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Caldora penicillata gen. nov., comb. nov. (Cyanobacteria), a pantropical marine species with biomedical relevance

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Abstract

Many tropical marine cyanobacteria are prolific producers of bioactive secondary metabolites with ecological relevance and promising pharmaceutical applications. One species of chemically rich, tropical marine cyanobacteria that was previously identified as *Symploca hydnoides* or *Symploca* sp. corresponds to the traditional taxonomic definition of *Phormidium penicillatum*. In this study, we clarified the taxonomy of this biomedically and ecologically important cyanobacterium by comparing recently collected specimens with the original type material and the taxonomic description of *P. penicillatum*. Molecular phylogenetic analyses of the 16S rRNA gene and the 16S-23S ITS regions showed that *P. penicillatum* formed an independent clade sister to the genus *Symploca*, and distantly related to *Phormidium* and *Lyngbya*. We propose the new genus *Caldora* for this clade, with *Caldora penicillata* comb. nov. as the type species and designate as the epitype the recently collected strain FK13-1. Furthermore, the production of bioactive secondary metabolites among various geographically dispersed collections of *C. penicillata* showed that this species consistently produced the metabolite dolastatin 10 and/or the related compound symplostatin 1, which appear to be robust autapomorphic characters and chemotaxonomic markers for this taxon.

Keywords

dolastatin 10; largazole; *Lyngbya*; natural products; *Phormidium*; phylogenetics; secondary metabolites; *Symploca*; symplostatin 1

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INTRODUCTION

During the last three decades, natural products (NPs) discovery efforts have successfully exploited collections of tropical marine cyanobacteria for biologically active secondary metabolites with potential pharmaceutical properties (Nunnery et al. 2010, Tidgewell et al. 2010). Tropical marine cyanobacteria with morphological resemblance to the genus *Symploca* Kützing ex Gomont (1892) are prolific producers of NPs (Luesch et al. 2001, Thacker and Paul 2004, Taori et al. 2008, 2009, Engene et al. 2013). Collections of tropical marine cyanobacteria identified as *S. hydnoides* and *Symploca* sp. have yielded several important natural products with promising pharmaceutical potential, including the metabolites: symplostatin 1 (Harrigan et al. 1998), dolastatin 10 (Luesch et al. 2001), largazole (Taori et al. 2008), janthielamide A, kimbeamides A-C, and kimbelactone A (Nunnery et al. 2012).

The cyanobacterial genus *Symploca* was first described from terrestrial humid habitats, with *Symploca meneghiniana* Kützing ex Gomont (1892) later designated as the type species (Gardner 1932: 283), but also including two temperate marine species *S. hydnoides* Kützing ex Gomont (1892) and *S. atlantica* Gomont (1892), and later on *S. funicularis* Setchell & N.L. Gardner in Gardner (1918). Traditionally, *Symploca* spp. have been defined as fine filamentous cyanobacteria with isodiametric cells and filaments clustered into fasciculated bundles (Gomont 1892, Geitler 1932, Komárek and Anagnostidis 2005). However, this taxonomic definition is somewhat ambiguous and *Symploca* spp. usually overlap morphologically with other filamentous cyanobacteria genera within Oscillatoriales (Castenholz 2001, Komárek and Anagnostidis 2005), such as *Microcoleus* Desmazičres ex Gomont (1892) and *Phormidium* Kützing ex Gomont (1892). The widespread genus *Phormidium* (*Ph. lucidum* Kützing ex Gomont 1892 as generitype) was also described from terrestrial humid habitats and has been considered as possibly the most problematic genus within Oscillatoriaceae (Komárek et al. 2014). In particular, *Symploca* and *Phormidium* are primarily differentiated based on their growth morphologies; *Symploca* spp. form fasciculated, erect thalli, whereas *Phormidium* spp. form thin mats (Komárek and Anagnostidis 2005). A prime example of the confusing morphological overlap and complicated nomenclatural history between these two genera is *Phormidium penicillatum* Gomont (1893), which was first collected and described from coral reef habitats of the island of Réunion in the Indian Ocean. Despite being included in *Phormidium*, this species has a very characteristic upright growth form that corresponds better with the characterization of *Symploca*. Nearly a century later, Hoffmann and Compère (1990) transferred *Phormidium penicillatum* to *Lyngbya* C. Agardh ex Gomont (1892) as *Lyngbya penicillata* (Gomont) L.R. Hoffmann nom. illeg., for which a new name, *Lyngbya penicilliformis* P. C. Silva was proposed (Silva, Basson and Moe 1996), since Hoffman's new combination was predated by *Lyngbya penicillata* (Kützing) Rabenhorst ex Forti (1907) based on a different type specimen.

