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Host *Artemisia*: what is on the phylogenetic menu of the endophytic fungi

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Abstract Endophytic fungi isolated from eleven different species of *Artemisia* were characterized using ITS region. Phylogenetic analysis of 97 endophytic fungal sequences using maximum likelihood based method and the hierarchical Bayesian method were performed. Both analyses gave essentially same results. Bayesian Markov chain Monte Carlo approach resulted in statistically well supported clades for all the species investigated. Four Bayesian inferences were performed grouping the sequences into i) Botryosphaeriales — Diaporthales taxa, ii) *Chaetomium — Biscogniauxia — Thielavia — Sordaria — Daldinia — Nigrospora* taxa, iii) Hypocreales — Glomerales — Microascales taxa and iiii) Pleosporales taxa. Using Botryosphaeriales — Diaporthales taxa*, Neofusicoccum*, *Botryosphaeria* and *Aplosporella* form a monophyletic clade (BPP = 0.91) depicting clear separation of these taxa. In the second phylogenetic analysis, Bayesian topology reconstructed the tree relating four main clades: clade 1 with *Nigrospora* and *Pestalotiopsis* sister clusters, clade 2 with *Sordaria* spp., *Chaetomium* spp. and *Thielavia* spp. as sister clusters, clade 3 with *Daldinia loculata* and clade 4 with *Biscogniauxia mediterranea*. The third Bayesian phylogenetic analysis represented two main clades: Clade 1 comprised of *Hypocreales*, *Glomerales* and *Microascales* members (BPP = 1) while Clade 2, reunited *Saccharomycetales* members as older as basal group (BPP = 1). The fourth Bayesian analysis exhibited several clades. Sequences of *Stemphylium* were associated but unclustered and recognised as *Stemphylium* section. *Curvularia* sequences formed a well-supported clade (BPP = 0.99) as a sister cluster of *Stemphylium* section. *Coniothyrium* - like taxa were also united in a cluster (BPP = 1).

Key words

phylogeny, endophytic fungi, *Artemisia*

Artemisia is a plant with raised interest in medicine and plant protection (3, 18, 20, 30, 36, 38). There has been an increased enthusiasm in isolating fungal endophytes in medicinal plants (1, 9, 32, 44, 59, 62, 63) and the primary scope is evaluating bioactivity potential. However studies on *Artemisia* as host for the endophytic communities, despite *A. annua*, are scarce. Overall, the identification of the fungal endophytes in *Artemisia* spp. is made based on morphological characterization and molecular analysis using nuclear ribosomal DNA sequences, including both the internal transcribed spacers and the 5.8S gene region. To the best of our knowledge, there are only two studies which investigate the phylogenetic analysis of the *Artemisia* spp. fungal endophytes (10, 26). Qian et al (41) isolated endophytic fungi from *Artemisia argyi* and found *Pleosporales* to be the most represented group, with three species of *Alternaria* present. It is worth mentioning that the authors reported the presence of *Rhodotorula* sp. and *Fusarium* sp. in *Artemisia argyi* for the first time. Myrchiang et al (37) investigated the endophytic fungi associated with *Artemisia nilagirica* isolated among the majority clade of *Ascomycota*, one strain of *Pythium intermedium* (*Oomycota*) and one strain of *Rhizopus oryzae* (Zygomycota). Huang et al (27) classified 108 fungal isolates obtained from three medicinal plant species *Artemisia capillaris*, *Artemisia indica* and *Artemisia lactiflora* using morphological identification. Multiple regions of the fungal rRNA genes have been used to study fungal taxonomy and diversity; which include small-subunit (SSU) and large-subunit (LSU) rRNA genes and an internal transcribed spacer (ITS) region separating these two rRNA genes (31, 39, 40). The heterogeneity and higher extent of variations are some of the useful properties of the ITS region (ITS1, 5.8S rRNA, and ITS2). Furthermore, growing ITS databases, has made this region more usable among mycologists for fungal identification (27). For taxonomic considerations, the sequences can be used to include related species into phylogenetic trees. We are interested in classifying cladistical because

genealogical relationships expressed in the classification reflect what occurs in nature. Furthermore, such classification can be used to make predictions about mating compatibility, evolution of secondary metabolites such as mycotoxins and morphological character states (35) and phylogeography (60). This study represents the work concerning the phylogenetic relationships of a morphologically and molecularly identified array of fungal endophytes from species of *Artemisia* with recovered sequences from the NCBI GenBank data base.

Material and Methods

Plants sampling

Plants of *Artemisia absinthium*, *A. vulgaris*, *A. austriaca*, *A. subulata*, *A. tangutica*, *A. lavandulifolia*, *A. argyi*, *A. brachyloba*, *A. scoparia*, *A. gorgonum* and *A. thuscula*, species were collected from Romania, Canary Islands (Fuerteventura, La Palma and Tenerife), China - Wuhan and Qichun (10) and Cabo Verde. In situ, plants were observed for their healthy appearance prior to the sampling and only those individuals which did not show symptoms of attack by pest or disease were selected. From each plant only stems segments were cut, labelled and kept in paper bags inside zip-locked bags at T = 4 - 5 °C until transported to the laboratory and then processed within 24 hours.

Fungal endophyte isolation

Established surface sterilization method was used in order to suppress epiphytic microorganisms from the plant and stem fragments were used to isolate endophytic fungi (11).

Morphological and molecular identification

Prior to taxonomic identification, a preliminary classification was made in order to avoid the selection of identical strains arising from the same plant individual, separating isolates into morphotypes. For the microscopic observations, a strain was inoculated onto a PDA Petri plate and a sterile cover slide was attached at a distance of two centimeters. Once the growth of the fungus partially covered the cover slide, the slide was removed, inverted on a slide with cotton blue (for the slightly coloured colonies) and observed under microscope. Several procedures of genomic DNA extraction were carried out due to impossibility of success with only one method (12). 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 (61). PCRs were performed in a total volume of 25 µl containing 10 ng genomic DNA, 0.5 µM primer, 200 µM dNTPs, 1X Buffer Taq, 0.0125U of Taq DNA Polymerase. For ITS sequences, PCR cycling parameters were carried out according to Shu et al (49) with slight modifications: 94 °C for 2.5 min; 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The final step was at 16 °C for 5 min. A total of 40 cycles were performed. All PCR products were detected by agarose gel electrophoresis (110V, 35 min, on 2% agarose gels, 1X TAE Buffer) loading 5 µl PCR product, 1 µl Loading Buffer (6X) and 2 µl SYBR Green I (dilution 1:10000). PCR and electrophoresis reagents were purchased from Sigma-Aldrich. 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). The sequences were run through the BLASTN search page using Megablast program (National Center for Biotechnology Information) where the most similar hits and their accession numbers were obtained.

