCH343	*Nigrospora sphaerica*	1	0	0; L

EF- endophyte; S.s.- *Sclerotinia sclerotiorum*, A.a.- *Alternaria alternata*, F.o.- *Fusarium oxysporum*; RDP- Rapid development and parasitism of the endophyte; RD- Rapid development of the endophyte; RDL- Rapid development of the endophyte and lysed mycelia of the pathogen; L-Opponent fungus presents lysed mycelia; P- Parazitism of the endophyte on the pathogen; 0- No apparent interaction; 1- Mycelia grow until touching each other and in the area where the contact is produced morphological changes occur / Slight growth inhibition of both fungi with narrow demarcation line (1-2 mm); 2- Pathogen growth is detained at a certain distance from the endophyte (> 2 mm)

In vitro bioactivity assays

Dual-culture assays

All isolates irrespective of their identity were assayed in dual culture; only active fungi results against at least one pathogen are shown. *S. sclerotiorum* (S.s.) was the most defeated pathogen as 6 isolates (*Nigrospora sphaerica*, *Curvularia spicifera*, *Penicillium chrysogenum*, *Alternaria alternata*, *Curvularia geniculata*, *Colletotrichum capsici*) presented the highest bioactivity (> 2mm) as antagonists; of which *P. chrysogenum* showed the highest activity against *A. alternata* (A.a.) also (Table 4). Also, two different isolates of *N. sphaerica* inhibited *A. alternata* at highest scores.

Table 5 Bioactivity screening of plants and endophytic fungi extracts against phytopathogenic fungi: PI% [1mg/ml] and EC_{50} [mg/ml] (confidence limits).

EF	Host plant	PI% EC_{50}	*A. alternata*	*F. oxysporum*	*S. sclerotiorum*
A. alternata (HCH321)	*A. brachyloba* CH9	PI%	29.81 [a]	33.2 [a]	42.4 [a]
		EC_{50}			>1
C. spicifer (HCH300)	*A. subulata* CH8	PI%	8.95	52.09 [a]	77.9
		EC_{50}			0.66 (0.34-4.67)
HCH316	*A. lavandulifolia* CH3	PI%	4.36	19.4 [a]	5.93[a]
B. dothidea HCH311	*A. lavandulifolia* CH3	PI%	85.79		22.04
		EC_{50}	0.38 (0.26-0.54)		>1
C. geniculatus (HCH325)	*A. lavandulifolia* CH6	PI%	91.09 [a]	31.5 [a]	100 [a]
		EC_{50}	0.03 (0.01-0.06)		
A. alternata (HCH273)	*A. tangutica* CH4		48.38 [a]	2.53	6.56
HCH264	*A. lavandulifolia* CH1		47.64 [a]	4.1	0
N. sphaerica (HCH285)	*A. argy* CH10		48.37 [a]	11.94[a]	24.92
D. hordei (HCH306)	*A. lavandulifolia* CH2			2.33	
	A. lavandulifolia CH1		42.44 [a]	0 [a]	5.22
	A. lavandulifolia CH2	PI%	0	11.62 [a]	8.17
	A. lavandulifolia CH3		53.22 [a]	10.98 [a]	6.43
	A. tangutica CH4		1	0 [a]	6.9
	Artemisia sp. CH5		49.47 [a]	8.68 [a]	0
	A. brachyloba CH6		48.53 [a]	11.59 [a]	5.16
	A. brachyloba CH7		54.51 [a]	12.90 [a]	16.40 [a]
	A. subulata CH8		37.91 [a]	18.88 [a]	0
	A. brachyloba CH9		7.55	26.85 [a]	14.65
	A. argy CH10		50.13 [a]	10.82 [a]	0
	A. scoparia CH11		88.05 [a]	51.78 [a]	0

a Mann Whitney test (2-tailed): statistically significant differences between control and PI% (p values<0.05)
[1mg/ml]- mg of extract/ml of medium

