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Phylogenetic Relationship and Secondary Metabolite Production of Marine Fungi Producing the Cyclodepsipeptides Scopularide A and B

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Abstract Strains originally affiliated to the genera *Scopulariopsis* and *Microascus* were compared regarding the scopularide production in order to investigate their ability to produce the cyclodepsipeptides and select the best suited candidate for subsequent optimisation processes. Phylogenetic calculations using available sequences of the genera *Scopulariopsis* and *Microascus* revealed that most of the sequences clustered within two closely related groups, comprising mainly *Scopulariopsis/Microascus brevicaulis* and *Microascus* sp., respectively. Interestingly, high yields of scopularide A were exhibited by three strains belonging to *S./M. brevicaulis*, while lower titres were observed for two strains of *Microascus* sp. Close phylogenetic distances within and between the two groups supported the proposed combination of both genera into one holomorph group. Short phylogenetic distances did not allow a clear affiliation at the species level on the basis of ribosomal DNA sequences, especially for *Microascus* sp. strains. Additionally, several sequences originating from strains assigned to *Scopulariopsis* exhibited a polyphyletic nature. The production pattern is in accordance with the phylogenetic position of the strains and significant production of scopularide B could only be observed for the *S./M. brevicaulis* strain LF580. Thus, the phylogenetic position marks the biotechnologically interesting strains and matters in optimisation strategies. In conclusion, the ability of all five strains to produce at least one of the scopularides suggests a distribution of the responsible gene cluster within the holomorph group. Setting the focus on the production of the cyclodepsipeptides, strain LF580 represents the best candidate for further strain and process optimisation.

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Introduction

Natural compounds remain the largest reservoir for the identification of new pharmacophors and the development of new drugs. The largely untapped potential of the marine environment in combination with the development of methods to expand the chemical space by studying the microbial diversity and microbial genomics, hold immense promise for the discovery and development of novel drugs and drug leads. However, setting the focus on the subsequent process development for biotechnological production, identification of known compounds of interest within the crude extract becomes the crucial step for choosing the best candidate for further optimisation. Dereplication as a basic technique in natural product research is on the one hand essential to exclude known compounds from further investigation during screening campaigns for new compounds (Ito and Masubuchi 2014), on the other hand helpful to find more effective producer strains of a known compound. Identification of strains being capable to produce the target compounds, e.g. faster, at a higher yield or exclusively contribute to the establishment of more robust and sustainable production processes. The most famous example represents the progress in production of penicillin: The initially identified producer strain *Penicillium notatum* strain NRRL 1249b21 showed a production titre of

1.2 mg L^{-1} (Barreiro et al. 2012). A more potent producer strain (*Penicillium chrysogenum* strain NRRL1951) was detected in 1943, with a production of 60 mg L^{-1} penicillin in submerged cultures. This wild-type strain was a better starting point for further strain improvements. Industrial strain improvement and process optimisation finally led to current yields of more than 50 g L^{-1} penicillin in fed batch cultures (Barreiro et al. 2012).

The producer strain of the two cyclodepsipeptides scopularide A and B LF580 was isolated from the inner tissue of the marine sponge *Tethya aurantium*. Based on its morphological characteristics, the strain was identified as *Scopulariopsis brevicaulis*. Sequence analysis of the 18S rRNA gene confirmed *S. brevicaulis* strain NCPF 2177 as next relative with a sequence similarity of 99.5 % (Yu et al. 2008). The genus *Scopulariopsis* is known since the late nineteenth century. At this time, the fungus was described as *Penicillium brevicaule* Sacc. At the beginning of the twentieth century, the fungus was renamed and reclassified as *S. brevicaulis* (Sacc.) Bainier. According to Abbott et al. (1998) a number of members of *S. brevicaulis* are considered to be the anamorphs of *Microascus* sp. This discovery established the definite link between the genus *Scopulariopsis* and the Microascaceae (Abbott and Sigler 2001; Abbott et al. 1998). Therefore, and in accordance to the official regulations of fungal names, the species *S. brevicaulis* was renamed into *Microascus brevicaulis* (Hawksworth 2011). The one-fungus-one-name rule (i.e. to name a genus by its teleomorph) was not consequently applied for all other members of these genera, despite the anamorph-teleomorph relationship was discussed to be valid for the whole genus *Microascus* (Issakainen et al. 2003). The removal of dual nomenclature was constantly considered and led finally to the one-fungus-one-name rule (Hawksworth 2012), but the practicability of renaming all members of a genus was doubted (Hawksworth et al. 2011). A prominent representative of this issue is the genus *Aspergillus*. For five of the six subgenera, one or more species are known with an equivalent teleomorph. In total, the genus *Aspergillus* is associated with eight teleomorph genera, which are quite distinct from each other (Pitt and Samson 2007). Quite recently in 2014, a comprehensive list for a unified system of nomenclature for this genus was published based on recent molecular studies, which overcome the bottlenecks of systematic identification based on phenotypes (Pitt and Taylor 2014). For the Microascaceae, such a retypification was not yet performed. However, anamorph-teleomorph relationships of *Scopulariopsis* and many *Microascus* species were demonstrated (Abbott and Sigler 2001; Morton and Smith 1963) but many *Scopulariopsis* species remained unconnected to teleomorphs.

