Aspergillus is monophyletic: Evidence from multiple gene phylogenies and extrolites profiles

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Abstract: Aspergillus is one of the economically most important fungal genera. Recently, the ICN adopted the single name nomenclature which has forced mycologists to choose one name for fungi (e.g. Aspergillus, Fusarium, Penicillium, etc.). Previously two proposals for the single name nomenclature in Aspergillus were presented: one attributes the name "Aspergillus" to clades comprising seven different teleomorphic names, by supporting the monophyly of this genus; the other proposes that Aspergillus is a non-monophyletic genus, by preserving the Aspergillus name only to species belonging to subgenus Circumdati and maintaining the sexual names in the other clades. The aim of our study was to test the monophyly of Aspergilli by two independent phylogenetic analyses using a multilocus phylogenetic approach. One test was run on the publicly available coding regions of six genes (RPB1, RPB2, Tsr1, Cct8, BenA, CaM), using 96 species of Penicillium, Aspergillus and related taxa. Bayesian (MrBayes) and Ultrafast Maximum Likelihood (IQ-Tree) and Rapid Maximum Likelihood (RaxML) analyses gave the same conclusion highly supporting the monophyly of Aspergillus. The other analyses were also performed by using publicly available data of the coding sequences of nine loci (18S rRNA, 5,8S rRNA, 28S rRNA (D1-D2), RPB1, RPB2, CaM, BenA, Tsr1, Cct8) of 204 different species. Both Bayesian (MrBayes) and Maximum Likelihood (RAxML) trees obtained by this second round of independent analyses strongly supported the monophyly of the genus Aspergillus. The stability test also confirmed the robustness of the results obtained. In conclusion, statistical analyses have rejected the hypothesis that the Aspergilli are non-monophyletic, and provided robust arguments that the genus is monophyletic and clearly separated from the monophyletic genus Penicillium. There is no phylogenetic evidence to split Aspergillus into several genera and the name Aspergillus can be used for all the species belonging to Aspergillus i.e. the clade comprising the subgenera Aspergillus, Circumdati, Fumigati, Nidulantes, section Cremei and certain species which were formerly part of the genera Phialosimplex and Polypaecilum. Section Cremei and the clade containing Polypaecilum and Phialosimplex are proposed as new subgenera of Aspergillus. The phylogenetic analysis also clearly shows that Aspergillus clavatoflavus and A. zonatus do not belong to the genus Aspergillus. Aspergillus clavatoflavus is therefore transferred to a new genus Aspergillago as Aspergillago clavatoflavus and A. zonatus was transferred to Penicilliopsis as P. zonata. The subgenera of Aspergillus share similar extrolite profiles indicating that the genus is one large genus from a chemotaxonomical point of view. Morphological and ecophysiological characteristics of the species also strongly indicate that Aspergillus is a polythetic class in phenotypic characters.

Key words: Aspergillus, Multigene phylogeny, Monophyly, Nomenclature, Teleomorphs.
Taxonomic novelties: Aspergillus subgenus Cremei, subgen. nov., Aspergillus subgenus Polypaecilum, subgen. nov., Aspergillago Samson, Houbraken & Frisvad, gen. nov.; New combinations: Aspergillago clavatoflava (Raper & Fennell) Samson, Houbraken & Frisvad, comb. nov., Penicilliopsis zonatus (Kwon-Chung & Fennell) Samson, Houbraken & Frisvad, comb. nov..

INTRODUCTION

The genus Aspergillus contains some of the most abundant and widely distributed organisms on earth, and comprises approximately 350 accepted species (Samson et al. 2014). It is one of the fungal genera with the highest economic importance in biotechnology (enzymes, organic acids, bioactive metabolites), but members of the genus are also frequently reported as foodborne contaminants (food spoilage and mycotoxin contamination), or as causal agents of human mycoses (pulmonary aspergillosis, otomycosis, keratitis). Aspergillus is also one of the oldest names in fungal taxonomy since it was applied by Micheli (1729), who gave it this name because the spore-bearing structure characteristic of the genus resembled an aspergillum (a device used by the Catholic church to sprinkle holy water). However this morphological characteristic resulted in a broad generic concept because it is associated to twelve quite different teleomorphs demonstrating the variation in physiological and morphological features (Houbraken & Samson 2011, Pitt & Taylor 2014). Houbraken et al. (2014) have reduced the number of teleomorphic names to ten (Petromyces, Neopetromyces, Saitoa, Fennellia, Emericella, Hemisartorya, Neosartorya, Neocarpenteles, Cristaspora, and Eurotium) and showed that the teleomorphs Warcupiella and Sclerocleista do not belong to the Aspergillus monophyletic clade.

The most important change in recent fungal nomenclature is the abandonment of dual nomenclature for pleomorphic fungi, following the decision taken at the International Botanical Congress in Melbourne (24–30 July, 2011). In the latest International Code of Nomenclature for algae, fungi and plants (ICN, McNeill et al. 2012), the single name nomenclature was adopted. This has forced mycologists to choose one name for each fungal genus (i.e Aspergillus, Fusarium, Penicillium, etc.). The ICN recommended that either the sexual or asexual name can be

chosen, in contrast to the earlier recommendation that the name of the sexual state should always be preferred. Several sexual names have priority over the asexual ones, but the final choice among the names should also be strongly supported by the (mycological) community. In general, the nomenclatural decision has been easily assigned for most fungal genera, but it sometimes became complicated for economically and socially important fungi having a well-established sexual and asexual name (Zhang *et al.* 2013). Even though taxonomy contains the rather independent disciplines such as classification, nomenclature and identification, decisions concerning nomenclature should take into account both the other two. In recent years cladonomy has having a more and more important impact on taxonomy, to a degree where monophyly is the overruling factor in deciding which taxa (clada) should be accepted and which names to give to them, rather than classificatory principles.

Phylogenetic approaches have helped to solve taxonomical and nomenclatural problems. A clear example is evident in the paper of Kepler *et al.* (2014) in which the robust monophyly of the genus *Metharrizum* included the majority of species recognized in *Metacordyceps* as well as the green-spored *Nomuraea* species and those in the more recently described genus *Chamaeleomyces*. In the same analysis *Pochonia* was shown to be polyphyletic and the description of *Metapochonia* gen. nov. was done to accommodate these species forming a separate clade. In this regard, a dispute on the asexual genus *Aspergillus* and its sexual generic names, started after the International Commission of *Penicillium* and *Aspergillus* (ICPA) discussed the single nomenclature and made a decision on April 12 2012 (www.aspergilluspenicillium.org).

Two proposals for the single name nomenclature in *Aspergillus* have been presented: one attributes the name "*Aspergillus*" to clades comprising ten different teleomorphic names, by supporting the monophyly of this genus (Houbraken & Samson 2011, Samson *et al.* 2014). In the second proposal *Aspergillus* is considered to be a non-monophyletic genus, and it recommends the preservation of the name *Aspergillus* only to species belonging to subgenus *Circumdati* while maintaining the sexual names in the other clades (Pitt & Taylor 2014, 2016, Taylor *et al.* 2016).

The first proposal considers the use of *Aspergillus* in a wide sense and preserves this large important genus, with the exclusion of some minor species with the anamorph of *Aspergillus* (i.e. *A. clavatoflavus*, *A. zonatus* and the *Sclerocleista* and *Warcupiella* teleomorphs) and the inclusion of some taxa lacking *Aspergillus* anamorph (*Polypaecilum* and *Phialosimplex*). As alternative to the "wide" *Aspergillus*, the second proposal suggests the non–monophyletic feature of *Aspergillus* and maintains existing teleomorph names (i.e. *Eurotium*, *Emericella*, *Neosartorya*, etc.) reducing *Aspergillus* mainly to species important for food fermentation, spoilage and mycotoxin contaminations. In this second proposal, as the type of *Aspergillus* belongs to the *Eurotium* clade, it was also proposed to move the type of *Aspergillus* to the subgenus *Circumdati*. In this respect, Taylor *et al.* (2016) provided data to suggest that if the genus *Aspergillus* should be considered monophyletic the *Penicillium* clade will belong within *Aspergillus* and the new nomenclatorial rules would lead, e.g., to *Aspergillus* subgenus *Penicillium*. Therefore, they propose to keep the sexual name *Eurotium* for subgenus *Aspergillus*, *Neosartorya* for subgenus *Fumigati*, *Emericella* for subgenus *Nidulantes* and *Chaetosartorya* for sect. *Cremei*. Additionally, they propose the retypification of *Aspergillus* with

A. niger and to maintain *Aspergillus* names for some economically relevant species in the subgenus *Circumdati*. However, this proposal is based on phylogenetic studies using the data set of Houbraken & Samson (2011), that was set up to resolve the phylogeny of the family *Trichocomaceae* and not specifically for the genus *Aspergillus*. In fact, their analysis did not show enough phylogenetic signals to unambiguously show the monophyly or paraphyly of the wide *Aspergillus* genus.