The taxonomic confusion between the two genera *Symploca* and *Phormidium* highlights some common problems with cyanobacterial systematics. Firstly, the morphological characters used to define taxa are typically ambiguous and only a limited number of morphological characters are available for distinguishing different taxa (Komárek 2006,

Sciuto et al. 2012, Dadheech et al. 2013). Many of these characters also show morphological plasticity and often vary depending on environmental conditions (Sumina 2006). As a result of these limitations in traditional mophology-based identification, modern cyanobacterial systematics is largely incorporating molecular data in order to construct monophyletic genera as a fundamental framework for biologically informative classification systems (Castenholz 2001, Hoffmann et al. 2005, Komárek and Anagnostidis 2005, Komárek 2006, Komárek et al. 2014). This polyphasic approach, combines genotypic (16S rRNA gene sequencing) and phenotypic data (morphological analysis, ultrastructural sections) (Sciuto et al. 2012, Dadheech et al. 2013, Strunecký et al. 2013, 2014), striving to find at least one autapomorphic character, in order to preliminarily characterize each genus (Komárek 2010).

However, a major obstacle with the use of a molecular phylogenetically-based system is to correlate the original type material used to describe a species with recently collected specimens used for genetic sequencing. Although cyanobacterial herbarium specimens have been used to obtain DNA for genetic sequencing (Palinska et al. 2006), many type specimens are lacking or no longer suitable for a complete polyphasic comparison (Sciuto et al. 2012,). As a consequence, reasonable presumptions are often necessary based on type locality and original descriptions to define reference strains. In regards to *Lyngbya* and *Symploca*, the cyanobacterial strains PCC 7419 (Castenholz et al. 2001a), and PCC 8002 (Castenholz et al. 2001b) have been used as reference strains for these genera, respectively, and CCALA 759 (Sciuto et al. 2012) has been designated as the epitype for *Phormidium irriguum* (Kützing) Anagnostidis & Komárek. Herein, we use molecular phylogenetic methods to resolve the phylogenetic relationships of the chemically rich tropical marine *Symploca* specimens that are responsible for several important NPs by comparing 16S rRNA gene sequences of recently collected specimens with sequences of the above-mentioned reference strains. We also show that these specimens are molecularly distinct (16S rRNA gene sequences with a similarity index of ±95% or less for generic definition; Komárek 2010) from *Phormidium*, *Lyngbya*, and *Symploca* specimens and instead form a separate lineage (previously described as Clade III in Engene et al. 2013). Furthermore, two of the analyzed sequences are highly similar (99-100% similarity on 16S rRNA gene sequences) to samples from the type locality of *Phormidium penicillatum* (Saint-Gilles, Réunion; Echenique-Subiabre et al. 2015). We conclude that those specimens traditionally identified in the NPs literature as *Symploca* spp., in fact, refer to *Phormidium penicillatum* Gomont (1893) and, consequently, we propose Clade III as the new genus *Caldora*, accommodating *Caldora penicillata* comb. nov. as the type species. Furthermore, we show that geographically dispersed collections of *C. penicillata* consistently produce the metabolite dolastatin 10 and/or the structurally related metabolite symplostatin 1. Thus, these compounds appear to act as autapomorphic characters and robust chemotaxonomic markers for the identification of this species.

MATERIALS AND METHODS

Cyanobacterial collection

Cyanobacterial specimens identified as *Lyngbya penicilliformis* or *Symploca* spp. were collected by SCUBA or snorkeling from various marine habitats in Bonaire, Curaçao,