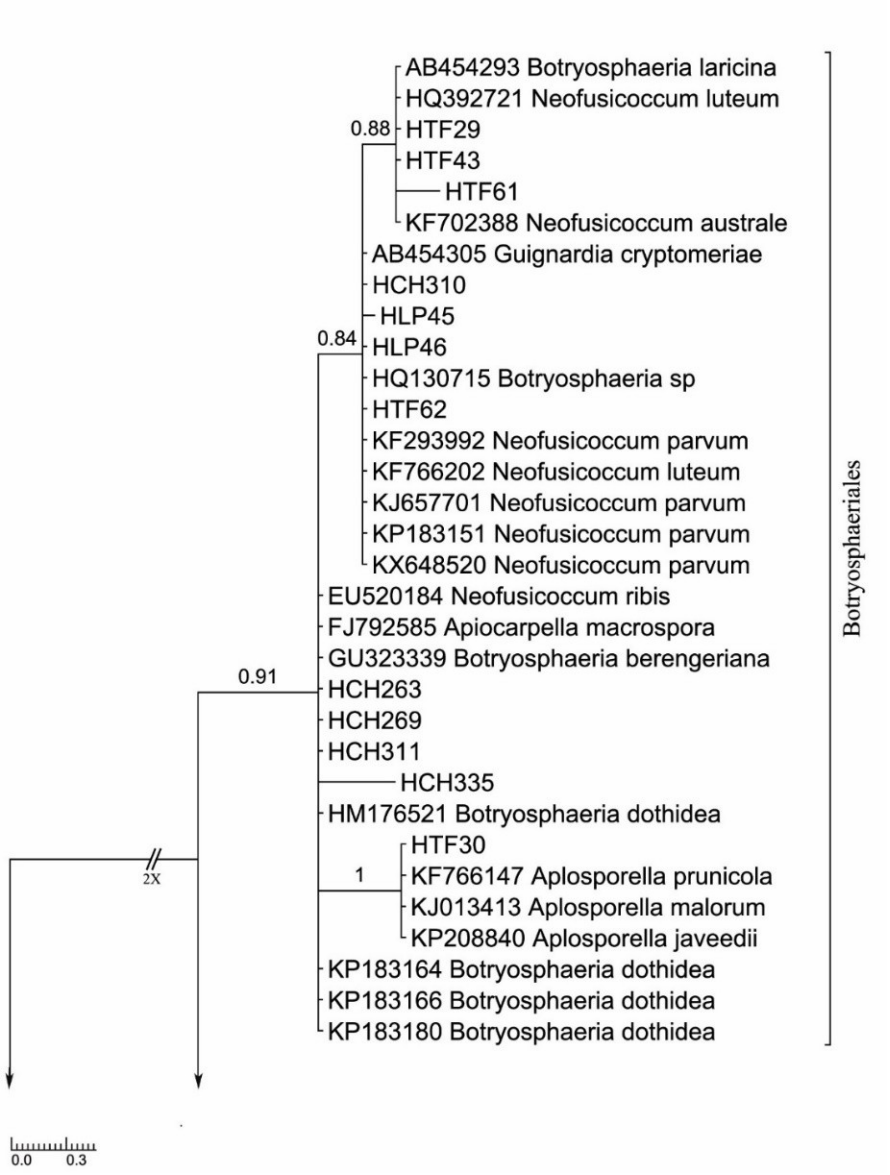
Phylogenetic analysis

ITS sequences (i.e. endophytic fungi and their most similar hits from GenBank) were aligned with the multiple alignment program ClustalW (55) as implemented in Mega 6.0 (54) and indels corrected manually to minimize alignment gaps (17). Designated outgroup was *Glomus* sp. (GenBank Accession Nº FJ164242.1). Because of the high number of indels, these were recoded as a binary matrix by means of the simple indel coding algorithm (50), appending the fragments to the nucleotide data as additional characters, as implemented in FastGap 1.21 (4). This "indel matrix" was used in all Bayesian analyses. Formerly, Gblocks program was used to eliminate poorly aligned positions and divergent regions (15). Best-fit models were compared in jModel test according to Bayesian Information Criterion (BIC) (14). Bayesian Inference analysis was conducted with MrBayes (28) and run for 1×10^7 generations with a sampling frequency of 100 generations. Of the resulting trees, the first 25,000 trees were discarded as burn-in and the following 75,001 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 (43). Convergence of the runs was indicated by an average standard deviation of split frequencies between duplicate runs of less than 0.01. In order to reconstruct the maximum likelihood trees both PhyML version 3.0 (hosted at http://www.atgc-montpellier.fr/phyml/) and TOPALI version 2.5 were used. For statistical reliability, non – parametric bootstrap samplings were carried out to estimate the support level for each internal branch, a total of 500 in PhyML and 100 in TOPALI (the maximum allowed by the software). The consensus trees were drawn using Treegraph software (52) and edited with Adobe Illustrator CS3.

Results and Discussions

Botryosphaeriales — Diaporthales taxa

26 sequences of endophytic fungal isolates of Botryosphaeriales spp. and Diaporthales spp. along with the correspondent two - three most similar hits from GenBank were used for these phylogenetic analyses. In total 80 sequences were used for this tree. Sequences used in the phylogenetic analyses provided by GenBank are embodied in Table 1 and the assigned species for the endophytic fungi are shown in Table 2. The data set consisted of 554 aligned bps; 161 conserved characters, 363 variable characters, 245 out of them parsimony informative and 116 singleton characters. Phylogenetic analyses of maximum likelihood and Bayesian inference were performed with PhyML version 3.0 and MrBayes using K80+G substitution model according to BIC. *Neofusicoccum*, *Botryosphaeria* and *Aplosporella* form a monophyletic clade (BPP = 0.91) depicting clear separation of these taxa. Relatively well supported, homogenous cluster of *Neofusicoccum* is shown (BPP = 0.84) by the Bayesian analysis. However, two external sequences of *Botryosphaeria* are included. When subjected to GenBank, sequence AB454293, identified as *Botryosphaeria laricina* has same results as its sister clustered sequence KF702388, identified as *Neofusicoccum australe* which is majoritarian *Neofusicoccum*. Also, sequence HQ392721, identified as *Neofusicoccum luteum*, when subjected to GenBank all first sequences producing significant alignments with higher "maximum and total score" were of *Neofusicoccum australe*. More seq. AB454305 identified as *Guignardia cryptomeriae* when subjected to GenBank has only one incidence with this species sequences while the rest of the results are mainly of *Neofusicoccum* spp. Despite all these interferences we have classified this cluster as *Neofusioccum* Section. The sister clade of *Neofusicoccum* Section reunites sequences of B*otryosphaeria* having similar branch lengths, except an endophytic fungal isolate HCH335 appearing with a higher substitution rate. Although identified as *Apiocarpella macrospora,* seq. FJ792585 when submitted to GenBank all relevant sequences in alignment were of *Botryosphaeria dothidea;* similarly was observed for *N. ribis* (sequence EU520184). Sequences of *Aplosporella* spp. cluster in the third sister clade and their close relation with *Neofusicoccum* and *Botryosphaeria* was previously shown (13). *Diaporthe* - *Phomopsis* (anamorph of *Diaporthe*) clade shows a series of multiple clusters although again ambiguous identifications combined with ITS based information solved only relatively the species aggrupation. Apparently ITS region in *Diaporthe* is evolving at higher rates than *TEF1* or *MAT* genes (45), therefore presenting a wider variation than advisable for species boundaries. Thus, a slowly evolving gene region should be used in order to establish species limits (56). Nevertheless, ITS sequence data can be used for reliable identification of phylogenetic relationships as long as they are interpreted with care (56). From the series of uncertain sequences, seq. JN854227 identified as *Diaporthe helianthi* when submitted to GenBank resulted among others in relevant similarity with the TYPE sequence of *Diaporthe novem* (seq. NR 111855) sharing same values of parameters with the main hits. External sequences identified as *Diaporthe phaseolorum* (AF001019, KX866874, and KJ590738) and *Diaporthe longicolla*, (KR709067 and JQ752971) can be interpreted as being polyphyletic. Also HCH330 and HCH337 identified as *Diaporthe longicolla* appear in sister clades. A resulting parsimonious tree of ITS sequences downloaded from GenBank shows that sequences of endophytic *Diaporthe longicolla* have paraphyletic origins (33). Nevertheless, the fact that *Diaporthe longicolla* (seq. KR709067) and *Diaporthe phaseolorum* (seq. KX866874) are clustered having same branch lengths is questionable in the present data set. We must admit that the genus *Phomopsis* contains more than 1000 species names therefore the traditional methods of identification are sometimes inadequate or unreliable (19, 46, 56). Being able to link the anamorph and teleomorph states through molecular sequence data regardless of whether the taxon in question expresses sexual or asexual structures (19, 24, 29, 48) is of great help as information related to clustering taxa and evolution pathways are offered, as well as an argument for recurring to a unique name for both states. Maximum likelihood analysis clustered the sequences in a similar manner as Bayesian analysis although the topology of the tree has changed. Yet, the backbone of the tree is not well supported, therefore irrelevant. Briefly, instead of two main clades in which Bayesian analysis resulted, likelihood analysis (Fig. 2) shows *Botryosphaeria* cluster as an older sister clade of *Neofusicoccum – Diaporthe* clade. In addition, *Aplosporella* cluster is drawn out. Moreover, it divides *Neofusicoccum* cluster, although again bootstrap supporting is low (BS = 36 and 54). *Diaporthe* sequences remain associated to a monophyletic clade (BS = 93) but are closely related to one of the *Neofusicoccum* clusters. Further, inner clustering inside D*iaporthe* clade remains essentially the same as in the Bayesian analysis.