Microascus and *Scopulariopsis* species do not seem to be very prolific producers of secondary metabolites: Up to date,

only ten compounds are known to be produced by strains of the genus *Microascus*. From the genus *Scopulariopsis* six additional metabolites were described including the two scopularides (Blunt and Munro 2012). Both scopularides were initially isolated from LF580 and their chemical structure does not belong to any existing group of natural cyclodepsipeptides (Yu et al. 2008). Due to their distinct activity against the pancreatic tumour cell lines Colo357 and Panc89 and the colon tumour cell line HT29 (Yu et al. 2008), the two scopularides A and B came into the focus of the EU project MARINE FUNGI. Scopularide A was chosen for hit-to-lead-development, and therefore, homologous biotechnological production was initiated. In order to identify the most suitable strain for optimisation of scopularide A and B production, we analysed the metabolite profiles of five strains being morphologically identified to belong to the genera *Scopulariopsis* or *Microascus* and available from the strain collection maintained at GEOMAR. Furthermore, the aim of the study was to consider the question whether scopularide production is limited to one representative of the genus *Microascus/Scopulariopsis* or if this gene cluster is distributed among this genus.

Results and Discussion

Morphological Comparison

Visual and microscopic examination was performed for all five strains. As growth behaviour and morphology of filamentous fungi strongly depend on the growth conditions (Harris et al. 2009), all strains were cultivated in parallel applying identical experimental conditions. Based on their morphology, all five strains were assigned to be members of *Scopulariopsis* or *Microascus*. Strains LF580, MF350 and MF351 formed one morphological group exhibiting short hyphal chains with large, subglobose, brown conidia produced from highly branched conidiospores with specifically formed annelides and were assigned to belong to *S. brevicaulis* (i.e. *Microascus brevicaulis*), while strains MF360 and MF361 additionally showed grey perithecia. These fruiting bodies indicate sexual reproduction.

The strain LF580, known as scopularide producer, was growing in pellets with a size of approximately 3-4 mm in diameter in WSP30 medium under shaken conditions. The colour of the pellets was yellow to beige; no colour change was observed in the surrounding medium. The shape of the pellets was compact. Strains MF350 and MF351 showed similar growth behaviour and morphology as LF580 (Fig. 1), though, somewhat smaller, pellets could be seen in MF350 and MF351. Comparing the growth behaviour of the strains assigned to the teleomorphs *Microascus* sp. (strains MF360 and MF361) and to the anamorphs (strains LF580, MF350
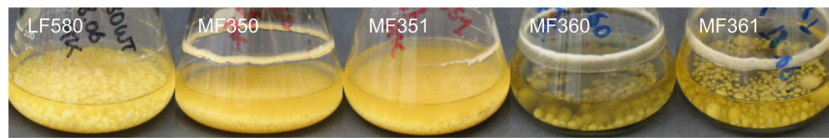
Fig. 1 Erlenmeyer flask with cultures of strains LF580, MF350, MF351, MF360 and MF361 after 7 days of cultivation. Pellet shape, size and number differed between the strains, dedicated to *Scopulariopsis/ Microascus brevicaulis* (LF580, MF350 and MF351) and the two *Microascus* sp. strains (MF360 and MF361) forming perithecia.

Greenish mycelia could be observed only for the strains MF360 and MF361 of *Microascus* (starting to appear after 4 days of cultivation). Scopularide A production could be observed for all five strains, though in significant lower titres for strains MF360 and MF361

and MF351), clear differences were visible: Fewer pellets were formed in the strains MF360 and MF361 (Fig. 1), showing non-homogeneous pellet sizes of 2–7 mm. The shape of the pellets was clearly defined and well differentiated, leading to a highly compact pellet structure. Strain MF361 showed the highest variation within one culture regarding pellet size. After 4 days of cultivation, a change in colour of mycelia and media was visible for both strains. The former beige mycelia and media turned into grey-green, reflecting the formation of perithecia and spores. This effect was most pronounced in cultures of strain MF360. The colour change was accompanied by an increase in pellet number and size.

Phylogenetic Analysis

Despite the discussion on the phylogenetic position of *Scopulariopsis* sp. in the fungal tree, only a few molecular taxonomy studies for a phylogenetic analysis of the family Microascaceae were available at the beginning of our study. Issakainen et al. (2003) performed a study with emphasis to give a better basic for further differential diagnosis of clinically occurring species. Ropars et al. (2012) study had its focus on relevant fungi in cheese manufacturing. With respect to secondary metabolite production and to the discussion on the relation between *Scopulariopsis* and *Microascus*, no detailed phylogenetic study was available. Identification of fungal strains is based on two main approaches: (a) Characterisation of the morphology of the hypha as well as the spores, accompanied with the description of their physiology, and (b) on the basis of sequence similarities of selected gene sequences. As the ITS1-5.8S rRNA-ITS2 region showed the highest score for strain identification, it was defined as the barcoding region for fungi and is now generally used for strain identification (Schoch et al. 2012). However, no ITS sequences of the strains LF580, MF350 and MF351 could be obtained by PCR techniques (data not shown). Ropars et al. (2012) reported similar difficulties in amplification of ITS sequences. ITS sequence of strain LF580 could be obtained from a recently performed genome sequencing project (Kumar et al. 2015). Hence, only the strains MF360, MF361 and LF580 were compared on the basis of the ITS1-5.8S rRNA-ITS2 fragment. Comparison for all five strains was performed on the basis of the 18S rRNA gene. Using all