Belize, Honduras, and the Florida Keys (see Table S1 in the Supporting Information for detailed collection information). Putative taxonomic identification and selection of the cyanobacterial specimens were performed in accordance with traditional classification systems (Geitler 1932, Castenholz 2001, Komárek and Anagnostidis 2005) and with reference to available field guides (Littler and Littler 2000, 2003). Specimens were cleaned immediately after collection under a dissecting microscope (Wild Heerbrugg, Heerbrugg, Germany) to remove other macroorganisms. All collections were preserved in RNA*later*® (Ambion, Austin, TX, USA) for genetic analysis, in seawater with 5% formalin for morphological analysis, and frozen at -20 °C for chemical analysis. The *Symploca* reference strain PCC 8002 was obtained from the Pasteur Culture Collection (PCC) for phylogenetic comparison (a comprehensive morphological characterization of strain PCC 8002 is available in Porta et al. 2003). The type specimen of *Phormidium penicillatum* (PC0167627; see Figures S1 and S2 in the Supporting Information), as well as other *P. penicillatum* specimens deposited in the Cryptogamy collection (PC) were used for taxonomic revision and morphological comparison. In addition, syntypes of *Lyngbya penicilliformis* were obtained from UC, and from the Drouet collection at US. Herbarium acronyms follow Thiers (2015). All collected specimens were deposited in US, under the Algal Collection numbers: 217964, 217968-217983. Due to the troubled taxonomic history of this taxon and the inability to obtain representative DNA sequences from the type material of *Phormidium penicillatum*, in addition to the absence of voucher specimens of recently sequenced samples from the type locality (Saint-Gilles, Réunion; Echenique-Subiabre et al. 2015; see Discussion), we selected the recently collected strain FK13-1 (N. Engene, 10 June 2013, Looe Key, Florida Keys) as epitype for future reference, designated under the provisions of Art. 9.8 and in accordance with Art. 9.20 and 9.21 of the International Code of Nomenclature for algae, fungi, and plants (McNeill et al. 2012).

Microscopy

Light microscopy was performed using an Olympus IX51 Leica epifluorescent microscope (Olympus, Tokyo, Japan) equipped with a Nikon Coolpix camera (Olympus, Tokyo, Japan). Morphological measurements were determined as: mean ± standard deviation (SD). Filament and cell size averages (adjacent cells of ten filaments) were calculated based on ten measurements (Table S2 in the Supporting Information). Samples for SEM were placed on glass slides that had been coated with a drop of Tissue TAC slide adhesive (DADE®) to facilitate adhesion. Samples were then fixed in 2.5% glutaraldehyde in 1X phosphate-buffered saline for 30 min and a secondary fix of 2% (water) osmium tetroxide for 30 min. Dehydration was achieved with a graded (20%, 50%, 70%, 90%, 100%, 100%) ethanol series. Samples were then critical point dried and sputter coated (200 A) with gold palladium. A Hitachi S-4800 SEM (Chiyoda, Tokyo, Japan) was used to image the samples. Images of *Caldora penicillata* were taken from strain FK12-26, which was collected in the same exact coordinates (N. Engene, 13 August 2012, Looe Key, Florida Keys) as the designated epitype FK13-1.

DNA extraction, multiple displacement amplification, and gene sequencing

For genetic purposes, live, pigmented filaments were scrupulously selected. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI,

USA), following manufacturer's specifications. A modified DNA extraction protocol by Sauvage et al. (2013) was also used to attempt obtaining DNA from the herbarium specimens. DNA concentration and purity was measured on a ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Whole-genome amplification was performed on bundles of filaments of the epitype strain FK13-1 that were isolated under a dissecting microscope followed by multiple displacement amplification (MDA). Filaments were washed twice in 2 μL filter-sterilized seawater and twice in 2 μL ddH_2O before transfer into 0.2 mL PCR-tubes. DNA was amplified from the single-filaments by MDA using the REPLI-g® UltraFast Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's specifications. All MDA reactions were performed in 20 μL reaction volume for 1.5 h at 30°C.

The 16S rRNA gene and the 16S-23S ITS regions were PCR-amplified using the 106F primer (Nübel et al. 1997) and the 340 primer (Iteman et al. 2000), respectively. Various primer sets were used in attempts to PCR-amplify nitrogenase reductase (*nif*H) genes, including *nifHf*/*nifHr*(Gugger et al. 2005), CDHPnif53F/CDHPnif723R and nifH1/nifH2 (Steward et al. 2004), and MC-nifHDK primers (Bolhuis et al. 2010). PCR protocols and products purification follow Engene et al. (2013). Subcloning was performed using the pGEM®-T Easy Vector system (Promega), following the manufacturer's specifications. Plasmid DNA was isolated using the QIAprep®Spin Miniprep Kit (Qiagen) and sequenced bidirectionally with M13 vector primers as well as the internal primers 359F, 785R, and 1509R (Nübe et al. 1997). Gene sequence anomalies, including chimeric sequences, were predicted using the Pintail software with the cut-off size set at >600 bp (Ashelford et al. 2005) and were manually confirmed by comparison of Neighbor Joining (NJ) phylogenetic trees for different regions (>300 bp) of the sequences. Genetic sequencing was performed at the Laboratories of Analytical Biology, NMNH. All gene sequences for the *Caldora penicillata* specimens are available in GenBank under the accession numbers (acc. nr.): KF746590-KF746608 (16S rDNA) and KF768777-KF768793 (16S-23S ITS regions).