AB454293 Botryosphaeria laricina
HQ392721 Neofusicoccum luteum
0.88
HTF29
HTF43
HTF61
KF702388 Neofusicoccum australe
AB454305 Guignardia cryptomeriae
HCH310
HLP45
0.84
HLP46
HQ130715 Botryosphaeria sp
HTF62
KF293992 Neofusicoccum parvum
KF766202 Neofusicoccum luteum
KJ657701 Neofusicoccum parvum
KP183151 Neofusicoccum parvum
KX648520 Neofusicoccum parvum
EU520184 Neofusicoccum ribis
FJ792585 Apiocarpella macrospora
GU323339 Botryosphaeria berengeriana
0.91
HCH263
HCH269
HCH311
HCH335
HM176521 Botryosphaeria dothidea
HTF30
1
KF766147 Aplosporella prunicola
KJ013413 Aplosporella malorum
KP208840 Aplosporella javeedii
KP183164 Botryosphaeria dothidea
KP183166 Botryosphaeria dothidea
KP183180 Botryosphaeria dothidea
2X
0.0
0.3
Botryosphaeriales

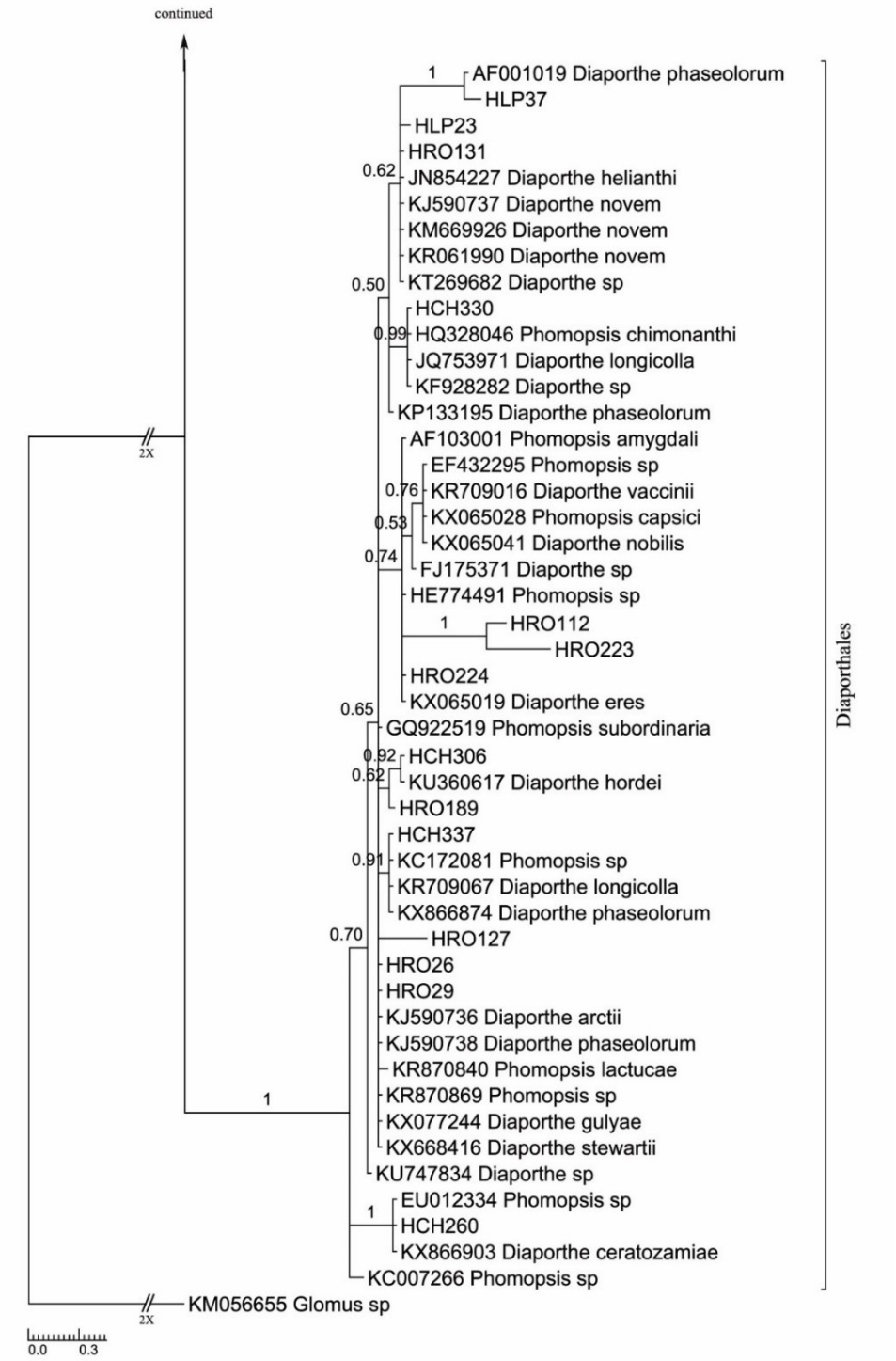
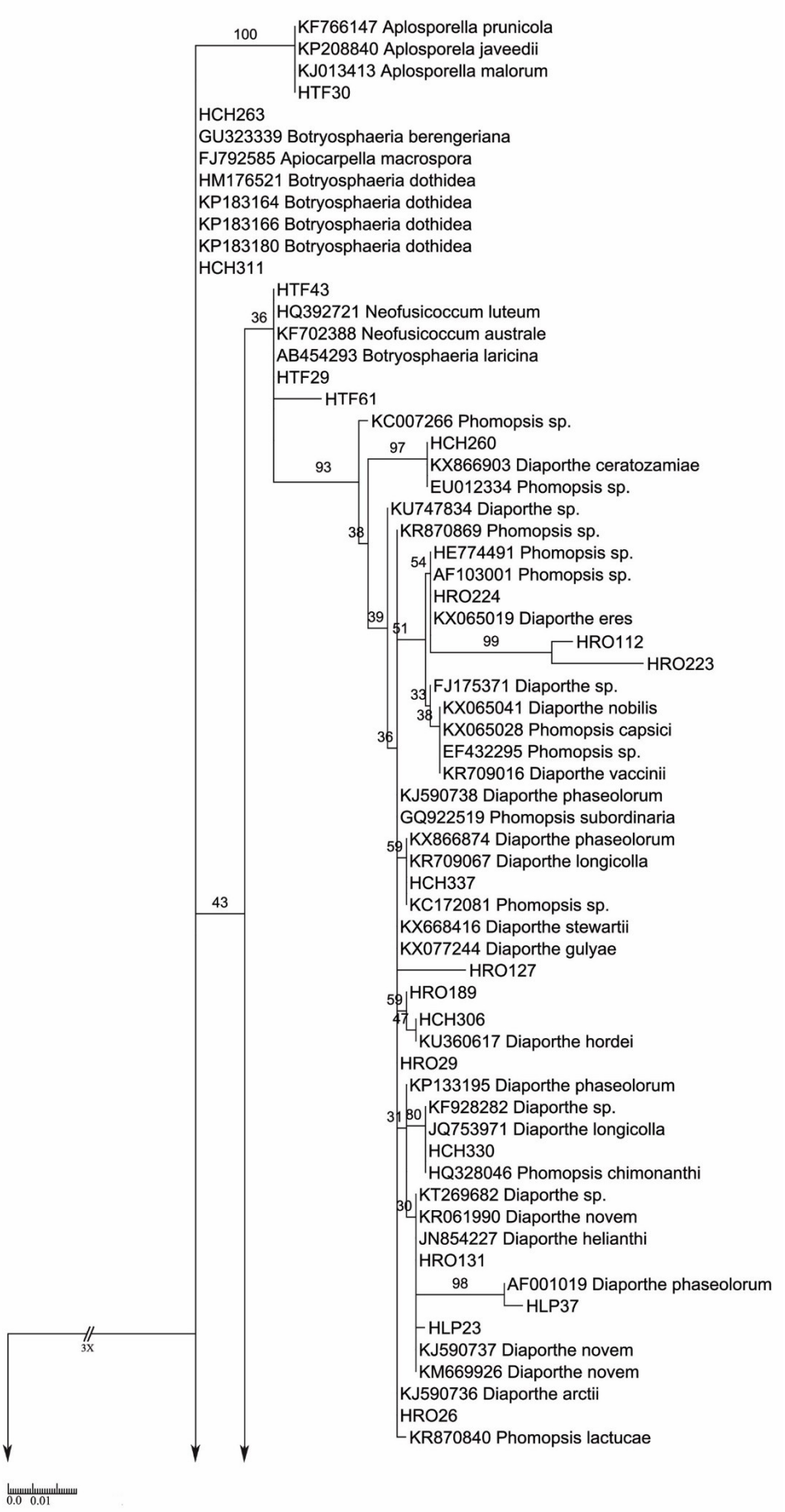


Fig. 1: Bayesian phylogenetic tree based on ITS rDNA sequence variants of the Botryosphaeriales — Diaporthales spp. The tree was rooted with *Glomus* sp. sequence as outgroup. Long branches were shortened by 50% as indicated with two diagonal slashes or by 75% indicated with three slashes. The Bayesian clade – credibility values (posterior probabilities) are indicated at internodes and the scale bar represents the expected changes per site.