To resolve the discussion of the two proposals it is important re-examining the phylogenetic analysis to assess the monophyly or paraphyly of this group of taxa with the "aspergillum" as the main spore-bearing structure. Therefore, the aim of our study was to test the monophyly of Aspergilli by a multilocus phylogenetic approach and this was achieved by two independent analyses. The phylogenetic analysis using six loci were performed by GP and DM at Bari, Italy whereas the nine loci analysis was carried out by SK, JV and GS at Szeged, Hungary.

MATERIALS AND METHODS

Phylogenetic analysis using six loci

Ninety six strains belonging to species of *Penicillium*, *Aspergillus* and related taxa were studied for their phylogenetic relationship by using their publicly available sequences of the following six loci: *RPB1* and *RPB2* genes coding for subunits of RNA polymerase II; *Tsr1*, coding for a putative ribosome biogenesis protein; *Cct8*, coding for the theta subunit of the TCP-1 chaperonin complex; *BenA* coding for the beta-tubulin protein, and *CaM* coding for the calcium binding protein calmodulin. The list of strains and the relevant sequences accession number used is reported in Supplementary Table 1.

DNA sequences of the six loci were singularly aligned with Muscle (for *RPB1*, *RPB2*, *CaM*, *BenA*, and *Cct8*) and ClustalW (for *Tsr1*) algorithms using the software MEGA7 (Kumar *et al.* 2016), manually optimized and trimmed to make sequences of equal length, and then concatenated. The alignment is deposited at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S20285). Successively, the Multiple Sequence Alignment (MSA) was evaluated for quality using Transitive Consistence Score (TCS) offered by the T-Coffee web server (Chang *et al.* 2015). The presence of rogue taxa in the set of data was evaluated through the RogueNaRok web server analysis, because the presence of these taxa can frequently have a negative impact on the results of a bootstrap analysis (e.g., the overall support in consensus trees, Aberer *et al.* 2013). Then the sequences were manually controlled and substituted if necessary to settle the MSA. JModelTest2 (v2.1.6) (Darriba *et al.* 2012) was used to find the preferred model of evolution for the concatenated dataset, PartitionFinder (v1.1.1) (Lanfear *et al.* 2012) was used to investigate the best-fit partitioning schemes and models of molecular evolution to be adopted in RaxML analysis of the partitioned dataset, models were selected according to Bayesian Information Criterion (BIC) for both tools. The different tools performed to infer the phylogenetic tree were as follows: a) MrBayes v3.2.6 (Ronquist *et al.* 2012) for posterior probabilities (Bpp) using models of evolution on concatenated dataset from JmodelTest; b) RAxML-HPC2 (v8.2.8) (Stamatakis 2014) for rapid bootstrap support (Rbs) using models of evolution defined by JmodelTest and PartitionFinder on concatenated and

partitioned dataset, respectively; c) IQ-Tree-omp (v1.4.1) (Minh et al. 2013, Nguyen et al. 2015, Chernomor et al. 2016) for UFML (Ultra Fast Maximul Likelihood) support (lbs).

The CIPRES Science Gateway V 3.3 (Miller et al. 2010) was used to perform MrBayes analysis, setting GTR + invgamma, 10^7 generations, sampling every 1 000 generations with a burnin fraction of 0.25; and RaxML analyses, setting GTR + GAMMA + P-Invar, executing 1 000 rapid bootstrap in-ferences and thereafter a thorough ML search, for the concat-enated and partitioned dataset respectively.

IQ-Tree analysis were done locally, setting GTR + I + G4 for the concatenated dataset and the calculated charpartition BIC (GTR + I + G4: *RPB1*, *RPB2*, *CaM*, *BenA*, and *Cct8*, TPM2 + I + G4: *Tsr1*) for the partitioned dataset, both analyses were run with 10^4 ultrafast bootstrap replicates.

Phylogenetic analysis using nine loci

Phylogenetic analyses were conducted using nine loci (18S rDNA, 5.8S rDNA, 28S rDNA (D1-D2), *RPB1, RPB2, CaM, BenA, Tsr1, Cct8*) with intron regions excluded from *CaM* and *BenA* sequences. The dataset primarily consisted of publicly available sequences which are listed in Supplementary Table 2. Additional *Cct8*, *RPB1*, *RPB2* and *Tsr1* loci of *Aspergillus* species were amplified and sequenced using the methods described previously by Houbraken & Samson (2011). Sequences were deposited into GenBank under the accession numbers KY006730-KY006827. All sequences were aligned by PRANK v.140603 (Löytynoja 2014) with default settings. Individual alignments were concate-nated by using SequenceMatrix 1.8 (Vaidya et al. 2011) and the dataset was partitioned by the nine loci. An initial maximum likelihood (ML) tree was generated from the dataset by raxmlGUI 1.5b1 (Silvestro & Michalak 2012) using the executables of RAxML 8.2.7 (Stamatakis 2014) under the GTR model with gamma-distributed rate heterogeneity with 500 rapid bootstrap replicates. Sequences encoding *Tsr1* are containing large num-ber of indels therefore this initial tree was used to refine the alignment of the *Tsr1* sequences by PRANK with the -F option. In the case of *SSU, RPB2* and *Tsr1* alignments FastGap 1.2 (Borchsenius 2009) was used to code the phylogenetic infor-mation of gaps as binary characters implementing the "simple indel coding" algorithm. The refined alignment of partial *Tsr1* sequences and the indel matrix was incorporated in the concatenated dataset. The final ML trees and branch supports were estimated by 1 000 thorough bootstrap replicates under the GTR + Γ model with ten partitions. Bootstrap support was map-ped on the ML tree using the SumTrees script of the Dendropy v4.2.0 package (Sukumaran & Holder 2010). The resulted best tree and the bootstrap replicates were submitted for rogue taxon identification by the RogueNaRok (http://rnr.h-its.org/, Aberer et al. 2013) web service. Bayesian analyses were performed on the partitioned dataset using MrBayes 3.2.6 (Ronquist et al. 2012) with GTR substitution model with gamma-distributed rate variation across sites for 10^7 generations with four chains and two replicates sampling every 1 000th genera-tions. The burnin proportion was set to 0.25. Convergence and ESS values of the runs were examined by Tracer 1.6 (Rambaut et al. 2014).

To test the phylogenetic hypotheses of the monophyly of Aspergilli, a constraint tree was generated by Mesquite v3.04 (Maddison & Maddison 2016). Per-site log likelihoods were calculated for 20 unconstrained and 20 constrained ML searches by using RAxML. To measure the support of the two hypotheses Approximately Unbiased (AU) test was conducted by CONSEL 0.1j (Shimodaira & Hasegawa 2001) with 10^5 replicates.

Tree space visualization of ML and Bayesian analyses was carried out by using the TreeSetVis v3.01 (Hillis et al. 2005) package for Mesquite and the RWTY v1.0.1 package for R v3.3.1 (R Core Team 2016). Sorting of the bootstrap replicates was conducted by PhySortR v1.0.7 (Stephens et al. 2016) package in R.

Branch support analysis

To verify the robustness of the six and nine-genes phylogeny the branch supports of the principal nodes depicting the *Aspergillus* and *Penicillium* monophyletic topology were evaluated. Three categories of branch support (Anisimova et al. 2011, Minh et al. 2013) were considered: parametric (Bpp, aLRT-Chi2, aBayes), nonparametric (Rbs, SH-aLRT) and hybrid (lbs).

To compute, aLRT-Chi2, SH-aLRT and aBayes branches support of the six-genes phylogeny, PhyML (v20130805) (Guindon & Gascuel 2003) and IQ-Tree-omp (v1.4.1) analyses were performed locally (Guindon et al. 2010, Anisimova et al. 2011). The single branch tests (SH-aLRT, aBayes) and ultra-fast bootstrap approximation of the nine-genes phylogeny were also conducted by using IQ-Tree v1.4.2 in 50.000 replicates under the GTR + Γ model.

Analysis of extrolites

Strains of species expected to be outside *Aspergillus* were analysed by HPLC-DAD (high performance liquid chromatog-raphy with diode array detection as described by Frisvad & Thrane (1987), using the agar plug method of Smedsgaard (1997), as updated by Nielsen et al. (2011).