available sequences from strains belonging to either *Scopulariopsis* or *Microascus* with a minimum length of 1300 nt for 18S rRNA gene (6 sequences) and 400 nt for the ITS1-5.8S rRNA-ITS2 region (69 sequences), phylogenetic trees were constructed.

In general, only a limited number of 18S rRNA gene sequences of both genera were available. Additionally, this gene has very often a poor species-level resolution in fungi (Schoch et al. 2012). However, specific subgroups that share phenotypic features can be observed. All five strains clustered in one group in the 18S rRNA tree (Fig. 2), indicating their close relationship. This group showed two distinct subclusters, which is in accordance with the assignment to *S. (Microascus) brevicaulis* (LF580, MF350, MF 351) and *Microascus* sp. (MF360 and MF361) and reflect the morphological differences observed. Strains LF580, MF350 and MF351 were identified as *S. (Microascus) brevicaulis* with the next relative of LF580 to be *S./M. brevicaulis* strain NCPF 2177 with similarities of the 18S rRNA gene sequence of 99.5, 99.4 and 99.7 %, respectively. Comparing the respective 18S rRNA gene sequences of the three strains to each other, strains MF350 and MF351 showed a similarity of 99.6 %. MF350 had a similarity of 99.5 % to the sequence of LF580, whereas, MF351 showed a slightly higher value of 99.7 %. In contrast, both sequences of the strains of *Microascus* sp., MF360 and MF361, exhibited lower similarities of 98.6, 98.5 and 98.8 % to the 18S rRNA gene sequences of the strains LF580, MF350 and MF351. The normalised sequences of the 18S rRNA gene of strains MF360 and MF361 showed similarities of almost 100 % to each other and to the 18S rRNA gene sequence of *Microascus cirrosus* (NCBI accession number M89994.1). Classification of the sequences to a specific species remains difficult, as the nuclear ribosomal small subunit has a poor species-level resolution (Schoch et al. 2012).

The tree based on ITS1-5.8S rRNA-ITS2 sequences (Fig. 3) supported the phylogenetic heterogeneity of the strains assigned to *Scopulariopsis* and *Microascus*. Even more, these molecular data indicate a polyphyletic nature of this group. Unfortunately, neither an ITS nor an 18S sequence is available for the *S./M. brevicaulis* type strain CBS127812. Concerning the strains of this study, subgroups that share phenotypic features were observed. Strain LF580 clearly affiliated to one group of *S. (Microascus) brevicaulis* strains ('brevicaulis' clade, marked in green in Fig. 3), as shown
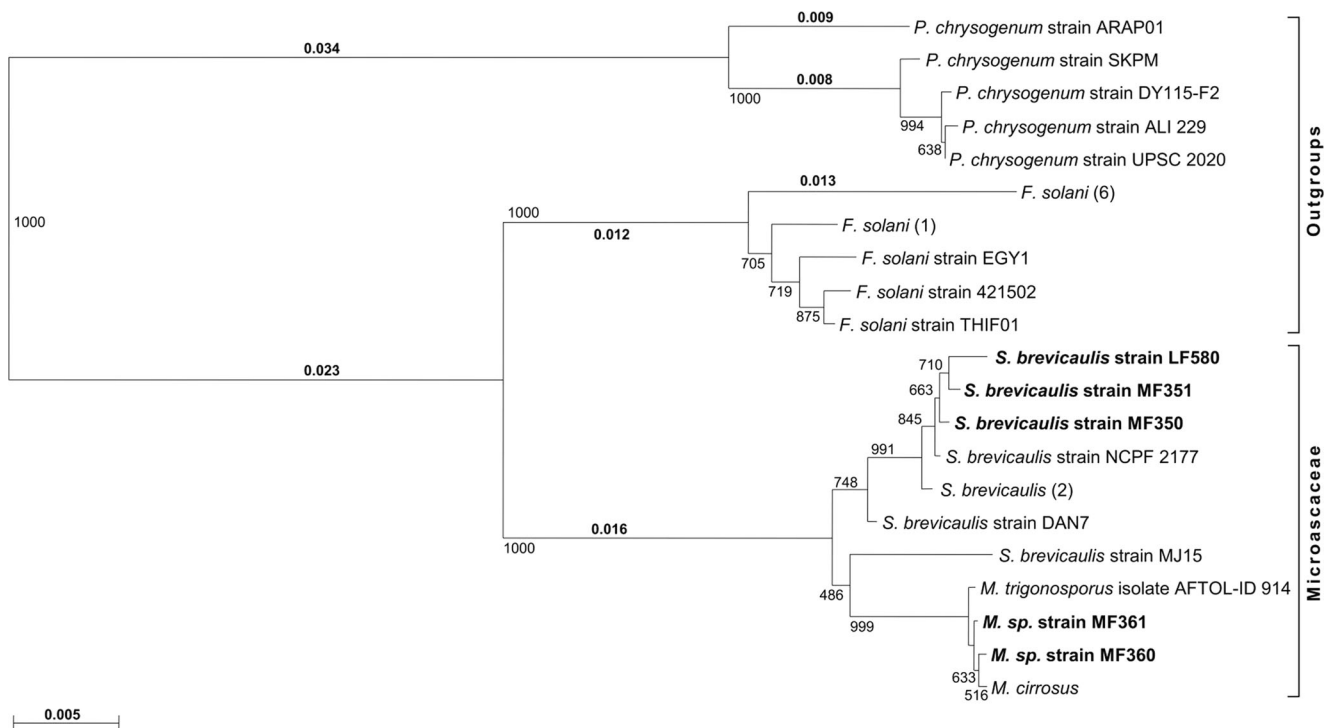
Fig. 2 Rooted tree of available *Scopulariopsis* and *Microascus* sp. 18S rRNA gene sequences with a minimum length of 1300 nt. *Bold letters* show *S./M. brevicaulis* strains LF580, MF350, MF351 and *Microascus* sp. strains MF360 and MF361. While the former clustered with other strains of *S./M. brevicaulis*, close to *S./M. brevicaulis* strain NCPF 2177, which revealed similarity of 99.5 % to strain LF580 as the closest relative (Yu et al. 2008), strains MF360 and MF361 clustered with sequences close to *M. trigonosporus* and *M. cirrosus*. Sequences of *Fusarium solani* and *Penicillium chrysogenum* strains were used as outgroups. Accession numbers of all sequences are given in the supplementary information (Table 1)