100
KF766147 Aplosporella prunicola
KP208840 Aplosporela javeedii
KJ013413 Aplosporella malorum
HTF30
HCH263
GU323339 Botryosphaeria berengeriana
FJ792585 Apiocarpella macrospora
HM176521 Botryosphaeria dothidea
KP183164 Botryosphaeria dothidea
KP183166 Botryosphaeria dothidea
KP183180 Botryosphaeria dothidea
HCH311
HTF43
36
HQ392721 Neofusicoccum luteum
KF702388 Neofusicoccum australe
AB454293 Botryosphaeria laricina
HTF29
HTF61
KC007266 Phomopsis sp.
97
HCH260
93
KX866903 Diaporthe ceratozamiae
EU012334 Phomopsis sp.
KU747834 Diaporthe sp.
38
KR870869 Phomopsis sp.
54
HE774491 Phomopsis sp.
AF103001 Phomopsis sp.
HRO224
39
KX065019 Diaporthe eres
51
99
HRO112
HRO223
33
FJ175371 Diaporthe sp.
38
KX065041 Diaporthe nobilis
KX065028 Phomopsis capsici
36
EF432295 Phomopsis sp.
KR709016 Diaporthe vaccinii
KJ590738 Diaporthe phaseolorum
GQ922519 Phomopsis subordinaria
59
KX866874 Diaporthe phaseolorum
KR709067 Diaporthe longicolla
HCH337
43
KC172081 Phomopsis sp.
KX668416 Diaporthe stewartii
KX077244 Diaporthe gulyae
HRO127
59
HRO189
47
HCH306
KU360617 Diaporthe hordei
HRO29
KP133195 Diaporthe phaseolorum
31
80
KF928282 Diaporthe sp.
JQ753971 Diaporthe longicolla
HCH330
HQ328046 Phomopsis chimonanthi
30
KT269682 Diaporthe sp.
KR061990 Diaporthe novem
JN854227 Diaporthe helianthi
HRO131
98
AF001019 Diaporthe phaseolorum
HLP37
HLP23
3X
KJ590737 Diaporthe novem
KM669926 Diaporthe novem
KJ590736 Diaporthe arctii
HRO26
KR870840 Phomopsis lactucae
0.0 0.01

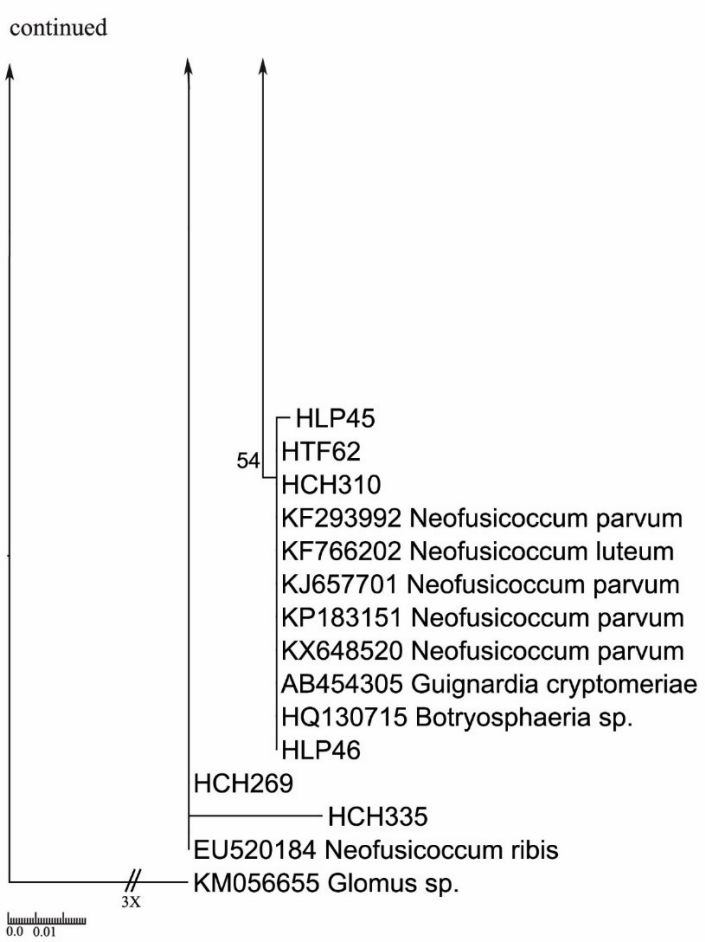


Fig. 2: Maximum likelihood tree based on ITS rDNA sequence variants of the Botryosphaeriales - Diaporthales spp. The tree was rooted with *Glomus* sp. sequence as outgroup. Long branches were shortened by 50% as indicated with two diagonal slashes or by 75% indicated with three slashes. Bootstrap values are indicated at internodes and the scale bar represents the expected changes per site.

Table 1

Botryosphaeriales — Diaporthales taxa: Accession No. of sequences provided by GenBank used in the phylogenetic analyses

AB454293.1	AB454305.1	AF001019.2	AF103001.1	EF432295.1
EU012334.1	EU520184.1	FJ175371.1	FJ792585.1	GQ922519.1
HE774491.1	HM176521.1	HQ130715.1	HQ328046.1	HQ392721.1
JN854227.1	JQ753971.1	KC007266.1	KC172081.1	KF293992.1
KF702388.1	KF766147.1	KF766202.1	KF928282.1	KJ013413.1
KJ590736.1	KJ590737.1	KJ590738.1	KJ657701.1	KM056655.1
KM669926.1	KP133195.1	KP183151.1	KP183164.1	KP183166.1
KP183180.1	KP208840.1	KR061990.1	KR709016.1	KR709067.1
KR870840.1	KR870869.1	KT269682.1	KU360617.1	KU747834.1
KX065019.1	KX065028.1	KX065041.1	KX077244.1	KX648520.1
KX668416.1	KX866874.1	KX866903.1	GU323339.1	