RESULTS

Phylogenetic analysis using six loci

The results of the six-gene phylogenetic analysis of the 96 strains belonging to species of *Penicillium*, *Aspergillus* and related taxa highly supported the monophyly of *Aspergillus* and its sister genus *Penicillium* in terms of Bayesian, UFML (IQ-Tree) and RAxML analyses. In particular, the six genes MSA consisted of 3 395 bps containing only the exons of each gene with the respective length of *RPB1* (767 bps), *RPB2* (963 bps), *Tsr1* (640 bps), *CaM* (150 bps), *BenA* (164 bps), and *Cct8* (711 bps). The number of conserved sites was 1 368, the number of vari-able sites was 2 008, with 1 755 parsimony informative sites. The Transitive Consistence Score (TCS) evaluate the robustness of the six-gene MSA with the high score of 996. No rogue taxa have been identified among the sequences of the strains used, con-firming the absence of taxa that could have a negative impact on the bootstrap analysis. The best model of evolution calculated with the JModelTest2 tool was the GTR + I + G (General Time Reversible + Invariant Site and Gamma Distribution) used for non-partitioned analysis in RAxML and MrBayes analysis. The best model of evolution for the RAxML partitioned analysis
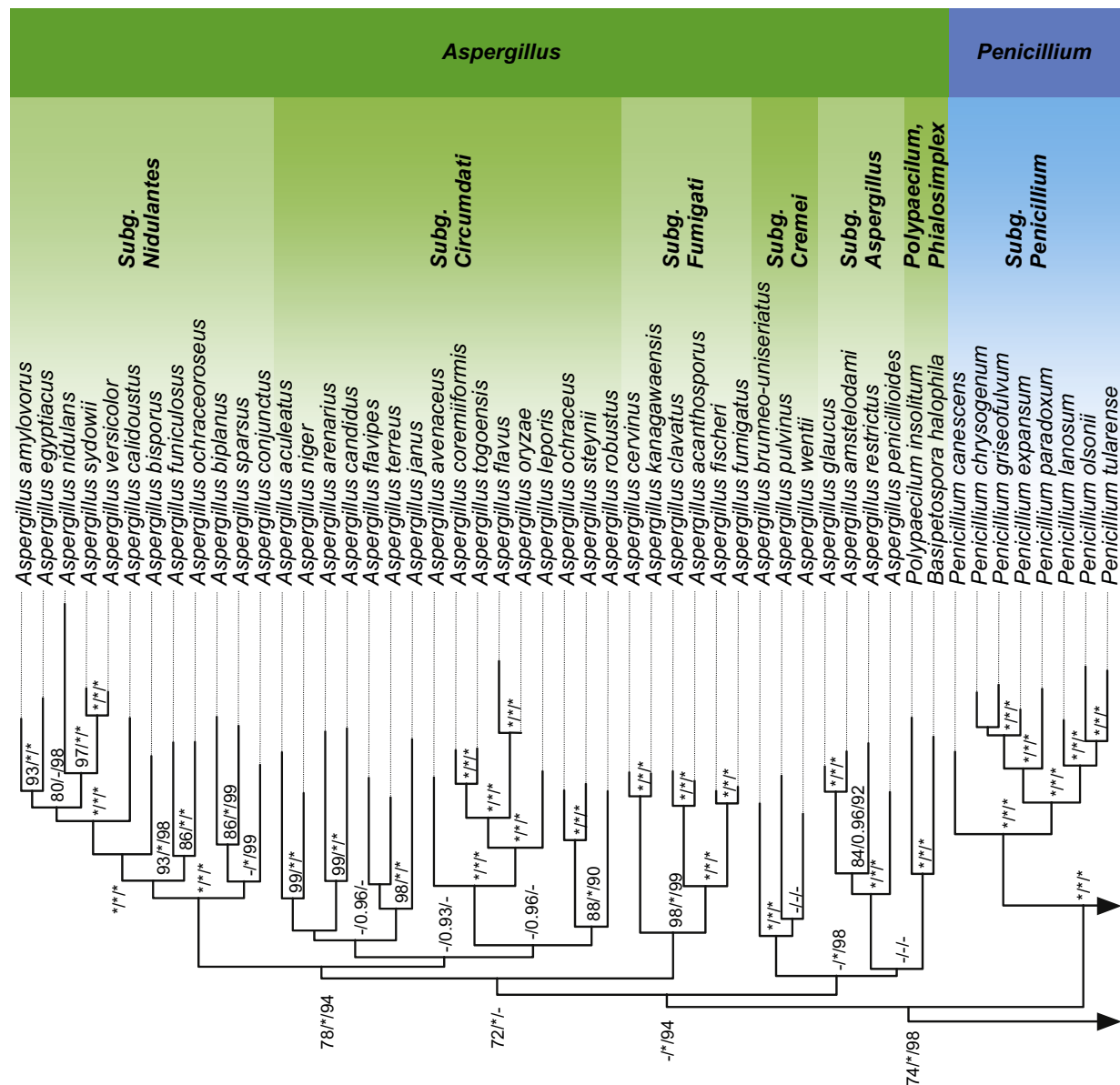
Fig. 1. Tree based on six genes. The tree shown is a rooted consensus tree inferred by maximum likelihood with partitioned dataset (IQ-TREE) and 10 000 bootstrap replicates, branch support values are given for two maximum-likelihood implementations and one Bayesian inference method (from left to right: RaxML bootstrap support; MrBayes posterior probabilities; IQ-TREE bootstrap support; respectively).

calculated from Partition Finder was confirmed as GTR + I + G for each partition of the six-gene MSA. The phylogenetic tree comprehensive of the ML analysis (RAxML and IQ-TREE) and the posterior probabilities Bayesian analysis with the same topology is represented in Fig. 1. All five phylogenetic trees supported the monophyly of the genus *Aspergillus* respectively with the higher bootstrap support of 94 % for the partitioned IQ-TREE, 1.0 for MrBayes and 63 % for RAxML not partitioned (see Fig. S1). Interestingly all the resolved trees highly supported (98 % IQ-TREE, 77 % RAxML and 1.0 MrBayes) the principal node clustering genera *Penicillium* and *Aspergillus* together. In addition, the five subgenera of *Aspergillus* are conserved in all the phylogenetic analysis with the same topology (Fig. 1).

The phylogenetic analysis clearly showed that *Aspergillus clavatoflavus, A. zonatus, Penicillium megasporum,* and *P. arenicola,* do not belong to their respective sister genera, being outside of the two lineages. In addition, the teleomorphic genera *Warcupiella* and *Sclerocleista,* formerly assigned with an

Aspergillus anamorph, were found to be outside the *Aspergillus* monophyletic clade.

Phylogenetic analysis using nine loci

The 204 species analysed in the concatenated alignment included 86 *Aspergillus*, 66 *Penicillium* and 52 species from other genera with 6 603 nucleic sites (18S rDNA: 1 792 sites, 5,8S rDNA: 161, 28S rDNA: 647 sites, *BenA*: 241 sites, *CaM*: 402 sites, *Cct8*: 718 sites, *RPB1*: 768 sites, *RPB2*: 983 sites, *Tsr1*: 891 sites) and 201 binary sites of indels. Phylogenetic trees obtained from both ML and Bayesian analyses (Figs 2 and S2, Fig 5B) were highly congruent and both analyses have shown that the genus *Aspergillus* is monophyletic with high support values. The results have evidenced that the genus *Aspergillus* can be divided into six subgenera comprising 22 sections. Maximum likelihood and Bayesian inference strategies recovered subgenus *Aspergillus* (100/1), *Polypaecili* (100/1), *Cremei*
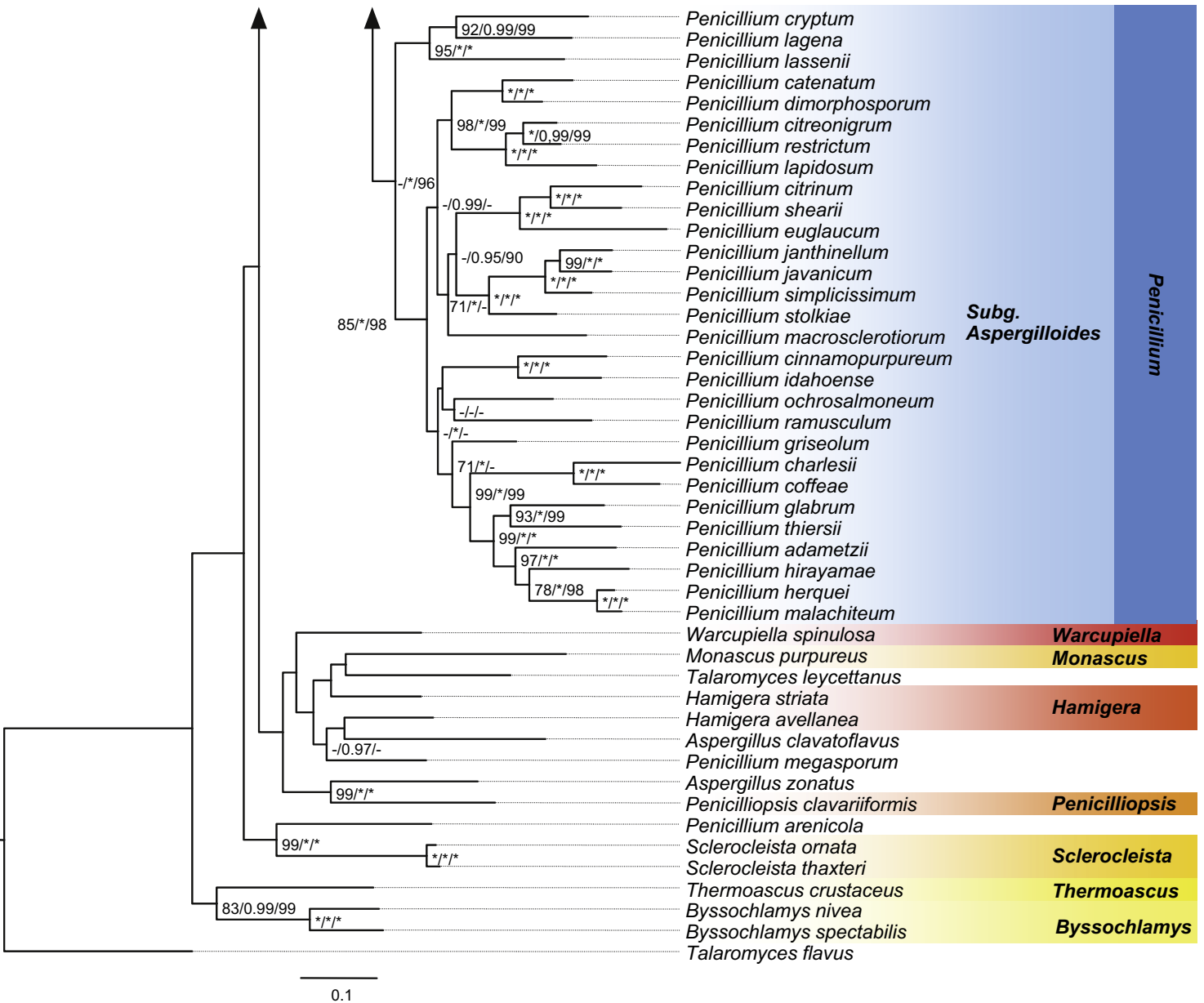
Fig. 1. (Continued).