already by 18S rRNA gene phylogeny (Fig. 2). The majority of sequences of this group belonged to *S./M. brevicaulis* but also included other *Scopulariopsis* spp. and one *Microascus manginii* sequence. *M. manginii* was shown to be the teleomorph of *Scopulariopsis candida* (Morton and Smith 1963). Based on the very close relationship of *M. manginii* to *S./M. brevicaulis* on morphological level, Morton and Smith (1963) included *M. manginii* into the clade of 'brevicaulis' within *Scopulariopsis*. The green cluster in the ITS tree (Fig. 3) would be the molecular representation of such a 'brevicaulis' clade. Sequences of most other *Microascus* sp. strains including MF360 and MF361 formed a separate cluster (Fig. 3, marked in orange) in close proximity to the 'brevicaulis' clade. Sequences of MF360 and MF361 were clustering with *Microascus trigonosporum* var. *trigonosporum* strain CBS 665.71 (indicated as *M. trigonosporum* 8 in Fig. 3) within the ITS tree and showed a sequence similarity of 99.2 %. Sequence similarity of MF360 and MF361 to *Microascus cirrosus* strain ATCC MYA-4885 was a little less, at 98.7 %. Comparison of the sequences of *M. trigonosporum* var. *trigonosporum* strain CBS 665.71 and *M. cirrosus* strain ATCC MYA-4885 among each other showed a similarity of the ITS1-5.8S rRNA-ITS2 sequence of 98.4 %. Detailed examination of the ITS1-5.8S

rRNA-ITS2 sequence of strains MF360 and MF361 revealed sequences to show a similarity of 100 % to each other. This is in accordance with similarities of the 18S rRNA sequences of both strains.

In general, the phylogenetic distances within the 'non-brevicaulis'-*Microascus* clade were extremely short despite the clade comprised a number of different species. Therefore, classification of strains MF360 and MF361 at species level on the basis of ribosomal DNA sequences is not possible. Even more, besides *Microascus* sp. strains, all sequences from *S. hibernica* and *S. chartarum* strains were clustering into this 'non-brevicaulis' clade (Fig. 3).