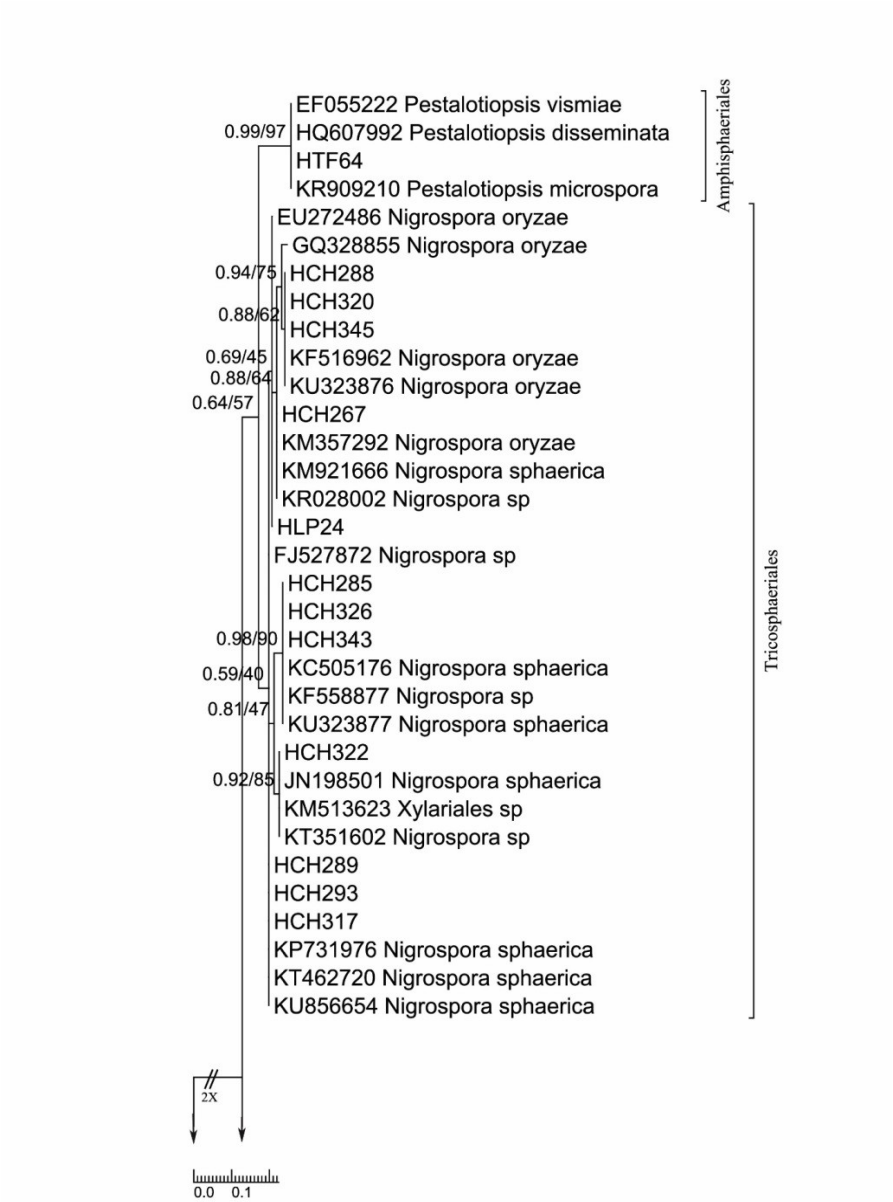
Table 2

Botryosphaeriales — Diaporthales taxa*: Artemisia* endophytic fungal strains used in phylogenetic analyses, codes and identities

Assigned species	Abbreviated strain code
Aplosporella prunicola	HTF30
Botryosphaeria dothidea	HCH263; HCH269; HCH311
Botryosphaeria sp. 1	HCH335
Diaporthe arctii	HRO26; HRO29
Diaporthe ceratozamiae	HCH260
Diaporthe eres	HRO224
Diaporthe hordei	HCH306; HRO189
Diaporthe longicolla	HCH330
Diaporthe novem	HLP23; HRO131
Diaporthe sp. 1	HCH337; HLP37
Diaporthe sp. 2	HRO127
Diaporthe sp. 3	HRO112
Diaporthe sp. 4	HRO223
Neofusicoccum australe	HTF29; HTF43; HTF61
Neofusicoccum parvum	HCH310; HLP45; HLP46; HTF62

Sordariomycetes taxa: *Chaetomium*, *Biscogniauxia*, *Thielavia*, *Sordaria*, *Daldinia* and *Nigrospora*

27 sequences of endophytic fungal isolates along with the correspondent two - three most similar hits from GenBank were used for these phylogenetic analyses. In total 65 sequences were used for the phylogenetic analyses. Sequences used in the phylogenetic analyses provided by GenBank are embodied in Table 3 and the assigned species for the endophytic fungi are shown in Table 4. The data set consisted of 537 aligned bps; 104 conserved characters, 417 variable characters, 241 out of them parsimony informative and 172 singleton. Phylogenetic analyses of maximum likelihood and Bayesian inference were performed with PhyML version 3.0 and MrBayes, using TrNef+G substitution model as suggested by BIC. Bayesian topology (Fig. 3) reconstructs the tree relating four main clades: clade 1 with Nigrospora and Pestalotiopsis sister clusters, clade 2 with *Sordaria* spp., *Chaetomium* spp. and *Thielavia* spp. as sister clusters, clade 3 with *Daldinia loculata* and clade 4 with *Biscogniauxia mediterranea*. Clade 1 reunites *Amphisphaeriales* (*Pestalotiopsis* spp.) and *Tricosphaeriales* (*Nigrospora* spp.) showing them in a closer relation than the one with the other two orders (i.e. *Sordariales* and *Xylariales*), although without relevant Bayesian posterior probability (BPP = 0.63). Clade 2 associates three genera of *Sordariales* - *Sordaria*, *Chaetomium* and *Thielavia* - (BPP = 0.89) which seem to constitute a monophyletic clade. For *Sordaria* cluster no species differentiation were shown, leaving all three species of external sequences and fungal endophytes with same branch lengths (BPP = 0.96). Conversely, for *Thielavia* sequences, two clusters are shown one for *Thielavia arenaria* and *Thielavia subthermophila* and one cluster for *Thielavia microspora.* Clade 3 associates two external sequences of which one is identified as *Daldinia loculata* and the other one was assigned to *Sordariomycetes* along with an endophytic fungal isolate (BPP = 0.97). We consider this clade as belonging to *Daldinia loculata*. Overall this phylogenetic analysis shows the monophyletic topology of four orders i) *Amphisphaeriales,* ii) *Tricosphaeriales,* iii) *Sordariales*, iiii) *Xylariales*, previously placed in *Sordariomycetes.* Previous studies on phylogenetic relations between members of this class were performed but not including all these four orders (64). Briefly, maximum likelihood sustains the topology made by Bayesian analysis, therefore only the Bayesian phylogenetic tree is shown with both BPP and BS values.


EF055222 Pestalotiopsis vismiae
0.99/97
HQ607992 Pestalotiopsis disseminata
HTF64
KR909210 Pestalotiopsis microspora
Amphisphaeriales
EU272486 Nigrospora oryzae
GQ328855 Nigrospora oryzae
0.94/75
HCH288
HCH320
0.88/62
HCH345
0.69/45
KF516962 Nigrospora oryzae
0.88/64
KU323876 Nigrospora oryzae
0.64/57
HCH267
KM357292 Nigrospora oryzae
KM921666 Nigrospora sphaerica
KR028002 Nigrospora sp
HLP24
FJ527872 Nigrospora sp
HCH285
HCH326
0.98/90
HCH343
0.59/40
KC505176 Nigrospora sphaerica
KF558877 Nigrospora sp
0.81/47
KU323877 Nigrospora sphaerica
HCH322
0.92/85
JN198501 Nigrospora sphaerica
KM513623 Xylariales sp
KT351602 Nigrospora sp
HCH289
HCH293
HCH317
KP731976 Nigrospora sphaerica
KT462720 Nigrospora sphaerica
KU856654 Nigrospora sphaerica
Tricosphaeriales
2X
0.0
0.1

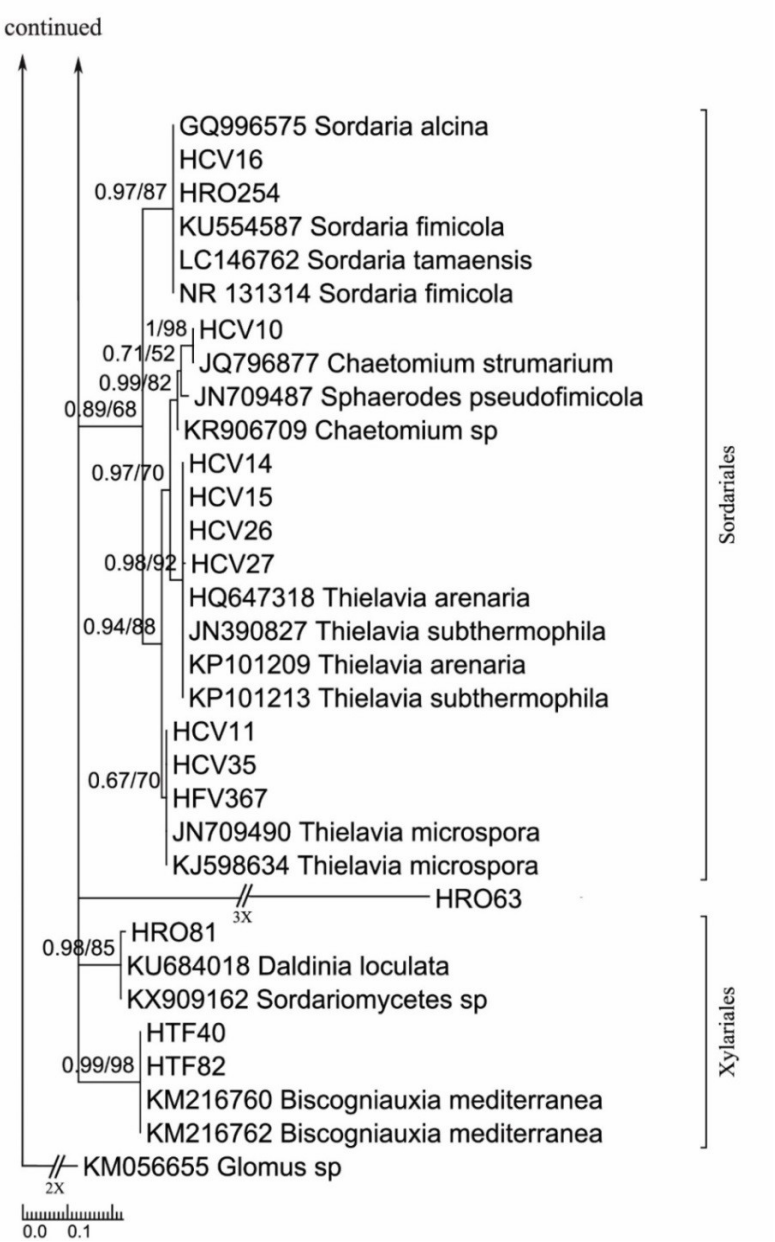


Fig. 3: Bayesian phylogenetic tree based on ITS rDNA sequence variants of the Sordariomycetes taxa. The tree was rooted with *Glomus* sp. sequence as outgroup. Long branches were shortened by 50% as indicated with two diagonal slashes or by 75% indicated with three slashes. The Bayesian clade – credibility values (posterior probabilities) and the ML bootstrap support values are indicated at internodes (BPP/BS). The scale bar represents the expected changes per site.