(90/1), *Fumigati* (100/1) and *Nidulantes* (100/1) as strongly supported clades with the exception of subgenus *Circumdati* (47/1), which was strongly supported by Bayesian analysis but had low support by the ML method.

The hypothesis of monophyly was tested using the constrained tree that is likely to be multifurcating to indicate uncertainty between the two competing hypotheses and let the algorithm find the most realistic ML solution for a given constraint. Our constrained tree was drawn in Mesquite 3.04 forcing the two genera, *Aspergillus* and *Penicillium* to be paraphyletic. Branches encompassing the members of genus

Penicillium were collapsed into polytomy as well as the members of sections *Terrei, Flavipedes, Jani, Nigri, Candidi, Flavi* and *Circumdati* in the *Aspergillus* clade. Altogether 20 constrained and 20 unconstrained topologies were compared using the approximately unbiased test with CONSEL. The test resulted in the complete rejection of the hypothesis of Taylor *et al.* (2016). The monophyly of the genus *Aspergillus* was accepted with p values ranging from 0.323 to 0.706, with mean of 0.45815. The hypothesis that genus *Aspergillus* is paraphyletic and *Penicillium* is a sister clade of subgenus *Nidulantes* was rejected with low p values in the range of 0.005–0.023, with mean of 0.0134.
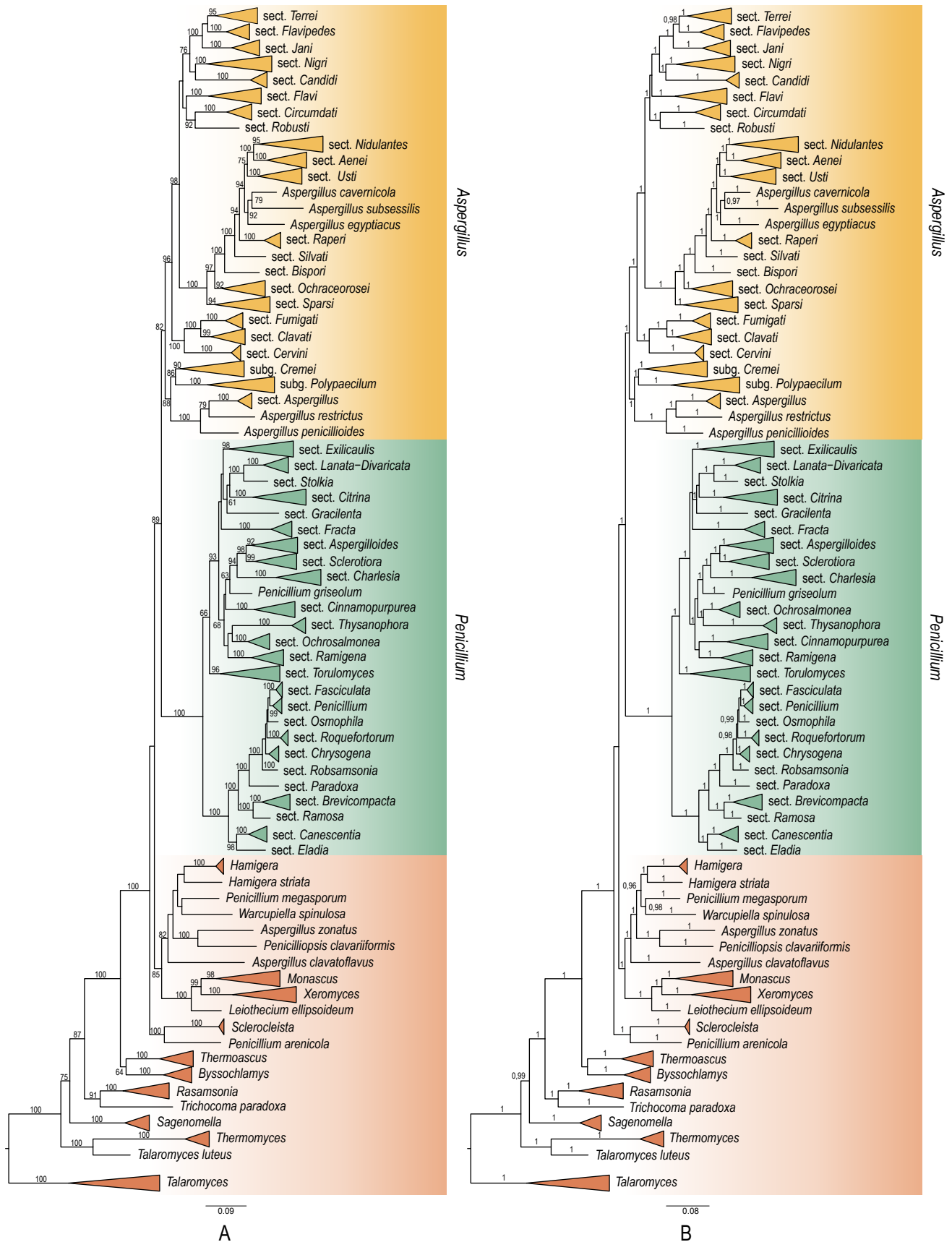
Fig. 2. Phylograms obtained by Maximum Likelihood (ML) and Bayesian analysis inferred from nine loci (18S rDNA, 5.8S rDNA, 28S rDNA (D1-D2), *RPB1*, *RPB2*, *CaM*, *BenA*, *Tsr1*, *Cct8*). Monophyletic groups are collapsed and shown as triangles. A. Best-scoring ML tree obtained by RAxML. B. 50 % majority rule phylogram of Bayesian analysis. Numbers above or below branches are bootstrap values (A) and posterior probabilities (B). Only support values greater than 60 % and 0.95 are shown.
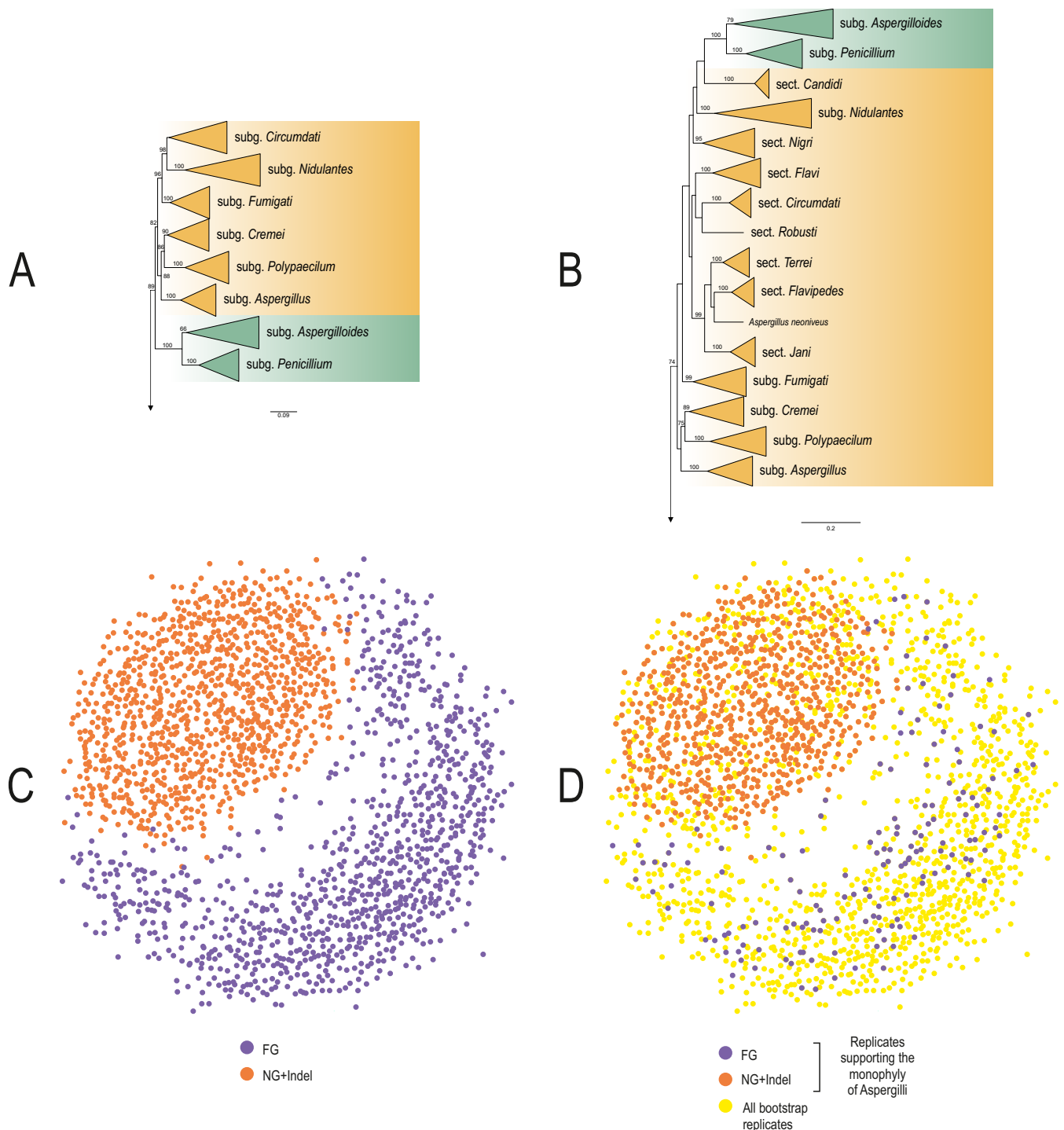
Fig. 3. Visualization of 1 000–1 000 bootstrap replicates obtained by using nine (NG) and four (FG) loci. Best-scoring ML tree using nine (A) and four loci (B) are shown with bootstrap support above branches higher than 60 %. Monophyletic groups are collapsed and shown as triangles. (C) Orange dots represent bootstrap replicates from the analysis encompassing nine genes, while purple dots are trees obtained with four genes. (D) Visualization of those replicates which support the monophyly of Aspergilli. Orange and purple dot are replicates from the nine-gene and the four-gene analysis respectively. Yellow dots represent the tree space occupied by all replicates from both runs.