The ITS tree clearly shows a monophyletic group of Microascaceae comprising the 'brevicaulis' clade and the 'non-brevicaulis' clade, in which the majority of strains was morphologically identified as *Microascus*. This finding is in accordance with other molecular phylogenetic studies, which identify the Microascaceae as monophyletic group (Abbott et al. 1998). Eleven ITS sequences of strains assigned to *Microascus/Scopulariopsis* did interestingly not cluster into the Microascaceae group and clearly should be considered as different species and genera. Concerning these strains (indicated by grey shading in Fig. 3), classification obviously needs revision. Recent advances in constructing a well-
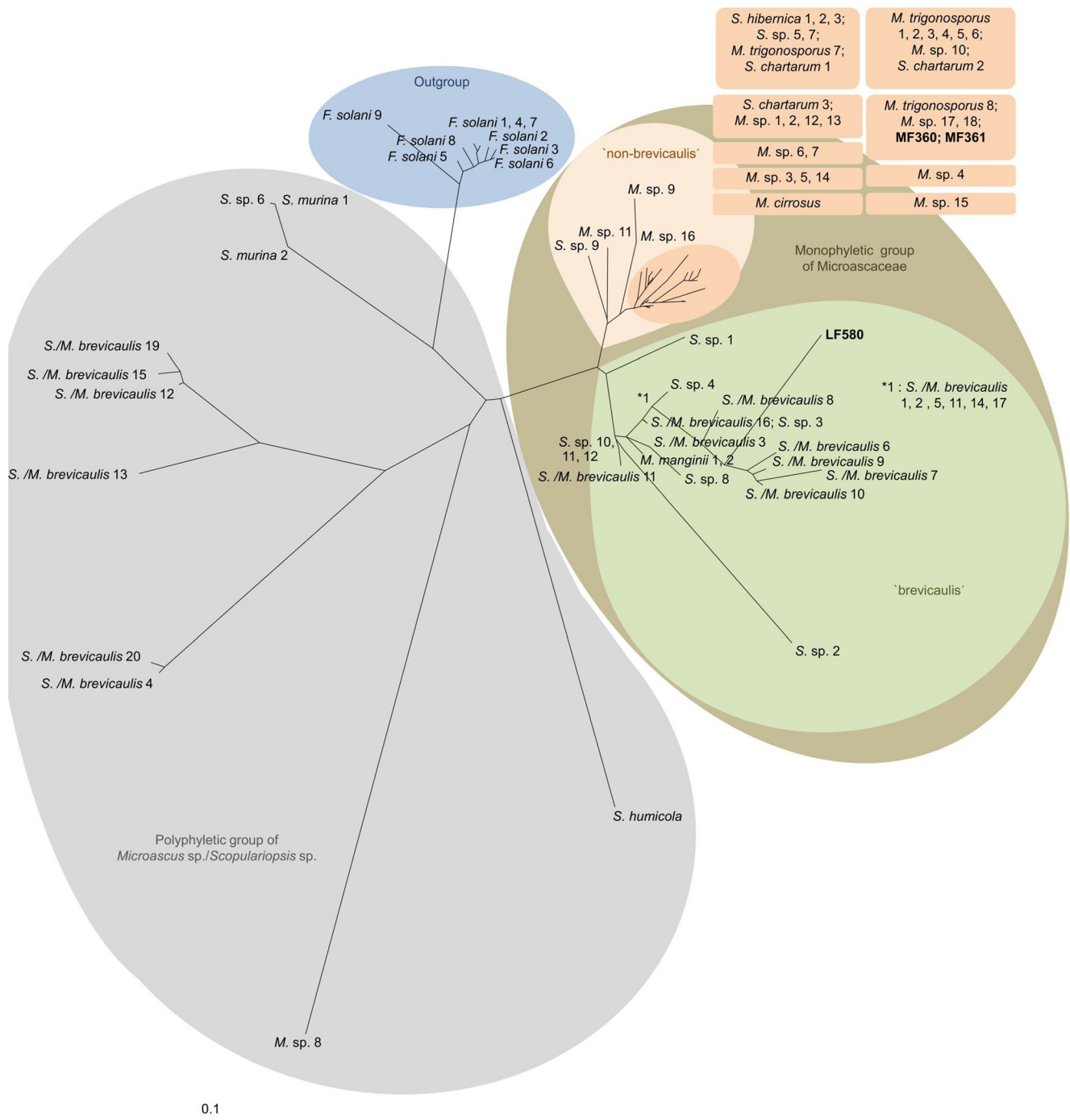
Fig. 3 Unrooted tree of available *Scopulariopsis* and *Microascus* sp. ITS1-5.8S rRNA-ITS2 sequences with a minimum length of 400 nt. Bold letters show *S./M. brevicaulis* strain LF580, *Microascus* sp. strains MF360 and MF361. Clusters were highlighted by colours for easy recognition of the different clades. Sequences labels within the *dark orange labelled subtree* were shown separately in the upper right. Strains MF360 and MF361 were found within this cluster. LF580 was clustering with several *S./M. brevicaulis* strains (clade marked in *green*). Sequences from *F. solani* strains were used as outgroup (*blue cluster*). A group of sequences of *Scopulariopsis* or *Microascus* strains, which did not cluster in the two groups, was shed in *grey*. Full strain names, strain numbers and accession numbers of all sequences are given in the supplementary information (Table 2)

supported fungal tree of life revealed the need for reconstruction and reclassification of strains, species and whole groups of uncertain phylogenetic position (Hibbett et al. 2007; McLaughlin et al. 2009).

Surprisingly, all strains originally identified to have perithecia and accordingly named as *Microascus* sp. clustered in the 'non-brevicaulis' *Microascus* clade. This was true for the two perithecia-forming strains of our study, MF360 and

MF361. In contrast, all strains originally found being asexual and accordingly named *Scopulariopsis* sp., clustered into the 'brevicaulis'-clade. MF350, MF351 and LF580, which clustered in this group, did not show any sexual state under any of the growth conditions tested.