Phylogenetic inference

The 16S rRNA gene (1,306 bp) and the 16S-23S ITS regions (480 bp) from a total of 16 specimens of *Caldora penicillata* were used for phylogenetic inference. Gene sequences from other cyanobacterial taxa were obtained from the National Center for Biotechnology Information (NCBI) web pages. DNA sequences of reference strains selected from Bergey's Manual (Castenholz 2001) and of species identified as type species from CyanoDB (Komárek and Hauer 2011) and AlgaeBase (Guiry & Guiry 2015) were included in the analyses. The reference unicellular cyanobacterium *Gloeobacter violaceus* PCC 7421 (GenBank acc. nr. NC005125) was included as an evolutionarily distant outgroup. All gene sequences were aligned using MUSCLE (Edgar 2004). The 16S rRNA gene sequence alignment was visually compared and refined using the SSU secondary structures model of *Escherichia coli* J01695 (Cannone et al. 2002). Mutation types and domains of the 16S rRNA genes were predicted based on superimposing of the secondary structures on the *E. coli* strain J01695 SSU model. All multiple sequence alignments are available at TreeBASE (http://www.treebase.org) under the submission IDs: 14952 (16S rDNA) and 14953

(16S-23S ITS regions). The 16S-23S ITS regions were analyzed for transfer RNA and transfer-messenger RNA genes using ARAGORN v1.2.36 (Laslett et al. 2004). Pairwise sequence divergences were calculated in MEGA 5.1 without model selection (Tamura et al. 2011). Appropriate nucleotide substitution models were compared and selected using uncorrected/corrected Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and the Decision-theoretic in jModeltest 0.1.1 (Posada 2008). Maximum likelihood (ML) inference was performed using PhyML (Guindon and Gascuel 2003) with the GTR+I +G model of evolution, assuming heterogeneous substitution rates and gamma substitution of variable sites (proportion of invariable sites (pINV) = 0.417, shape parameter (α) = 0.382, number of rate categories = 4). Bootstrap resampling was performed on 1,000 replicates. Bayesian inference (BI) was conducted using MrBayes 3.1 under the GTR+I+G model (Ronquist and Huelsenbeck 2003). Four Metropolis-coupled Markov chain Monte Carlo (one cold and three heated) were run for 1,000,000 generations and the first 100,000 generations (10%) were discarded as burn-in and the following data sets were sampled every 100 generations. *Secondary metabolite screening.* Algal biomass of each specimen was lyophilized and extracted twice with ethyl acetate: methanol (1:1). Extracts were analyzed for chemical compositions by low-resolution liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) system with a linear trap quadrupole (LTQ) Advantage Max spectrometer (Thermo Finnigan, Waltham, MA, USA). Each sample (10 μL) was injected and separated on a reversed-phase HPLC column as outlined in Engene et al. (2013). MS and tandem MS (MS/MS) spectra and retention time of each peak were recorded using the positive and negative ion detection modes. Identification of secondary metabolites required support of predicted isotope patterns, corresponding MS/MS fragmentations, and conserved retention times that were compared with previously characterized secondary metabolites (Engene et al. 2013). Abundances of molecules were roughly estimated based on their ion abundance.

RESULTS

Variability in morphology

A total of 17 cyanobacterial specimens corresponding with the taxonomic definitions of *Lyngbya penicilliformis* and *Symploca* spp. were collected and analyzed from various geographically dispersed tropical and subtropical marine locations in Florida and the Caribbean Sea (Table S1). Collectively, the overall morphologies of *Caldora penicillata* specimens were soft and mucilaginous in appearance, erect and amorphic (Fig. 1). However, morphology varied from various puffball-shaped clumps (Fig. 1, a and b) to feathery shapes (Fig. 1, c-f). Size of *Caldora penicillata* specimens also varied from several centimeter sized clumps to extensive mats with up to 20 cm long finger-like projections. A shared feature among all specimens was a soft, mucilaginous internal portion, which was always colorless or whitish in color. The external portions varied widely in colors and ranged from various shades of orange (Fig. 1, a and b), red (Fig. 1, c-e), and green (Fig. 1f). These external colorations often reflected in the overall coloration of the specimens.