Tree space of the bootstrap replicates

To investigate the background of the high dissimilarity between the results of Taylor *et al.* (2016) and our results we analysed the tree space of the bootstrap replicates and the trees obtained from Bayesian MCMC analysis by multi-dimensional scaling.

We reduced our dataset to *Cct8, RPB1, RPB2* and *Tsr1* genes without removing taxa to have only those genes that had been used in the analysis of Taylor *et al.* (2016). The dataset was un-partitioned without a binary matrix of indels. Both ML and

Bayesian analysis were conducted with the same settings as applied on the nine-gene dataset. Our results with the four-gene dataset differed from those of Taylor *et al.* (Fig. S3). Briefly, the genus *Aspergillus* was a sister group and paraphyletic to the genus *Penicillium* and subgenus *Circumdati* was not recovered as a monophyletic clade. The most closely related group to Penicillia was section *Candidi*. Subgenus *Nidulantes* formed a well-defined monophyletic clade with a sister clade of the members of section *Nigri*. Other sections from the subgenus *Circumdati* were clustered together with high support except
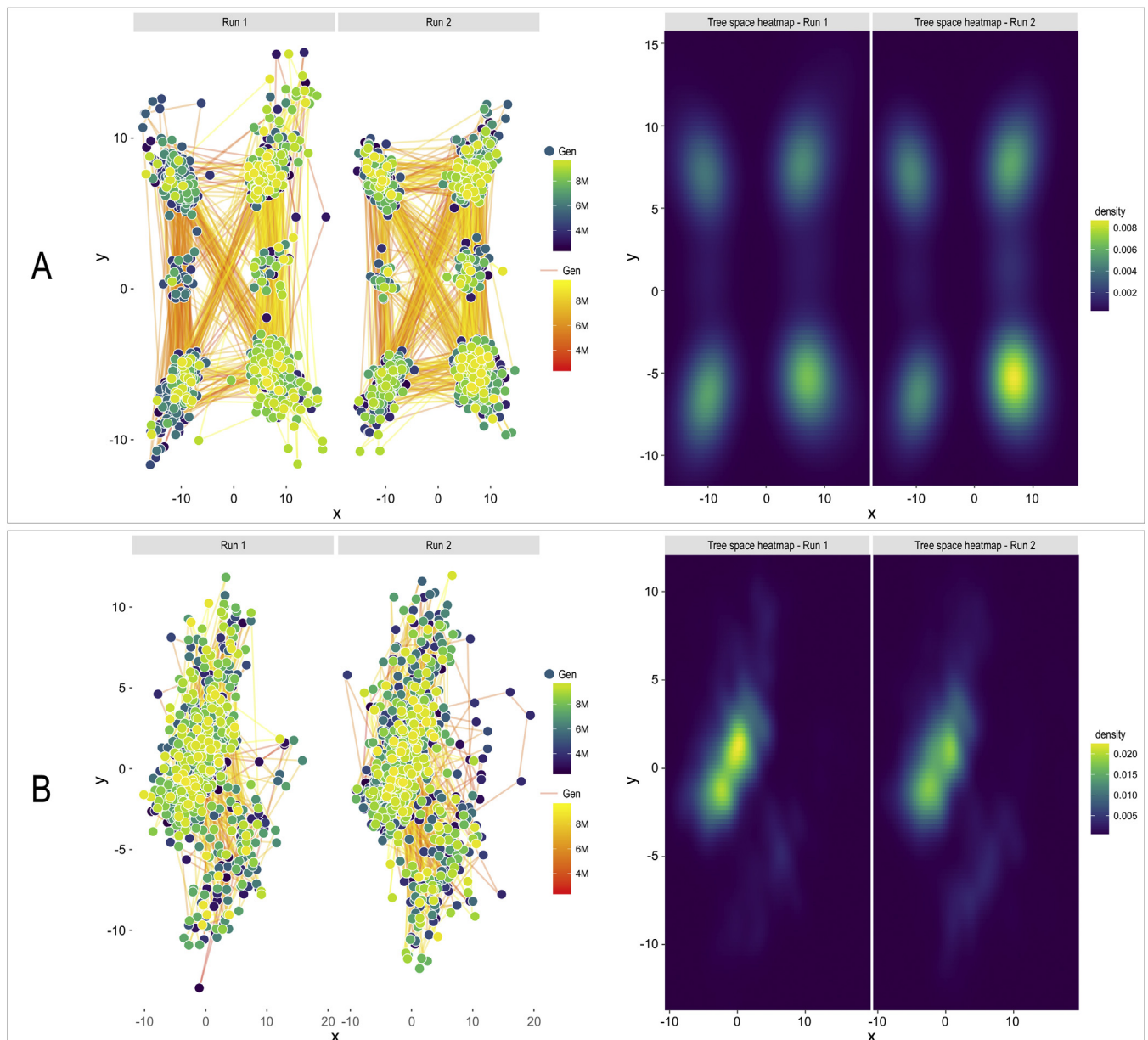
Fig. 4. Post-burnin tree space plots of 1 000 trees of Bayesian analysis with four (A) and nine (B) loci. Lines represent the connections between the subsequent generations while dots represent the two-dimensional place of the trees in the space. The colour of the lines and dots represents the generations. On the heat map green coloured areas represent the space occupied by larger number of trees.

sect. *Circumdati* however, the deeper branching was not statistically supported. Members of subgenera *Fumigati*, *Cremei* and *Aspergillus* formed monophyletic clades with moderate to high support, but deeper nodes were poorly supported.

The results of the Bayesian analysis were similar to the results of the ML analysis. The relationship between Aspergilli and Penicillia was the same as in the ML analysis. Five subgenera formed well-defined clades with high statistical support, while sections in subgenus *Circumdati* were not monophyletic (Fig. S3). We re-analysed the dataset of Taylor *et al.* (2016) without any modification, and the resulting trees were highly congruent to the ones obtained with our reduced dataset. We were not able to obtain a tree with a monophyletic clade containing all sections from subgenus *Circumdati* regardless the use of Bayesian or ML approaches. However, this difference from the tree shown in the article of Taylor *et al.* (2016) can be the result of the different parsimony starting tree between the two analyses,

as different seeds will generate different starting trees, which can have an impact on the final ML tree.