Very short phylogenetic distances between strains being described as *Scopulariopsis* sp. (green marked cluster in Fig. 3) and strains originally identified as *Microascus* sp. (orange marked cluster in Fig. 3) were observed in our ITS-based sequence analysis, clearly indicating the affiliation of both to the same genus/taxon within the Microascaceae.

Secondary Metabolite Profile and Scopularide Production

Secondary metabolite profiles are strongly depended on several growth parameters and can vary for the same strain under different cultivation conditions (Bode et al. 2002). However, adaptation to identical environmental conditions as well as phylogenetic relatedness can lead to consistent secondary metabolite profiles. Moreover, the presence of genes for the biosynthesis of a given type of secondary metabolites may or may not be limited to phylogenetic groups, as known e.g. for the species-specific secondary metabolite production in the genus *Penicillium* (Smedsgaard and Frisved 1996). Therefore, metabolite profiles of available strains of the genus *Scopulariopsis/Microascus* were compared with focus on the production of scopularides A and B. The culture conditions were adapted from previous experiments with strain LF580 (Kramer et al. 2014), and all experiments were performed simultaneously in triplicates to ensure constant conditions for all strains. A solvent extraction was done at crucial time points for scopularide production known from previous experiments with strain LF580. The resulting extracts were applied to HPLC-UV/MS analyses to elucidate the metabolite spectrum and for dereplication purposes. All five strains were able to produce scopularide A, strains MF350 and MF351 at a level comparable to strain LF580 (Fig. 4). The production of the derivative scopularide B was limited to strain LF580. Production increased in correlation to cultivation time: The scopularide A yield was higher after 7 days of cultivation indicating similar growth and production behaviour of the three *S./M. brevicaulis* strains. This was in correlation with similarities in pellet formation. In contrast, the two strains MF360 and MF361 showed only minimal production of scopularide A, accompanied by a lower number of much bigger and compact pellets as describe in "Morphological comparison" section. A dependence of pellet size and morphology to productivity is known from several filamentous growing organisms (Krull et al. 2013). Such a correlation was hypothesised for strain LF580, respectively for its mutant strain LF580-M26 (Kramer et al. 2015). Furthermore, Tamminen et al. (2014) demonstrated a correlation between biomass and scopularide production for strain LF580. It is expected that this dependencies regarding the scopularide production hold true for

the others strains, in particular for strains MF350 and MF351. However, due to the different sexual state of MF360 and MF361, other aspects may have to be taken in account. Interestingly, all tested strains showed production of scopularide A, but only for strain LF580, a production of scopularide B was detected. A visual observation for all strain was done daily. The different growth behaviour of MF360 and MF361 was clearly visible, as described above (Morphological comparison section). Analytical data were determined at two time points. In accordance with the production profile of scopularide A, the morphology showed clear differences, which could be as well correlated with the phylogenetic distribution of the strains. However, the differences in the production yield of MF360 and MF361 compared to LF580, MF350 and MF351 (less than 1 % of scopularide production in MF360 and MF361 compared to LF580, based on MS peak area) were extremely high; therefore, it is presumed, that the sexual lifestyle of MF360 and MF361 must be taken into account. Finally, scopularide B production is limited to strain LF580, which confirms LF580 as the best starting point for further optimisation processes.

Distribution of Scopularide Producers in the Phylogenetic Tree

Even if the production of scopularide A was less than 1 % for the *Microascus* sp. strains MF360 and MF361, all strains obviously share the gene cluster for scopularide A production. No other genera are known from the literature producing the scopularides. This suggests an almost mutual exclusive distribution of the scopularide A production in the genus of *Scopulariopsis/Microascus* and may imply a strong phylogenetic and ecological component. Hence, this molecule could serve as phylogenetic marker for the *Scopulariopsis/Microascus* group. Recently, the putative biosynthetic gene cluster of scopularide A was identified based on the sequenced genome of strain LF580 (Lukassen et al. 2015). This molecular information can be used for more detailed expression studies in the future. Comparing the chemosynthetic properties to the phylogenetic distribution, all five strains were clustering in the monophyletic group of Microascaceae (Figs. 2 and 3). Production of scopularide A at high levels could be related to the asexual lifestyle as high production titres were found only in the 'brevicaulis' clade (green cluster in Fig. 3). Significant production of scopularide B was found only in the 'brevicaulis' clade (asexual) but not in the 'non-brevicaulis' clade (sexual). Whether the responsible biosynthetic genes remain silent or are not present cannot be deduced from our data, as the biosynthesis route for scopularide B is still unknown. Isaka et al. (2007) demonstrated that metabolites not dependent on the sexual form yielded same production levels in both, the anamorphs and the teleomorphs.