Specimens with dissimilarity in shape and coloration were collected from the same sites and compared to verify that the different morphotypes represented the same species.

Specifically, strains NAB11-28 and NAB11-29 from Bonaire (Lac Bay) displayed puffball and feathery growth morphologies, respectively. Moreover, puffball-shaped specimens of the strains of NAC11-67 and NAC11-68 from Curaçao (Porto Marie) were greenish and red-orange, respectively. In these cases, the specimens were identical in gene sequences (see section below) and in the production of secondary metabolites (Table 1), highlighting the wide morphological variation in appearance in the field.

Sections of both the internal and external parts of strain FK12-26 were analyzed by both scanning electron microscopy (SEM) and light microscopy (Fig. 2, a-e). The internal base was shown to be composed primarily of residues of empty polysaccharide sheaths (Fig. 2b), and the external section contained mainly live and pigmented filaments (Fig. 2c). Filaments were thin (4-6 μm) and often entangled in bundles. Cells were barrel-shaped or occasionally cylindrical with no cross-wall constrictions and enclosed by thin, barely visible polysaccharide sheaths (Fig. 2, b-d). Notably, both the empty polysaccharide sheaths and the sheaths of live filaments were almost completely clean from associated biofilm-forming microorganisms (Fig. 2, b-d).

Evolutionary divergence

The SSU (16S) rRNA gene was sequenced from all recently collected specimens of *Caldora penicillata* and used for phylogenetic inferences (Fig. 3). Phylogenetic analysis revealed that *C. penicillata* sequences formed a monophyletic clade that was most closely related to those of the genera *Symploca* (p-distance = 4.5%), *Coleofasciculus* Siegesmund, J.R. Johansen & Friedl (2008) (p-distance = 6.5%), and *Moorea* Engene, Rottacker, Kastovsky, Byrum, Choi, Ellisman, Komárek & Gerwick (2012; p-distance = 6.6%). Moreover, *C. penicillata* sequences formed a tight clade with a mean intra-generic sequence divergence of only 0.9% (Fig. 3). The taxon that was most closely related to *C. penicillata* was a cyanobacterium originally identified as *Leptolyngbya* sp. (p-distance = 2.1%; strain PAC-10-03; GenBank acc. nr. KC207936) collected from the Coiba National Park of the Pacific coast of Panama (Medina et al. 2008, Engene et al. 2013).

Interestingly, sequencing of multiple clones from libraries generated from the two strains BCBC11-25 and HMC13-9 showed that these two specimens contained two slightly genetically different copies of their 16S rRNA genes (Fig. 3). The highest degree of intra-genomic gene sequence divergence was observed in BCBC11-25, where the two gene copies (i.e. BCBC11-25 *rrn*A and BCBC11-25 *rrn*B in Figs. 3 and 4) varied in 16 nucleotide positions of 1319 nucleotides of the sequenced genes (1.2%). Secondary structure modeling revealed that the 16 nucleotide substitutions were all either: (i) located in loop-regions (7 bp), (ii) were C↔T substitutions located in stem-regions resulting in U-G bonds (3 bp), (iii) or resulted in compensatory substitutions in stem-regions (6 bp). Thus, these substitutions of the 16S rRNA gene were all likely true mutations, rather than PCR or sequencing artifacts, since the overall RNA structure was conserved in both gene copies.

Despite the general absence of associated heterotrophic bacteria as shown by SEM (Fig. 2d), *Caldora penicillata* specimens were shown microscopically and genetically to often form assemblages with other filamentous cyanobacteria (Fig. 2f). These associated filamentous cyanobacteria varied greatly in morphologies and likely belonged to a variety of taxonomic

groups. Two of the predominant cyanobacterial taxa associated with *C. penicillata* were cf. *Spirulina* sp. (e.g., GenBank acc. nr. KJ439051) and *Hormoscilla spongeliae* (Gomont) Anagnostidis & Komárek (e.g., GenBank acc. nr. KJ439052). Moreover, these cyanobacterial consortia varied greatly in complexity and overall proportions among the different cyanobacterial components. Although cyanobacterial complexity was only determined from representative microscopic sections, all specimens analyzed appeared to have *C. penicillata* as the dominant component, and all the clone libraries generated from the *C. penicillata* specimens contained gene sequences belonging to the *C. penicillata* clade. Whole-genome amplification was performed by MDA to pinpoint the gene sequences that corresponded to the correct species filaments in the epitype strain FK13-1. A bundle of morphologically uniform *C. penicillata* filaments were micromanipulated from the strain FK13-1 and their genomic-DNA amplified by MDA. The 16S rRNA gene PCR-amplified from the MDA-amplified DNA (GenBank acc. nr. KF746604) was identical to the gene sequence obtained from the genomic DNA (GenBank acc. nr. KF746603), which verified the identity of the specimens of *C. penicillata* (i.e., FK13-1 MDA in Fig. 3).