We used the TreeSetViz package for Mesquite to investigate the distribution of the bootstrap replicates in the tree space of our and the reduced dataset. To visualize the tree space 1 000 bootstrap replicates were used from both runs. The topological distances between all replicates were measured by the calculation of pairwise unweighted Robinson-Foulds (Robinson & Foulds 1979, 1981) distances. The distribution of the replicates was visualized in two dimensions by multidimensional scaling (MDS) (Lingoes *et al.* 1979, Young & Hamer 1987, Borg & Groenen 1997). The MDS search was run until no major changes were observed in the value of the stress function to minimize the distortion between the true distance and the two-dimensional distance. The analysis showed that the bootstrap replicates of the nine-gene dataset were grouped together in a well-defined island, while the replicates of the
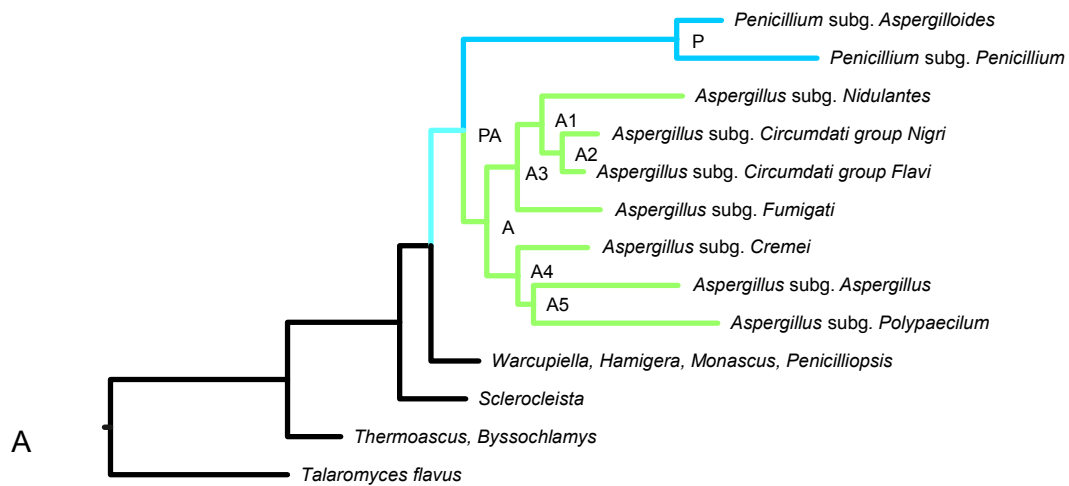
Clades	Node	PhyML aBayes	IQ-Tree aBayes	IQ-Tree-p aBayes	Bpp	PhyML aLRT-Chi2	PhyML SH-aLRT	IQ-Tree SH-aLRT	IQ-Tree-p SH-aLRT	Rbs	Rbs-p	lbs	lbs-p
Aspergillaceae	PA	1	1	1	1	0,999	0,967	0,965	0,965	77	74	94	98
Penicillium	P	1	1	1	1	0,999	1	1	1	100	100	100	100
Aspergillus	A	1	1	1	1	0,999	0,922	0,897	0,958	63	55	92	94
	A1	1	1	1	1	0,999	0,951	0,958	0,956	70	78	95	94
	A2	0,93	0,714	0,834	0,927	0,949	0,427	0,141	0,274	35	45	71	75
	A3	1	1	1	1	0,999	0,957	0,957	0,943	68	72	89	88
	A4	1	1	1	0,999	0,999	0,939	0,965	0,960	64	60	90	93
	A5	0,70	-	0,485	0,860	0,731	0,375	-	0,02	-	37	-	43
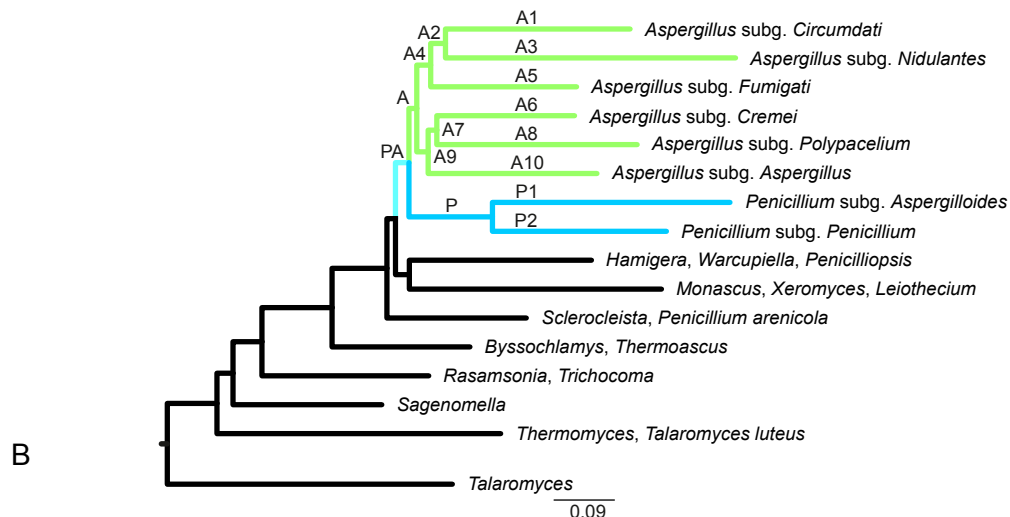
Clades	Node	Rbs-p	lbs-p	IQ-Tree-p SH-aLRT	Bpp-p	IQ-Tree-p aBayes
Aspergillaceae	PA	89	98	98.1	1	1
Penicillium	P	100	100	100	1	1
	P1	66	94	81.3	1	1
	P2	100	100	100	1	1
Aspergillus	A	82	99	94.9	1	1
	A1	47	90	58.2	1	0.997
	A2	98	100	99.8	1	1
	A3	100	100	100	1	1
	A4	96	100	99.6	1	1
	A5	100	100	99.9	1	1
	A6	90	100	93.3	1	1
	A7	86	97	73.2	1	0.999
	A8	100	100	100	1	1
	A9	88	98	95.9	1	1
	A10	100	100	100	1	1

Fig. 5. Collapsed phylograms showing the support values of the principal nodes involved in the monophyly of *Aspergillus* based on six (A) and nine (B) genes. The tables are summarizing the values of these nodes obtained by different methods (Bpp – Bayesian posterior probabilities, Rbs – RAxML bootstrap support, lbs – IQ-Tree UFBoot support). Single branch tests (aBayes, SH-aLRT and aLRT-Chi2) were conducted with PhyML and IQ-Tree. The use of partitioned data set is indicated by -p in the tables.

four-gene dataset were much more widely distributed in the tree space (Fig. 3C). This indicates that the variation between the bootstrap samples in the reduced dataset is higher, suggesting that the alignment used in the analysis has substantially lower phylogenetic signal, which is not strong enough to resolve all clades with high confidence and by the addition of more genes and partitioning the dataset the signal became more balanced.

Replicates which support the monophyly of Aspergilli were sorted out from both analyses by PhySortR and mapped on the tree space of all bootstrap samples. The bootstrap samples supporting the monophyly of Aspergilli from the dataset encompassing nine genes were distributed uniformly suggesting that there is no high variability in the branching patterns between the replicates (Fig. 3D). Samples sorted out by the same criterion from the four genes analysis were more distinct to each other suggesting that the uncertainty of the dataset is not exclusive to those clades that contains Aspergilli.

The results of Bayesian analysis were examined by using Tracer and the RWTY package. The ESS values were above 200 for all parameters in all runs. The topological convergence for each run was assessed using the cumulative split frequency plots of RWTY package (Fig. S4) examining the split frequencies of the worst 40 clades. With minor movements all split frequencies reached stationarity during the run indicating that all chains reached convergence. Tree space visualisation of the MCMC analysis showed high similarity to those obtained from the bootstrap samples. Altogether 1 000 trees were visualized after removing 25 % of the generations as burnin. In the case of the four-gene analysis the posterior distribution of tree topologies were not concentrated into one region. It is common that during the MCMC analysis the trees are moving through the tree-space from regions with low optimum to regions with high likelihood scores, but in an analysis with stable data this region should form a single, well-defined island in the tree space. Our data (Fig. 4A) show that the dataset with four genes has four almost equally optimal solutions and these are present in the later generations. These observations suggest that the phylogenetic signal in the dataset is not strong enough to have a well-defined set of trees and therefore, this dataset is not suitable to draw conclusions regarding the phylogenetic relationship of Aspergilli and Penicillia. The MCMC analysis of the dataset with nine genes resulted in a more compact set of trees occupying the tree space (Fig. 4B). The earlier generations showed relatively high movements in the space, but after the initial search the trees settled down in a more compact region with optimal solutions close to each other, suggesting that the phylogeny obtained with this dataset is more reliable than the results of the four-gene dataset.

Branch support analysis

The test of branch support for the six-genes phylogeny, by SH-aLRT, aLRT-Chi2 and aBayes values, give additional strength to the principal nodes depicting *Penicillium* and *Aspergillus* monophyletic topology (Fig. 5A, nodes P, A and PA). The lower bootstrap support observed in some nodes is generally balanced by high branch supports, except for the A2 node where the monophyly of subgenus *Circumdati* is not supported strongly. The A5 node resulted not supported due to the variable position

of the *Polypaecilum* clade, clustering with subgenus *Aspergillus* or with section *Cremei*, as it is clearly visible when comparing partitioned to non-partitioned trees (Fig. S1). Single branch tests conducted with the nine-gene dataset support the monophyly of *Aspergillus*, confirming the subdivision of the genus into six subgenera with high values except subgenus *Circumdati* (Fig. 5B).