Table 3

Sordariomycetes taxa: Accession No. of sequences provided by GenBank used in the phylogenetic analyses

EF055222.1	EU272486.1	FJ527872.1	GQ328855.1	GQ996575.1
HQ607992.1	HQ647318.1	JN198501.1	JN390827.1	JN709487.1
JN709490.1	JQ796877.1	KC505176.1	KF516962.1	KF558877.1
KJ598634.1	KM056655.1	KM216760.1	KM216762.1	KM357292.1
KM513623.1	KM921666.1	KP101209.1	KP101213.1	KP731976.1
KR028002.1	KR906709.1	KR909210.1	KT351602.1	KT462720.1
KU323876.1	KU323877.1	KU554587.1	KU684018.1	KU856654.1
KX909162.1	LC146762.1	NR_131314.1		

Table 4

Sordariomycetes taxa: *Artemisia* endophytic fungal strains used in phylogenetic analyses, codes and identities

Assigned species	Abbreviated strain code
Biscogniauxia mediterrranea	HTF40; HTF82
Chaetomium strumarium	HCV10
Daldinia loculata	HRO81
Nigrospora oryzae	HCH267; HCH288; HCH320; HCH345; HLP24
Nigrospora sp. 2	HLP38
Nigrospora sphaerica	HCH285; HCH289; HCH293; HCH317; HCH322; HCH326; HCH343; HRO63
Pestalotiopsis sp.	HTF64
Sordaria fimicola	HCV16; HRO254
Thielavia arenaria	HCV14; HCV15; HCV26; HCV27
Thielavia microspora	HCV11; HCV35; HFV367

Hypocreales — Glomerales — Microascales taxa

Ten sequences of endophytic fungal isolates along with the correspondent three or four most similar hits from GenBank were used for these phylogenetic analyses. In total 35 sequences were used for the phylogenetic analyses. Sequences used in the phylogenetic analyses provided by GenBank are embodied in Table 5 and the assigned species for the endophytic fungi are shown in Table 6. The data set consisted of 572 aligned bps; 187 conserved characters, 377 variable characters, 320 out of them parsimony informative and 57 singleton. Phylogenetic analyses of maximum likelihood and Bayesian inference were performed with TOPALi and MrBayes using TPM1+G substitution model as suggested by BIC. Both Bayesian and maximum likelihood (Fig. 4) phylogenetic analyses showed two main clades, as expected. Clade 1 comprises Hypocreales, Glomerales and Microascales members (BPP = 1) while Clade 2, reunites Saccharomycetales members as older in basal group (BPP = 1). Clade 1 is monophyletic and is divided into four clusters. Interestingly, members of *Hypocreales* are separated in sister clades except a closer relation which is observed for *Stachybotrys – Sirastachys* (*Stachybotryaceae*) and *Nectria – Sarocladium – Corallomycetella* (*Nectriaceae*), which are united in a cluster (BPP = 0.99). *Corallomycetella repens* sensu stricto is considered to be restricted to specimens from Asia while *Corallomycetella elegans* (i.e. synonym of *Nectria mauritiicola*) is resurrected for specimens from Africa and America (25). Therefore, the fungal endohytic isolate HTF23 is more probable to be considered *Nectria mauritiicola*. *Colletotrichum – Glomerella* sequences are clustered (BPP = 0.80) but no clear and doubtless classification regarding species could be obtained. It has been estimated that approximately 86% of named sequences of *Colletotrichum gloeosporioides* in GenBank do not align with the epitype (5, 33). It is interesting that in this data set no closer relations were obtained for the Hypocreales members, leaving for instance *Purpureocillium lilacinum* unclustered. More, the close relation of *Hypocreales* with *Glomerales* is underlined.

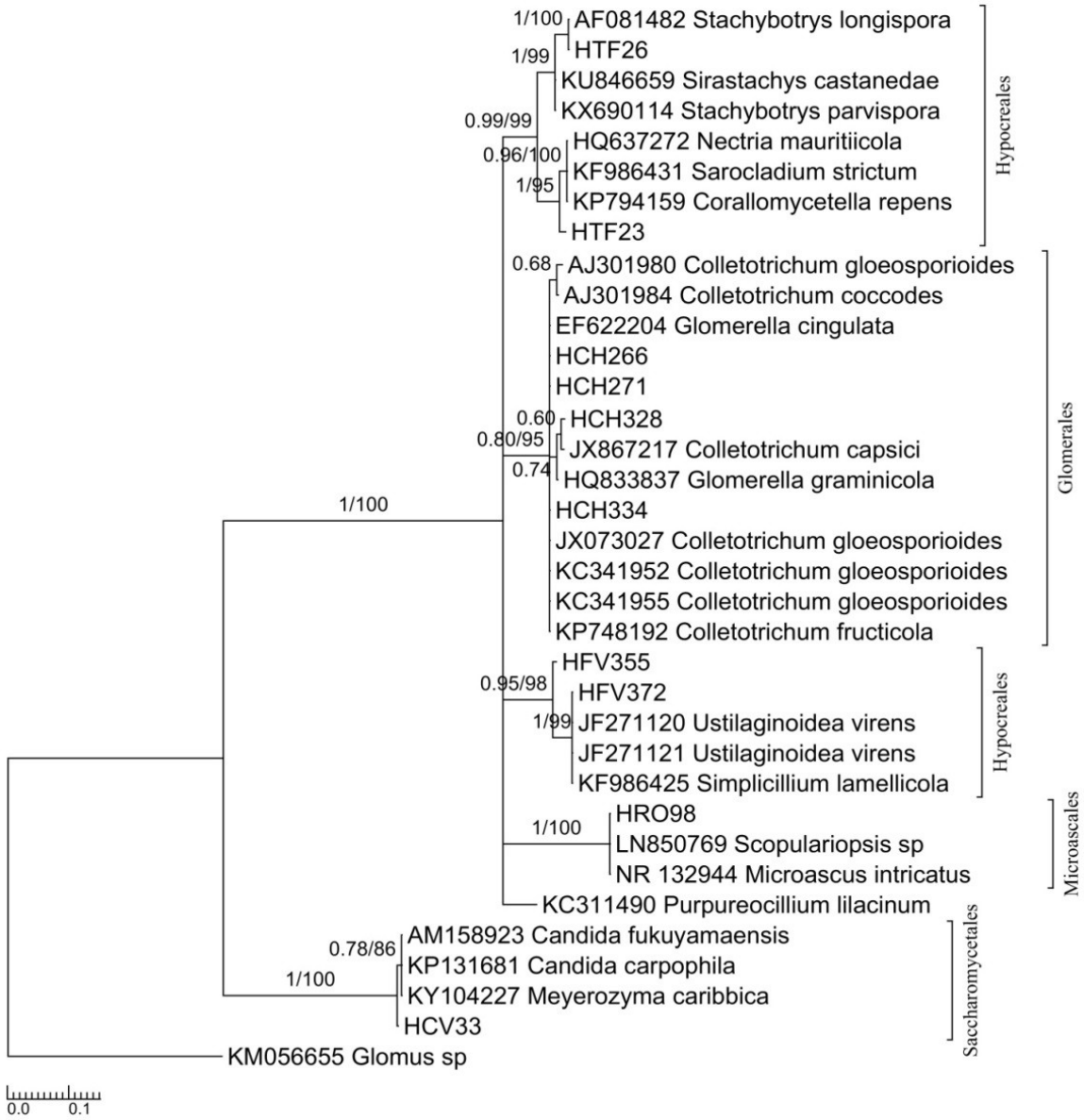


Fig. 4: Bayesian phylogenetic tree based on ITS rDNA sequence variants of the Hypocreales - Glomerales – Microascales taxa. The tree was rooted with *Glomus* sp. sequence as outgroup. The Bayesian clade – credibility values (posterior probabilities) and the ML bootstrap support values are indicated at internodes (BPP/BS). The scale bar represents the expected changes per site.