The genetic potential for the biosynthesis of the scopularides may or may not be limited to the phylogenetic
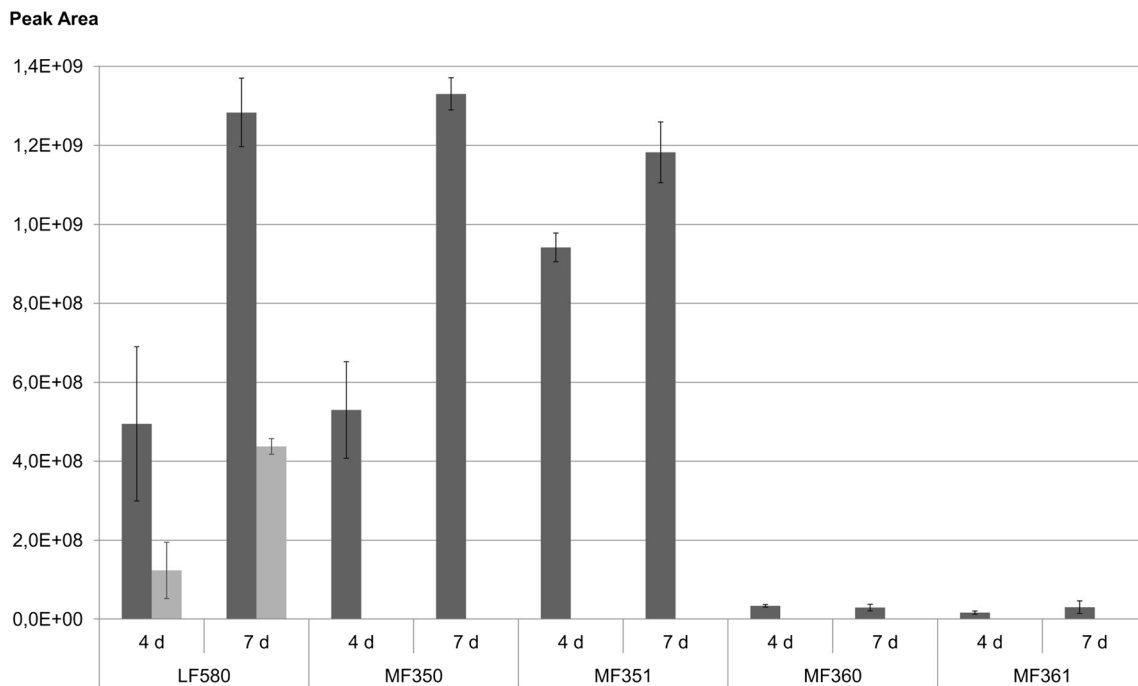
Peak Area

Fig. 4 Production level of scopularide A and B by the different strains belonging to Microascaceae . Production level is given in peak area of MS data of scopularide A (*dark grey*) and B (*light grey*) by the different strains *Scopulariopsis/Microascus brevicaulis* strains LF580, MF350, MF351, *Microascus* sp. strains MF360 and MF361 after cultivation for 4 and 7 days. MF350 and MF351 produced scopularide A at a level comparable to LF580. Production of the strains MF360 and MF361 was less than 1 % of the scopularide A yield of LF580. Scopularide B production was limited to LF580

group of Microascaceae. Adaptation to identical environmental conditions in a context of evolutionary relatedness can produce consistent secondary metabolite profiles: Though, no chemical data are available for other described *Scopulariopsis/Microascus* strains, our data suggest that scopularide production is a feature of the Microascaceae and depends on the life style of the respective strain.

Experimental Section

Strains

S./M. brevicaulis strains LF580, MF350, and MF351 and *Microascus* sp. strains MF360 and MF361 were taken from the strain collection of marine fungi maintained at GEOMAR as cryoconserved material.

Isolation and Identification

DNA extraction was carried out in innuSPEED Lysis Tubes S (Analytik Jena) with 400 μL of DNase-free water (Carl Roth). Homogenisation of cell material was performed two times for 45 s using a PreCellys 24 device (Bertin Technologies) at 6500 rpm. Suspension was centrifuged at 8000*g* and 15 °C for 10 min. The supernatant was stored at −20 °C until further use.

Fungal specific PCR by amplifying the ITS1-5.8S rRNA-ITS2 fragment was carried out using DreamTaq Green PCR Master Mix (2×) (Fermentas) with the primer pair ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990). PCR was conducted as follows: Initial denaturation (2 min at 94 °C), 35 cycles of primer denaturation (40 s at 94 °C), annealing (40 s at 55 °C), and elongation (1 min at 72 °C) followed by a final elongation step (10 min at 72 °C). PCR products were sequenced at the Institute of Clinical Molecular Biology in Kiel using the primer ITS1.

For the 18S rRNA gene region the isolation was performed as described above with the primer pair NS1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and FR1 (5′-AIC CAT TCA ATC GGT AIT-3′) (Gomes et al. 2003; Vainio and Hantula 2000). PCR was conducted as follows: Initial denaturation (8 min at 94 °C), 35 cycles of primer denaturation (30 s at 94 °C), annealing (45 s at 48 °C), and elongation (3 min at 72 °C) followed by a final elongation step (10 min at 72 °C) (Gomes et al. 2003). NS1, FR1 and in addition 470f (5′-CAG CAG GCG CGC AAA TTA-3′) (Dyková et al. 2008) were used for sequencing.