Phenotypic data supporting taxonomy and cladonomy

Species in *Aspergillus* subgenus *Circumdati* have most extrolites in common with the other subgenera/sections in *Aspergillus*, indicating that *Aspergillus* is one large genus. Subgenus *Nidulantes* is closely related to *Circumdati*, but even subgenus *Fumigati* and subgenus *Aspergillus* have several extrolites or heteroisoextrolites (Frisvad & Larsen 2016) in common. Data listed in Table 1 shows that at least xanthocillins, terphenyllins and emodin are in common within all the subgenera of the genus *Aspergillus*. Heveadrides are common also in section *Aspergillus* (Slack *et al.* 2009).

An important example of chemical and morphological relationships in *Aspergillus* is *A. cejpii* (subgenus *Fumigati*). This species has a polypaecilum-like asexual morph, but it is phylogenetically placed "between" section *Clavati* and *Fumigati*, two sections in which all species have uniseriate aspergilla. *Aspergillus cejpii* is phylogenetically placed into an intermediate position between *Fumigati* and *Clavati* (Varga *et al.* 2007, Houbraken & Samson 2011), and thus had to be transferred from *Dichotomomyces* (anamorphs had been named both *Polypaecilum* and *Talaromyces*) to *Aspergillus* (Samson *et al.* 2014). In subgenus *Aspergillus, A. pisci* (formerly *Polypaecilum pisci*) is placed in a sister-clade to *Aspergillus* section *Aspergillus*, containing species with phialosimplex-like and polypaecilum-like morphs, while in the clade based on *A. wentii*, a species with a penicillium-like morph is placed as *A. inflatus* (Samson *et al.* 2014). Most, if not all species in the subgenus *Aspergillus* are species able to grow well at very low water activities, while species in subgenus *Fumigati* are adapted to higher water activities. Yet species with polypaecilum-like morphs are placed in both subgenera. *Aspergillus cejpii* has heat resistant ascospores in common with species in section *Fumigati* with neosartorya-like morphs (Jesenska *et al.* 1992, 1993), while *A. pisci* has salt tolerance in common with most species in subgenus *Aspergillus*. Thus one can predict that if a fungus in subgenus *Fumigati* produces ascospores, those ascospores are heat-resistant, while if a new species is found to belong to subgenus *Aspergillus*, one can predict that it can grow under conditions with very low water activity, despite the differences in micro-morphology.

Regarding extrolites, *A. cejpii* also has an intermediate position between sections *Fumigati* and *Clavati*, while the species also show some chemical similarities with subgenus *Aspergillus*, and even with subgenus *Circumdati*. *A. cejpii* has been shown to produce gliotoxins and fiscalin B in common with *A. fumigatus* and *A. fischeri* (Varga *et al.* 2007, Frisvad & Larsen 2015, Harms *et al.* 2015a, Rodrigues *et al.* 2015, Fan *et al.* 2016), xanthocillins (Kitahara & Endo 1981, Harms *et al.* 2015b) in common with *A. fumigatus* (Zuck *et al.* 2011), showing several chemical similarities between *A. cejpii* with its phylogenetic sister group

Table 1. Isoextrolites and heteroisoextrolites in *Aspergillus* subgenera (see Frisvad & Samson 2004; Samson *et al.* 2004; Nielsen *et al.* 2009; Frisvad & Larsen 2015, 2016; Ma *et al.* 2016[1]).

	Aspergillus and *Cremei*	*Fumigati*	*Nidulantes*	*Circumdati*
Pseurotins	–	+	–	+
Kojic acid	–	–	+	+
Terrein	–	–	+	+
Asperphenamate	+	–	–	+
Sterigmatocystin	+	–	+	+
Cyclopiazonic acid	–	+	–	+
Malformins	–	+	–	+
Fumitremorgins	–	+	+	+
Emodin (as precursor)	+	+	+	+
6-Methylsalicylic acid (as precursor)	–	+	–	+
Itaconic acid	+	–	–	+
Viridicatins	–	+	+	+
Penicillins	–	+	+	+
Notoamides	–	–	+	+
Aflavinins	–	+	+	+
Echinulins	+	+[2]	–	+[2]
Diketopiperazines	+	–	–	+
Polythiodiketopiperazines	–	+	+	+
Kotanins/desertorins	+	–	+	+
Falconensin type azaphilones	–	+	+	+
Xanthocillins and terphenyllins	+	+	+	+
Mycophenolic acid	+	+	–	–
Heveadrides	+	+	–	–
Patulin	+	+	–	–

[1] Even though Ma *et al.* (2016) identified their strain as *Aspergillus tamarii*, their strain was clearly an *A. fumigatus*.
[2] While *Aspergillus* subgenus *Aspergillus* species produce echinulins and neoechinulins, species from *Fumigati* and *Circumdati* produce the related cycloechinulin.

section *Fumigati*. Furthermore, indoloterpenes, such as JBIR-03, emeniveol, emindol SB, emindole SB mannoside, asporyzin A-C, 27-O-methylasporyzin C (Ogata *et al.* 2007, Qiao *et al.* 2010a, b, Harms *et al.* 2014) can be also found in common with species in subgenus *Circumdati* and *Nidulantes* (Nozawa *et al.*1988, Kimura *et al.* 1992). Finally, tryptoquivalones in common with species of section *Clavati* and *Fumigati* (Varga *et al.* 2007, Frisvad & Larsen 2016), while asporyergosterols and similar bioactive sterols (Qiao *et al.* 2010a, Harms *et al.* 2015b) in common with several Aspergilli, and heveadrides in common with *Aspergillus* section *Aspergillus* (Slack *et al.* 2009, Harms *et al.* 2015a) have also been found. *Aspergillus arxii* (formerly *Cristaspora arxii*) was found to produce heveadrides, in common with *Aspergillus cejpii* (new data provided here) and *Aspergillus* species in section *Aspergillus* (Table 1). Thus, *A. cejpii* has several physiological, chemical and phylogenetic similarities with other species of *Aspergillus*.

DISCUSSION

In our study we compared 96 and 204 species using six and nine genes phylogenies, respectively. The involved species covered all sections from genus *Aspergillus*, except sections *Tanneri* and *Petersonii* (Samson *et al.* 2014, Hubka *et al.* 2014, Jurjevic *et al.* 2015), all accepted sections from the genus *Penicillium* except section *Turbata* (Visagie et al. 2014, Houbraken *et al.* 2015) and species from other genera of the family *Aspergillaceae*, *Thermoascaceae* and *Trichocomaceae* (Peterson *et al.* 2010, Houbraken & Samson 2011, Yilmaz *et al.* 2014). Both phylogenetic studies supported the monophyly of the genus *Aspergillus* using Bayesian and ML approaches. These findings are contradictory to those of Pitt & Taylor (2014), as well as Taylor *et al.* (2016), while they are in agreement with the previous studies of Houbraken & Samson (2011), and Houbraken *et al.* (2014).

Both results are in accordance regarding the subgenus *Circumdati* as this clade was resolved with low support values in all analyses except the Bayesian approaches. In the ML analysis all sections formed monophyletic groups with moderate to high support except for species previously assigned to section *Usti* and *Restricti*. Both the ML and Bayesian approach divided section *Usti* into two separate groups in which *A. amylovorus*, *A. subsessilis* and *A. egyptiacus* formed a well-defined clade with high posterior probabilities and ML bootstrap values (1/92). Members of section *Restricti* did not form a separate clade however; this can be due to the inadequate taxon sampling as a recent phylogenetic analysis across species diversity in the subgenus *Aspergillus* strongly supported monophyly of both, sect. *Aspergillus* and sect. *Restricti* (unpublished data). Both analyses rendered the genus *Penicillium* as a monophyletic

sister group to Aspergilli with high support (100/1). The genus can be divided into two subgenera: *Aspergilloides* and *Penicillium* comprising 25 sections with high statistical support obtained by Bayesian analysis. The results of the ML analysis were largely congruent with those of Bayesian approach except for the moderate support (66) for the subgenus *Aspergilloides*. Regarding the basal genera the topology of the tree was mainly in agreement with previous studies (Peterson 2008, Houbraken & Samson 2011).

Taylor *et al.* (2016) tested several hypotheses regarding the monophyly of Aspergilli, however most of these tests did not reflect the current knowledge on Aspergilli. Their tests rejected the inclusion of *A. penicilliformis*, *A. zonatus*, *Sclerocleista ornata* and *S. thaxteri* in the genus *Aspergillus*. Previous studies (Peterson 2008, Houbraken & Samson 2011, Samson *et al.* 2014) have proven that these species are phylogenetically distinct from the Aspergilli and therefore the rejection of these hypotheses is in agreement with recent phylogenies. The inclusion of *A. clavatoflavus* was not rejected but the p value of the hypothesis did not indicate strong support for the inclusion of this species to the Aspergilli. However, the taxonomic position of this species remained unclear. Several studies have demonstrated that *A. clavatoflavus* is not a member of the genus *Aspergillus* (Peterson 2008, Peterson *et al.* 2010, Houbraken & Samson 2011, Samson *et al.* 2014). The reason of this contradictory result can be that the dataset used in their study had low resolving power restricting the estimation of a well-established phylogeny. On the tree obtained by Taylor *et al.* (2016), the deeper clades were poorly supported; therefore the inclusion of *A. clavatoflavus* may not have altered the overall likelihood value of the constrained tree substantially.