Table 5

Hypocreales — Glomerales — Microascales taxa: Accession No. of sequences provided by GenBank used in the phylogenetic analyses

AF081482.1	AJ301980.1	AJ301984.1	AM158923.1	EF622204.1
HQ637272.1	HQ833837.1	JF271120.1	JF271121.1	JX073027.1
JX867217.1	KC311490.1	KC341952.1	KC341955.1	KF986425.1
KF986431.1	KM056655.1	KP131681.1	KP748192.1	KP794159.1
KU846659.1	KX690114.1	KY104227.1	LN850769.1	NR_132944.1

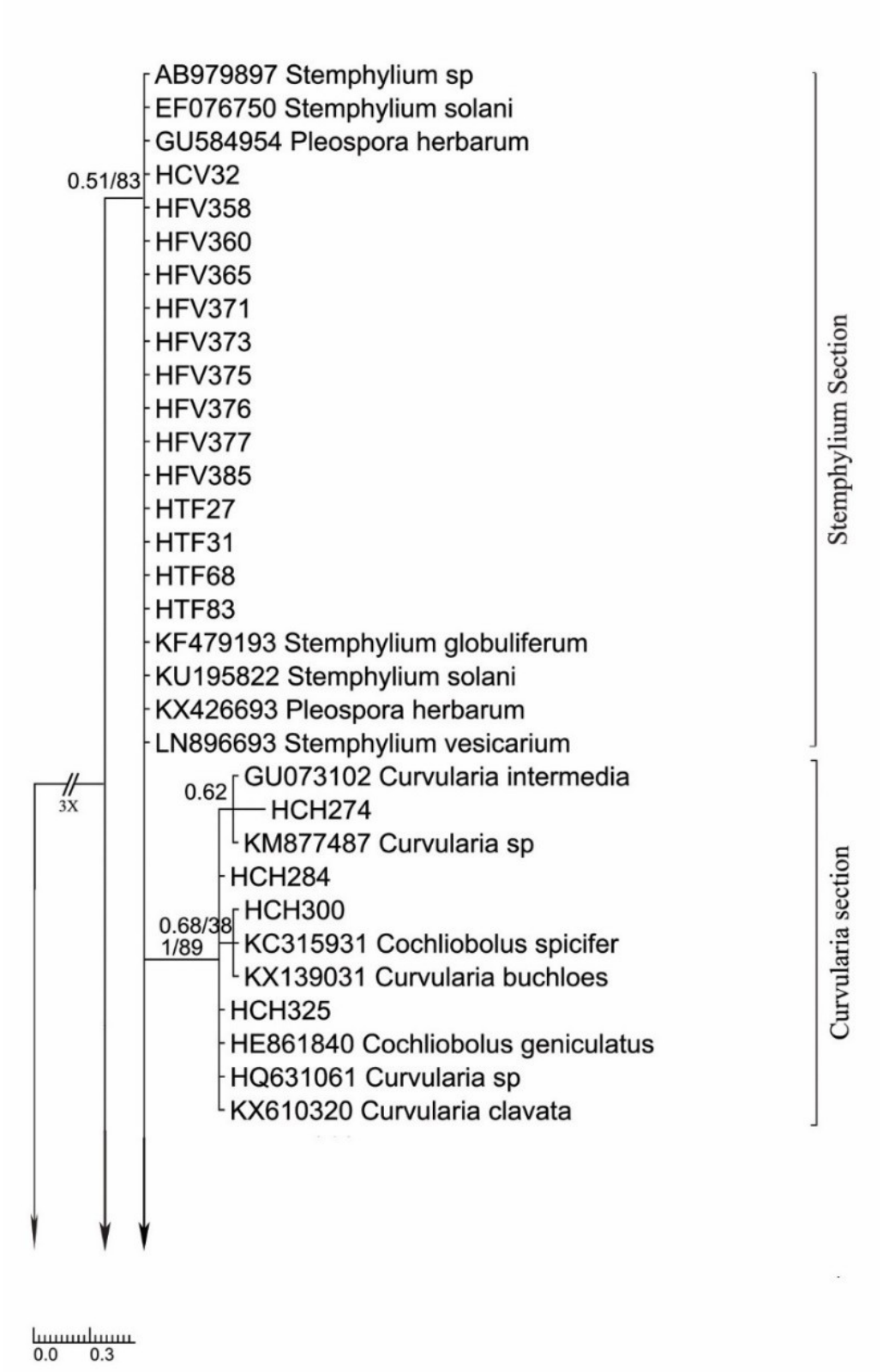
Table 6

Hypocreales – Glomerales – Microascales taxa: *Artemisia* endophytic fungal strains used in phylogenetic analyses, codes and identities

Assigned species	Abbreviated strain code
Candida sp. 1	HCV33
Colletotrichum capsici	HCH328
Colletotrichum gloeosporioides	HCH266; HCH271; HCH334
Microascus intricatus	HRO98
Nectria mauritiicola	HTF23
Simplicillium lamelicola	HFV355; HFV372
Stachybotrys longispora	HTF26

Pleosporales taxa

34 sequences of endophytic fungal isolates along with the correspondent three or four most similar hits from GenBank were used for these phylogenetic analyses. In total 85 sequences were used for the phylogenetic analyses. The data set consisted of 469 aligned bps; 205 conserved characters, 234 variable characters, 154 out of them parsimony informative and 79 singleton. Sequences used in the phylogenetic analyses provided by GenBank are embodied in Table 7 and the assigned species for the endophytic fungi are shown in Table 8. Phylogenetic analyses of maximum likelihood and Bayesian inference were performed with PhyML and MrBayes, using TrNef+G as substitution model according to BIC. Bayesian analysis (Fig. 5) resulted in several clades, mainly separating taxa as expected. Sequences of *Pleospora* - *Stemphylium* (anamorph of *Pleospora*) were associated but unclustered and recognised as *Stemphylium* section. *Cochliobolus* - *Curvularia* (anamorph of *Cochliobolus*) sequences formed a well-supported clade (BPP = 0.99) as a sister cluster of *Stemphylium* section. *Cochliobolus* and *Pleospora* have been previously described as clustered in a phylogeny of *Phoma* sections, *Pleosporaceae* (21). *Coniothyrium* - like taxa were also united in a cluster (BPP = 1) with various inside separations either according to the species/genus, for instance *Camarosporium brabeji* cluster or heterogeneously selected association of *Tremateia* - *Leptosphaerulina* cluster (BPP = 1)/*Microsphaeropsis* - *Coniothyrium* (anamorph of *Paraphaeosphaeria)* - *Microdiplodia* cluster (BPP = 0.99). Finally, miscellaneous strains of *Coniothyrium*, *Paraconiothyrium*, *Microdiplodia* and *Paraphaeosphaeria* were comprised in the large Clade *Coniothyrium* - like taxa. Previous clustering of *Paraphaeosphaeria* - *Coniothyrium* - *Microsphaeropsis* taxa was indicated in a study on *Coniothyrium* - like members of *Pleosporales* and their relatives (57). Similar topology of the phylogenetic lineage of *Pleospora*, *Microsphaeropsis, Coniothyrium, Paraconiothyrium, Paraphaeosphaeria* was previously described (58). Interestingly the cluster comprising *Phoma* - like taxa was revealed as an inner cluster of *Coniothyrium* – like Clade, although not well supported (BBP = 0.55). Clustering in sister clades members of *Coniothyrium* – like, *Phoma* – like, *Paraphoma* and *Epicoccum* was previously only partially studied (21, 22, 34, 57, 58). Finally a cluster containing members of two genera of *Phoma* – like morphology, *Neoplatysporoides* and *Libertasomyces* is well supported (BPP = 0.98); and it also shows inner cluster of a fungal endophytic isolate HLP44 and the external seq. of *Neoplatysporoides aloicola* (BPP = 0.90). Recent molecular phylogenetic studies focussing on sexual and asexual genera of Pleosporales have demonstrated that *Coniothyrium* and *Microsphaeropsis*, and also the ubiquitous and speciose coelomycete genus *Phoma*, are polyphyletic, with species occurring in several clades of the order *Pleosporales*, which are now being used as a firm basis for redefining families (2, 21, 23, 42, 47, 51, 57, 58, 65). The position of the type species *Microsphaeropsis olivacea* was confirmed within the family *Didymellaceae* and that of *Coniothyrium (Coniothyrium palmarum)* within the *Leptosphaeriaceae*. Several *Coniothyrium* species were grouped in the well-supported clade of *Montagnulaceae*, together with *Paraphaeosphaeria* (66). *Paraphaeosphaeria* was established to accommodate species similar to *Phaeosphaeria* which have a *Coniothyrium* - type (conidia brown, non-septate) anamorph (16). Yet, some anamorphs are *Coniothyrium*-like whereas others are more typical of *Microsphaeropsis* (53). Previous work demonstrated that *Paraphaeosphaeria* is polyphyletic (6–8). Maximum likelihood analysis revealed similar topology, maintaining the main clusters, and only slightly changing the segregation inside clades. Therefore only the Bayesian phylogenetic tree is shown with both BPP and BS values.