The 16S-23S internal transcribed spacer (ITS) regions were also sequenced and independently used to infer the phylogenetic relationships among *C. penicillata* specimens (Fig. 4). The 16S-23S ITS regions varied in sequence length between 429 and 480 nucleotides among the specimens. However, the gene regions all included a conserved 79-nucleotide gene stretch encoding tRNA-Il. In addition to the 16S-23S ITS regions available for strains of *Moorea* and *Coleofasciculus*, the *Symploca* reference strain PCC 8002 was obtained from PCC and its ITS region was sequenced. Phylogenetic analyses of the 16S-23S ITS regions were congruent with the 16S rRNA gene in placing *C. penicillata* as an independent clade, moderately supported, and sister to *Symploca* (p-distance = 30.6%) and *Moorea* (p-distance = 32.0%), with *Coleofasciculus* somewhat distantly related (p-distance = 32.6%).

Several unsuccessful attempts were also made to PCR-amplify nitrogenase reductase (*nif*H) genes from *C. penicillata* specimens as well as the SSU (16S) rRNA gene from the *Lyngbya penicilliformis* herbarium specimens using various nonspecific primers.

Characteristic secondary metabolites

All *C. penicillata* collections were extracted and analyzed by ESI-LC-MS for their production of bioactive secondary metabolites, and abundance of each molecule was estimated based on its ion abundance (Table 1). Notably, all 16 specimens produced dolastatin 10, and this secondary metabolite often formed the major component of the crude extracts. The presence of dolastatin 10 was often complemented by the structurally related analog symplostatin 1, which typically was present in lower abundance. In addition, 10 of the specimens also produced the metabolite largazole (Table 1; Fig. 5).

DISCUSSION

In this study, we applied a combined molecular, morphological and chemical approach to evaluate the taxonomy of the marine cyanobacteria specimens traditionally identified as *Lyngbya penicilliformis* and *Symploca* spp. (Luesch et al. 2001, Thacker and Paul 2004,

Taori et al. 2008, 2009, Engene et al. 2013), which are herein assigned to the new genus *Caldora*, designating *C. penicillata* as generitype (accommodating *Phormidium penicillatum*; see below under *Taxonomic conclusions*).

In many coral reef habitats surveyed, *Caldora penicillata* was one of the most common and abundant cyanobacteria present. *Caldora penicillata* often formed large populations attached to different solid substrata, such as hard bottoms, dead corals, or gorgonians. Our field observations suggest that the most suitable habitats for *C. penicillata* are exposed reefs with high water circulation. The depth range varied greatly from 1-30 m, but *C. penicillata* appeared to be most prevalent at depths of 3-12 m. Although *C. penicillata* was found all year round in tropical marine locations, a greater abundance was typically observed during seasons with warmer water temperatures (> 25°C).

In this study, we sampled cyanobacterial specimens from the Florida Keys and various Caribbean Sea locations such as Belize, Netherlands Antilles (Bonaire and Curaçao), and Honduras. In addition, specimens corresponding morphologically to *Caldora penicillata* and with <1% 16S rRNA gene sequence divergence to the designated epitype strain FK13-1have also been reported from several Pacific locations, including Papua New Guinea (strain PNG06-65.1; GenBank acc. nr. HM072001; Choi et al. 2010), Guam (strain VP377; GenBank acc. nr. AF306497; Harrigan et al. 1998), and Palau (strain VP642b; GenBank acc. nr. AY032933; Luesch et al. 2001) (Fig. 3). Furthermore, recent studies have reported 99% and 100% similarity of our *C. penicillata* 16S rRNA gene sequences from Palau (VP642b) and Papua New Guinea (PNG06-65.1), respectively, with OTU03 from Réunion Island, corresponding to samples collected in the type locality of *Phormidium penicillatum* (Saint-Gilles) and nearby (ca. 25 km from Saint-Gilles; Etang Salé) (Echenique-Subiabre et al. 2014). Molecular results of our collections from various Caribbean and Pacific locations (at depths ranging between 2-20 m), together with the above-mentioned molecular records from Réunion indicate that this taxon is widely distributed in tropical and subtropical ocean basins globally. The tropical marine, coral reef habitats of the collected specimens also agree well with the environment of the type material of *Phormidium penicillatum*. Moreover, our collections correspond phenotypically with the original type material and taxonomic description of *P. penicillatum* by Gomont (1893), which shows the distinctive barrel-shaped filament cells (see Fig. S2) as in the specimens of *Caldora penicillata* used in this study. Thus, based on molecular, ecological and phenotypic characteristics we find it most likely that our collected specimens correspond with the originally description of *Phormidium penicillatum*.