Our main concern about the tests conducted by Taylor *et al.* (2016) is that it is not clear whether they had used multifurcating or fully resolved constraints for estimating ML trees before the calculation of the site-wise likelihoods. Using fully resolved trees as constraints can lead to the underestimation of the probabilities of hypotheses, which can explain the unexpectedly low p values in some of their analyses. In our experiments the hypothesis of Taylor *et al.* (2016) was rejected with a mean p value of 0.0134, when a constrained tree containing polytomies was used. When the ML likelihood search was conducted with the completely resolved best tree obtained by RAxML the approximately unbiased test in CONSEL also rejected the hypothesis but with values very close to zero.

The exclusion of subgenus Polypaecilum from a monophyletic *Aspergillus* clade was also rejected indicating that the species of this section are members of the genus *Aspergillus*. Moreover, when this section was included in a monophyletic *Aspergillus* clade, the hypothesis was accepted. This finding is in agreement with the previous results of Houbraken & Samson (2011), Samson *et al.* (2014) and our recent findings.

Additional evidences of the robustness of our analysis with respect to that of Taylor *et al.* (2016) could be retrieved from the recently guidelines published on IMA Fungus for introducing new genera of fungi (Vellinga *et al.* 2015). The authors proposed six criteria; our analysis is in accordance with all the criteria but in particular two of these criteria are fully in accordance with our results and not with those of Taylor *et al.* (2016). They have assessed that: 1) all genera that are recognized should be monophyletic, not only the one that is the focus of the study, but also the group from which it is separated and the group to which it is added (the reciprocal monophyly criterion), 2) the branching

of the phylogenetic trees should have sufficient and strong statistical support. Finally, also the extrolite data support the clustering of the wide *Aspergillus* genus evidencing that at least xanthocillins, terphenyllins and emodin are in common within all the subgenera of the genus (Table 1). In particular, some species that have been shown to be outside *Aspergillus*, despite having an *Aspergillus* conidiophore, appear to be unique chemically: *Aspergillus clavatoflavus* has been analysed chemically and produced a series of unique secondary metabolites never found in any species of *Aspergillus* and does not produce kojic acid, produced by all species in *Aspergillus* section *Flavi* except *A. avenaceus* and *A. togoensis* (Varga *et al.* 2011). *Aspergillus zonatus* was reported to produce aszonalenin and aszonapyrone (Kimura *et al.* 1982a, b, Katsube *et al.* 1985, Bhat *et al.* 1993), but several chemical analysis of the ex-type strain of this fungus showed that it only produces some few unique extrolites, and that aszonalenin and aszonapyrone was not among them (Frisvad, unpublished). Aszonalenin and aszonapyrone was found in several species in *Aspergillus* section *Fumigati* (Larsen *et al.* 2007, Frisvad *et al.* 2009, Frisvad & Larsen 2016) however, indicating that the culture of *A. zonatus* was contaminated with an isolate from section *Fumigati*. Also Throckmorton *et al.* (2015) did not find biosynthetic gene clusters coding for aszonapyrone when examining the genome sequenced isolate of *A. zonatus*, but they did find a PKS Aspzol_2112764 coding for an unknown non-reduced polyketide. Aflatoxin B_1 was also reported from a strain of *A. zonatus* (El Kady *et al.* 1994), but this was obviously a mistake.

Sclerocleista ornata and *S. thaxteri* produce viriditoxin in common with both *Paecilomyces variotii* and *Aspergillus* section *Fumigati* species such as *A. viridinutans*, and citrinin in common with *Monascus* spp. and *Aspergillus* sections *Flavipedes* and *Terrei*. Apart from this, they produce at least two types of secondary metabolites not yet found in any *Aspergillus* section. Given that at least *S. thaxteri* occupies a dung habitat; it is interesting to note that the two *Sclerocleista* species grow very poorly on media containing sucrose, thus making them pretty unique. It is recommended to use the genus name *Sclerocleista* for those two closely related species. Thus, the phenotyping data confirm the grouping of the wide *Aspergillus* genus with the exclusion of *A. clavatoflavus* and *A. zonatus* species, and of the *Warcupiella* and *Sclerocleista* clades, previously treated as *Aspergillus* subgenera.

TAXONOMIC DISCUSSION AND CONCLUSIONS

The phylogenetic analyses show that the *Polypaecilum* clade and section *Cremei* are strongly supported therefore should be treated as subgenera:

Aspergillus subgenus Cremei Samson, Houbraken & Frisvad, **subgen. nov.** MycoBank MB819182.

Etymology: named after the epithet of the type species.

Diagnosis: Conidia *en masse* grey-green to yellow brown, globose to subglobose, biseriate or uniseriate conidial heads, metulae and phialides produced synchronously, except in *A. inflatus*, where they are produced successively. Species are moderately osmophilic and halophilic (Wheeler & Hocking 1993).

Type species: *Aspergillus cremeus* Kwon-Chung & Fennell

Aspergillus subgenus Polypaecilum Samson, Houbraken & Frisvad, **subgen. nov.** MycoBank MB819184.

Etymology: named after the genus *Polypaecilum*.

Diagnosis: Conidia formed on reduced phialides (as in *Phialosimplex salinarum*, Greiner *et al.* 2014, appearing as phialide collula only), small phialides with long collula often with a thickened centre part (like in *Phialosimplex caninus*, Sigler *et al.* 2010) or on polyphialides (as in *Polypaecilum insolitum*, Smith 1961), with the common theme of a thin, long collulum producing chains of conidia that are large compared to the diameter of the collulum. Aspergilla are not produced. The species are halophilic or osmophilic (Wheeler *et al.* 1988, Wheeler & Hocking 1993, Greiner *et al.* 2014, Piñar *et al.* 2015, 2016). The subgenus *Polypaecilum* contains species of the previously known genera *Polypaecilum* and *Phialosimplex*.

Type species: *Polypaecilum insolitum* G. Sm. = *Aspergillus insolitus* (G. Smith) Houbraken, Visagie & Samson

Our analysis shows that *A. zonatus* does not belong to *Aspergillus,* which was already demonstrated by Peterson (2008), and Houbraken & Samson (2011). Together with *Penicilliopsis clavariiformis* the taxon forms a strongly supported clade. *Penicilliopsis* is typified by *P. clavariiformis* and characterized by seed-borne, stipitate stromata often occurring in tropical forests. The anamorph genera *Pseudocordyceps, Sarophorum* and *Stilbodendron* are phenotypically related (Samson & Seifert 1985, Hsieh & Ju 2002). The former two genera have conidiogenous structures similar to those of *Penicillium* and the latter has *Aspergillus*-like conidiogenous structures. Therefore it is possible that *A. zonatus* belongs to *Penicilliopsis*. The type culture of *A. zonatus* was found a sample of forest soil in Costa Rica and *Penicilliopsis* occurs in a similar habitat. Since *A. zonatus* is known only from this type culture the accommodation in a new genus might be premature until more material is collected. For the time being the species is recombined in *Penicilliopsis*.

Penicilliopsis zonata (Kwon-Chung & Fennell) Samson, Houbraken & Frisvad, **comb. nov.** Mycobank MB819185.

Basionym: *Aspergillus zonatus* Kwon-Chung & Fennell, The Genus *Aspergillus*: 377 (1965) [MB#326666]

A detailed description of the species is provided by Raper & Fennel (1965: 377).

Aspergillus clavatoflavus described from rain forest soil, collected in Australia, is also not related to *Aspergillus*. Our analyses confirm its position outside *Aspergillus* as it was already demonstrated by Peterson (2008), and Houbraken & Samson (2011) without any closely related taxon. Although the species is only known from its ex-type culture the erection of a new genus is proposed herein:

Aspergillago Samson, Houbraken & Frisvad, **gen. nov.** MycoBank MB819186.

Etymology: Resembling *Aspergillus*

Diagnosis: Morphologically resembles *Aspergillus* by its typical aspergillum, but phylogenetically distant.

Type species: *Aspergillus clavatoflavus* Raper & Fennell, Gen *Aspergillus*: p. 378 (1965).

Aspergillago clavatoflava (Raper & Fennell) Samson, Houbraken & Frisvad, **comb. nov.** MycoBank MB819187.

Basionym: *Aspergillus clavatoflavus* Raper & Fennell, Gen *Aspergillus*: p. 378 (1965)

For a full description, see Raper & Fennell (1965: 378–381).

Raper & Fennell (1965) proposed *A. clavatoflavus* as a new taxon because it resembled the morphology of *A. clavatus* and *A. flavus*. However, the conidiophores were produced in loose synnemata, a feature not observed in *Aspergillus*. In that respect the synnematous conidiophores of *A. clavatoflavus* resembles those of *Stilbothamnium* which is considered to be a synonym of *Aspergillus* (Varga *et al.* 2011, Samson *et al.* 2014).

CONCLUSION

From our extensive and independent phylogenetic multilocus analyses of 96 and 204 species respectively, it can be concluded that there is no phylogenetic evidence to split *Aspergillus* into several genera and the name *Aspergillus* can be used for all the species which have been proven taxonomically to belong to *Aspergillus.* The monophyly of the genus *Aspergillus* supports the use of *Aspergillus* in a wide sense.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.simyco.2016.11.006.

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