AB979897 Stemphylium sp
EF076750 Stemphylium solani
GU584954 Pleospora herbarum
0.51/83
HCV32
HFV358
HFV360
HFV365
HFV371
HFV373
HFV375
HFV376
HFV377
HFV385
HTF27
HTF31
HTF68
HTF83
KF479193 Stemphylium globuliferum
KU195822 Stemphylium solani
KX426693 Pleospora herbarum
LN896693 Stemphylium vesicarium
Stemphylium Section
3X
0.62
GU073102 Curvularia intermedia
HCH274
KM877487 Curvularia sp
HCH284
0.68/38
1/89
HCH300
KC315931 Cochliobolus spicifer
KX139031 Curvularia buchloes
HCH325
HE861840 Cochliobolus geniculatus
HQ631061 Curvularia sp
KX610320 Curvularia clavata
Curvularia section
0.0
0.3

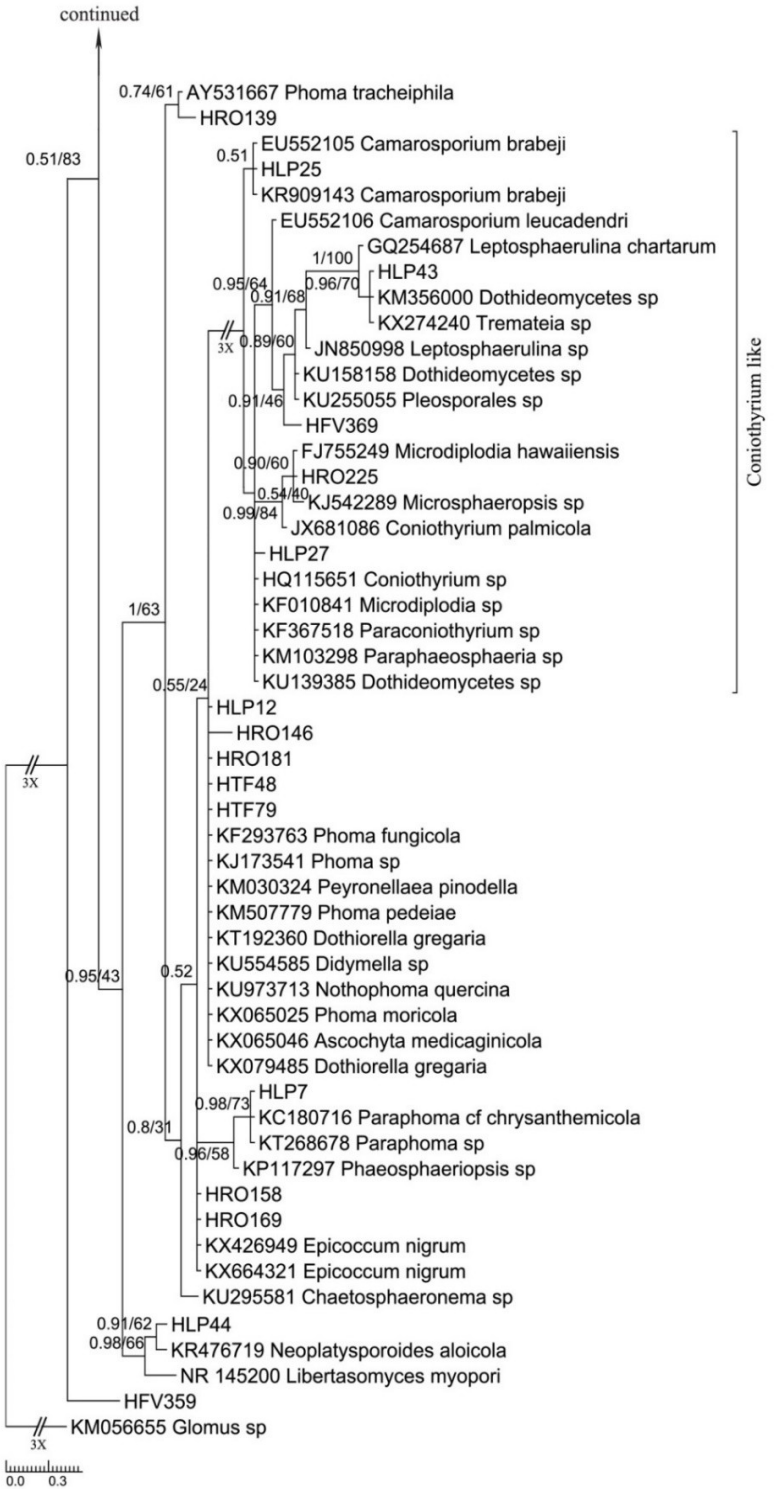


Fig. 5: Bayesian phylogenetic tree based on ITS rDNA sequence variants of the Pleosporales taxa. The tree was rooted with *Glomus* sp. sequence as outgroup. Long branches were shortened by 50% as indicated with two diagonal slashes or by 75% indicated with three slashes. The Bayesian clade – credibility values (posterior probabilities) and the ML bootstrap support values are indicated at internodes (BPP/BS). The scale bar represents the expected changes per site.

Table 7

Pleosporales taxa: Accession No. of sequences provided by GenBank used in the phylogenetic analyses

AB979897.1	AY531667.1	EF076750	EU552105.1	EU552106.1
FJ755249.1	GQ254687.1	GU073102.1	GU584954.1	HE861840.1
HQ115651.1	HQ631061.1	JN850998.1	JX681086.1	KC180716.1
KC315931.1	KF010841.1	KF293763.1	KF367518.1	KF479193.1
KJ173541.1	KJ542289.1	KM030324.1	KM056655.1	KM103298.1
KM356000.1	KM507779.1	KM877487.1	KP117297.1	KR476719.1
KR909143.1	KT192360.1	KT268678.1	KU139385.1	KU158158.1
KU195822.1	KU255055.1	KU295581.1	KU554585.1	KU973713.1
KX065025.1	KX065046.1	KX079485.1	KX139031.1	KX274240.1
KX426693.1	KX426949.1	KX610320.1	KX664321.1	LN896693.1
NR_145200.1				

Table 8

Pleosporales taxa: *Artemisia* endophytic fungal strains used in phylogenetic analyses, codes and identities

Assigned species	Abbreviated strain code
Camarosporium brabeji	HLP25
Coniothirium sp. 1	HLP27
Curvularia geniculata	HCH284; HCH325
Curvularia intermedia	HCH274
Curvularia spicifera	HCH300
Epicoccum nigrum	HRO158; HRO169
Leptosphaerulina sp. 1	HFV369
Microdiplodia hawaiiensis	HRO225
Neopolatysporoides aloicola	HLP44
Paraphoma chrysantemicola	HLP7
Phoma sp. 1	HTF48; HTF79
Phoma sp. 2	HRO146
Phoma sp. 3	HLP12; HRO181
Phoma tracheiphila	HRO139
Stemphylium solani	HFV358; HFV359; HFV360; HFV365; HFV371; HFV373; HFV375; HFV376; HFV377; HFV385; HTF27; HTF31; HTF68; HTF83
Stemphylium sp. 1	HCV32
Tremateia sp. 1	HLP43

Conclusions

Phylogenetic analyses revealed clustering between the endophytic fungi sequences and the external selected hits from NCBI GenBank without any proof of stronger relations between the endophytic fungi of the same species.

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