Phormidium penicillatum has undergone several nomenclatural changes, including a taxonomic revision and transfer from the genus *Phormidium* to the genus *Lyngbya* (Hoffmann and Compère 1990). This transfer was followed by a name replacement to *Lyngbya penicilliformis* (Silva et al. 1996). However, our DNA-based phylogenetic analyses reveal that specimens traditionally identified in the natural products literature as *L. penicilliformis* and *Symploca* spp. are evolutionarily unrelated to the reference strains of both *Lyngbya* and *Phormidium*. Instead, these specimens form a separate lineage sister to *Symploca* and closely related to *Moorea* and somewhat to *Coleofasciculus*. The uncorrected 16S rRNA gene sequence divergences between these groups were approximately 5%. This

value corresponds with the threshold recommended for distinguishing genera in cyanobacteria (Komárek 2006, Tindall et al. 2010). Thus, based on evolutionary and ecological divergence, we propose the new genus *Caldora* (see below under *Taxonomic conclusions*). It is also likely that additional species will be included in this genus. For example, the closely related specimen, a cyanobacterium originally identified as a *Leptolyngbya* sp., shared many morphological features with *C. penicillata* (Medina et al. 2008). This specimen also produced the bioactive secondary metabolite coibamide A that shares the N,N-dimethyl valine motif with dolastatin 10 (Medina et al. 2008). Further research is needed to elucidate whether the coibamide A-producing cyanobacterium should also be included in the genus *Caldora.*

All our collected specimens of *Caldora penicillata*, despite coming from geographically dispersed regions, produced the secondary metabolite dolastatin 10, a potent microtubule inhibitor effective against various types of tumors (Luesch et al. 2001). The majority of *C. penicillata* specimens also produced the analog symplostatin 1, and structural similarities between these molecules suggest that the same genetic pathway is involved in the biosynthesis of these two metabolites (Harrigan et al. 1998). The stable production of dolastatin 10 and/or symplostatin 1 in all analyzed specimens highlights the potential of these metabolites as reliable chemotaxonomic markers for rapid and robust identification of *C. penicillata.* Several specimens also produced the cytotoxic metabolite largazole (Taori et al. 2008). Interestingly, this compound was irregularly produced in specimens from the same geographic regions, suggesting that environmental factors might affect its production. At this point, it is unclear if largazole is produced by associated microbes or if the biosynthetic pathway encoding this secondary metabolite shows differential gene expression. It should also be noted that several secondary metabolites, including janthielamide A, kimbeamides A-C, and kimbelactone A (Nunnery et al. 2012), have also been reported from geographically localized collections of *C. penicillata* as verified by gene sequence data. However, none of these metabolites were detected in any of our *C. penicillata* specimens. Future genomic sequencing efforts will likely give better insights into the metabolic origins of these metabolites.

Taxonomic conclusions

The diagnosis of the new genus *Caldora* and a detailed description of *Caldora penicillata* follows:

Caldora Engene, Tronholm et V.J. Paul, **gen. nov.**

Diagnosis—Thalli habit from amorphic feathery or wispy to puffball clumps, sometimes mat-like. Coloration varies from red to orange and green. Each filament composed of entangled, thin, unbranched filaments; trichomes cylindrical, lacking heterocysts and other specialized cells, surrounded by barely visible polysaccharide sheaths. Cells barrel-shaped or sometimes cylindrical.

Etymology—From the Latin word *caldor*, meaning “heat, warmth” referring to the pantropical distribution of the genus.

Type species—*Caldora penicillata* Engene, Tronholm et V.J. Paul, comb. nov.