Bioinformatics

All five strains were compared on the basis of 18S rRNA gene sequences. Available sequences of *Microascus* and *Scopulariopsis* strains with a minimum length of 1300 nt

obtained from NCBI were used for tree calculation. For strain LF580 the full 18S rRNA gene sequence was used. Five 18S rRNA gene sequences of *Fusarium solani* and *Penicillium chrysogenum* were taken as outgroups. The alignments were generated by ClustalX (Thompson et al. 1997). Tree calculations were performed using ClustalX2 and W2 and bootstrapping values were generated with the neighbour-joining options of ClustalX. The trees were adjusted using TreeView (Page 1996) and NJplot (Perriere and Gouy 1996).

In addition, strains MF360, MF361 and LF580 were compared on the basis of the ITS1-5.8S rRNA-ITS2 fragment. Sequences of MF360 and MF361 were obtained by PCR. The sequence of LF580 was obtained from the genome data of LF580 (Kumar et al. 2015). ITS1-5.8S rRNA-ITS2 sequences with a minimum length of 400 nt of *Scopulariopsis* and *Microascus* strains available from NCBI were used for comparison and tree calculation. Nine ITS1-5.8S rRNA-ITS2 sequences of *Fusarium solani* strains were taken as outgroup. All NCBI accession numbers are provided in the supplementary information (Table 1, 2, will be provided during review process).

Cultivation

Cultivation was carried out in 300-mL Erlenmeyer flask, containing 100 mL modified Wickerham medium (WSP30) (1 % glucose × H_2O, 0.5 % soy peptone, 0.3 % malt extract, 0.3 % yeast extract, 3 % NaCl) (Wickerham 1951), for 4 and 7 days, at 28 °C and 120 rpm, in the dark. The cultivation was carried out in triplicates for each time point. Pre-cultures were cultivated on WSP30 agar plates, 2 % agar, at room temperature, for 4 days, in the dark. Agar pieces with 5 mm diameter of 4-day-old cultures were used as inoculum.

Extraction of Secondary Metabolites

The samples were extracted after 4 respectively 7 days of cultivation. A homogenisation of the samples were performed using an Ultra-Turrax at 16,000 rpm (T25 basic IKA® Werke) until complete homogenisation was obtained (after approximately 20 s). Twenty milliliters of the suspension were transferred to a 50-mL tube. Twenty milliliters ethylacetate were added, followed by a second homogenisation. Samples were centrifuged at 4000 rpm for 10 min. Remaining biomass was obtained by centrifugation at 4700 rpm for 15 min and subsequent drying at 60 °C. The solvent was evaporated employing a vacuum centrifuge. The dry extracts were then resolved in 1 mL of methanol and filtered through a 0.2-mm PTFE filter (Rotilabo-syringe filters, ROTH) via a syringe.

LC-MS Analysis

HPLC-UV/MS analyses were performed on a VWR Hitachi Elite LaChrom system coupled to an ESI-ion trap detector (Esquire 4000, Bruker Daltonics), using a RP-C18 column (Phenomenex Onyx Monolithic C18, 100 × 3.00 mm) with a H_2O/MeCN-gradient (0 min 5 % B, 4 min 60 % B, 6 min 100 % B; with a flow of 2 mL min^{-1}) with 0.1 % formic acid added to A and B. For comparison of the yield of the scopularides, the peak areas with the respective *m/z*-values were automatically integrated using DataAnalysis Version 3.3 (Bruker Daltonics GmbH). The arithmetical mean and standard deviation of the peak area values were calculated.

Conclusions

The production of scopularide A of five strains of the Microascaceae were compared to identify the best candidate for subsequent strain optimisation in the context of hit-to-lead development of the anti-cancer active scopularide A. *S./M. brevicaulis* strain LF580 was confirmed as the best producer of scopularides A and B. Two other closely related strains, MF350 and MF351, yielded an adequate amount of scopularide A, but no significant production of scopularide B. In addition, the production of scopularide A could be observed for two perithecia forming *Microascus* strains, MF360 and MF361, phylogenetically distinct from *S./M. brevicaulis*. Because no other producer strains of the scopularides are known from literature, strain LF580 represents the best producer strain available for strain (Kramer et al. 2014) and process optimisation (Tamminen et al. 2014).

A monophyletic group of Microascaceae was revealed from a phylogenetic analysis of the five strains and comparison with sequences available from databases referred to *Scopulariopsis* sp. and *Microascus* sp. This group comprised one subgroup with mainly *S./M. brevicaulis*, exhibiting an asexual lifestyle and a second subgroup comprising *Microascus* sp. strains, shown to exhibit a sexual lifestyle. Sequences of a number of other strains assigned to *S. brevicaulis* were distantly related to the first group and their systematic assignment certainly needs revision.

The identification of strains at species level remains quite difficult on the basis of molecular phylogenetic markers. For example, an accurate affiliation of the strains MF360 and MF361 was not possible using 18S rRNA or ITS1-5.8S rRNA-ITS2 sequences, because of the short phylogenetic distances.

researching the ITS1-5.8S rRNA-ITS2 fragment. Arlette Wenzel-Storjohann performed DNA isolation and conducted PCR for sequences analysis.

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Compliance with Ethical Standards

Conflict of Interest   The authors declare that they have no conflict of interest.

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