The above mentioned isolates exhibited apparent parasitism (P) together with *Diaporthe longicolla* and *Rhizoctonia solani*, the latter being reported as parasitizing fungi in soil (Buttler 1957). *N. sphaerica* and *R. solani* also maintained the inhibition response against *F. oxysporum* and *A. alternata*. *Rhizoctonia binucleata* has been reported in antagonism interaction evidenced by

competition of nutrients or infection sites, antibiosis and hyperparasitism (González García et al. 2006). No correlation between similar *Nigrospora* species and values could be made and neither between same plants species as hosts for endophytic species of *Nigrospora* and their antagonistic values. Yet, the most important antagonistic interaction was shown by 3 different strains of *N. sphaerica* (different host plants) against *A. alternata* and *S. sclerotiorum*. In this study *P. chrysogenum* acted as a powerful antagonist against *A. alternata* and *S. sclerotiorum.* The two isolates of *C. spicifer* and *C. geniculatus,* respectively maintained their previous activity from dual culture assay in the extracted secondary metabolites '*in vitro*' assays with the lowest EC_{50} of 0.03 mg/ml registered by *C. geniculatus* against *A. alternata*. Three interactions with pathogen's mycelia lysis (L) (explained by Gindrat 1979)**,** were observed with *C. geniculatus*, *Nigrospora* sp. and *N. sphaerica* as antagonists. Manifestation of rapid development and apparent parasitism (RDP) were observed in the interactions of: *Aspergillus aculeatus* on *S. sclerotiorum* and *Aspergillus flavus* on *F. oxysporum* and *A. alternata.*

Antifungal and antibacterial activity of endophytic extracts

All host plants and eight different fungal extracts have been assayed against bacterial and fungal pathogens in a screening (Table 5). No effect was recognized on bacteria (data not shown) though antibacterial activity was reported before with several of the assayed endophytic species in this study (Rekha & Shivanna 2014). Furthermore, lower concentrations were used only for some of the fungal extracts: HCH321, HCH325, HCH311 and HCH300. There were two *Curvularia* species with high activity against two specific pathogens (*S. sclerotiorum* and *A. alternata*) found in this study. One previous study shows various mycotoxins isolated from species of *Cochliobolus* amongst which were ophiobolins (terpinoids), which possess inhibitory effects against fungi (Manamgoda et al. 2011). Xiao et al. (2014) studied *B. dothidea* isolated from *Melia azadirachta* and found it to be active against some pathogens, unveiling inside the chemical profile chaetoglobosins, a family of cytochalasans with wide range of biological activities targeting cytoskeletal processes. This reinforces the activity of *B. dothidea* (HCH311) against *A. alternata*. From all the plant extracts, only *A. scoparia* has highly inhibited *A. alternata* (PI%= 88.05 at 1mg/ml), although none of the six hosted isolates has shown any antagonistic quality in dual culture assays.

Conclusions

Only two fungal species were found in both locations suggesting different diversities. This study offers arguments suggesting that fungal specificity is dependent on geographical area and microenvironment. As for colonization frequency per total, *Nigrospora* species are to be mentioned as their occurrence inside *Artemisia* has been considerably less referred to. Two genera and two new species were reported as endophytes in *Artemisia* spp. (*Nigrospora*, *Curvularia*, *Neofusicoccum parvum* and *Penicillium chrysogenum*). With respect to preliminary bioactivity, species of *Nigrospora* had various interactions as lysis and parasitism as well as presenting the highest values against all pathogens. Thus, isolates of this genus should be closely observed due to their variation in expression within an interaction with another fungus and last but not least because of their lack of occurrence inside *Artemisia* sp. Moreover, *Curvularia* with its two different species represents a target for the chemical profile as their EC_{50}s were the lowest found in all isolates. In order to optimize secondary metabolite production of endophytic fungi under laboratory conditions, a deeper understanding is needed of both: host-endophyte relationships at the molecular and genetic levels of biogenetic gene cluster regulation and the effects of the environmental changes and culture conditions on gene expression. Further research at advanced molecular level may offer better insights into endophyte biodiversity and the regulation of fungal secondary metabolism.

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