Basionym—*Phormidium penicillatum* Gomont, Bull. Soc. Bot. France, ser. 2, 15: LXXXVIII (1893).

Synonym—*Lyngbya penicilliformis* P. C. Silva in Silva, Basson & Moe. Univ. Calif. Publ. Bot. 79: 39 (1996).

Epitype—strain FK13-1, US 217964. Looe Key, Florida (24°35’400”N, 81°31’100”W), 10 June 2013, epizoic on corals and epilithic, 10 m depth, leg. N. Engene.

Caldora penicillata Engene, Tronholm et V.J. Paul, **comb. nov**.; Figures 1 and 2

Description—Thalli vary in shape from amorphic feathery, undulating or wispy to puffball-shaped clumps, often forming thick, undulating, digital projections; specimens up to 20 cm long; basal parts composed of empty polysaccharide sheaths, colorless or whitish, and mucilaginous; exterior composed of living, pigmented, filaments that vary in coloration such as red, orange, and green; filaments unbranched with individual sheaths, typically entangled into fasciculated bundles; filaments (4) 5-6 (7) μm in diameter; trichomes cylindrical, surrounded by thin (>0.5 μm), barely visible, polysaccharide sheaths; cells barrel-shaped or cylindrical (4) 5-6 (7) μm broad and (5) 6-8 (10) μm long, with no constrictions at cross-walls; terminal cells of filaments rounded; trichomes lack heterocysts or other specialized cells; producing secondary metabolites dolastatin 10 and/or symplostatin 1, and sometimes largazole; acc. nr. KF746590-KF746608 (16S rDNA), KF768777-KF768793 (16S-23S ITS regions).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ITS	internal transcribed spacer
PKS	polyketide synthases
NRPS	nonribosomal peptide synthetases

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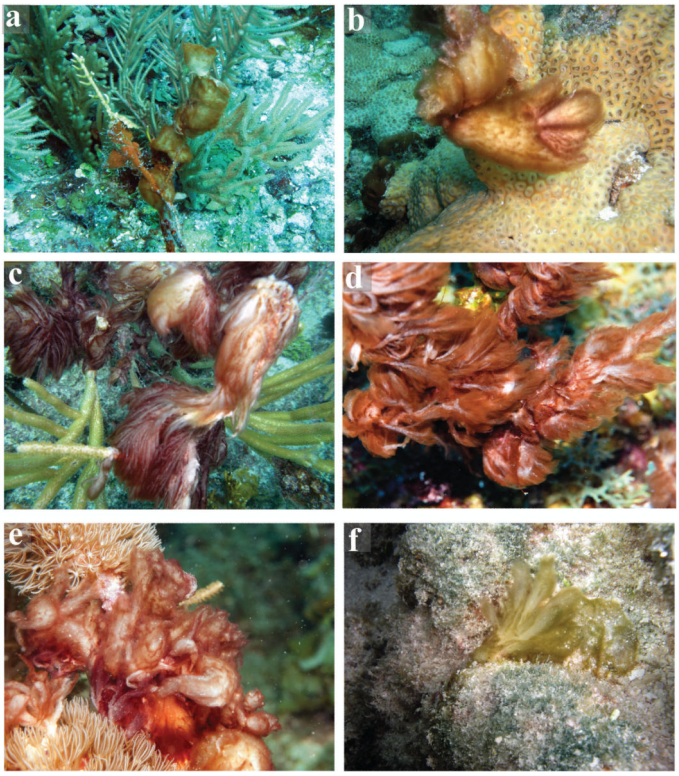

Fig. 1.

Environmental pictures illustrating morphological variability in shape and coloration between different *Caldora penicillata* specimens. Note external parts of specimens vary in color between reddish, greenish, to orange, while internal bases are always whitish or colorless. (a) Amorphic puffball-shaped specimen (BCBC11-25) growing on gorgonians of South Water Cay, Belize. (b) Small puffball-shaped tufts of *C. penicillata* attached to corals. (c) *C. penicillata* with feathery morphology and red coloration growing on shallow coral reef at Looe Key, FL (courtesy of B. Lapointe). (d) *C. penicillata* on coral reef at Carrie Bow Cay, Belize (courtesy of A. Wood). (e) *C. penicillata* on gorgonians of Miskito keys, Honduras (courtesy of Z. Foltz). (f) Greenish *C. penicillata* specimen (NAB11-29) on hard-bottom on a shallow-water reef of Lac Bay, Bonaire.

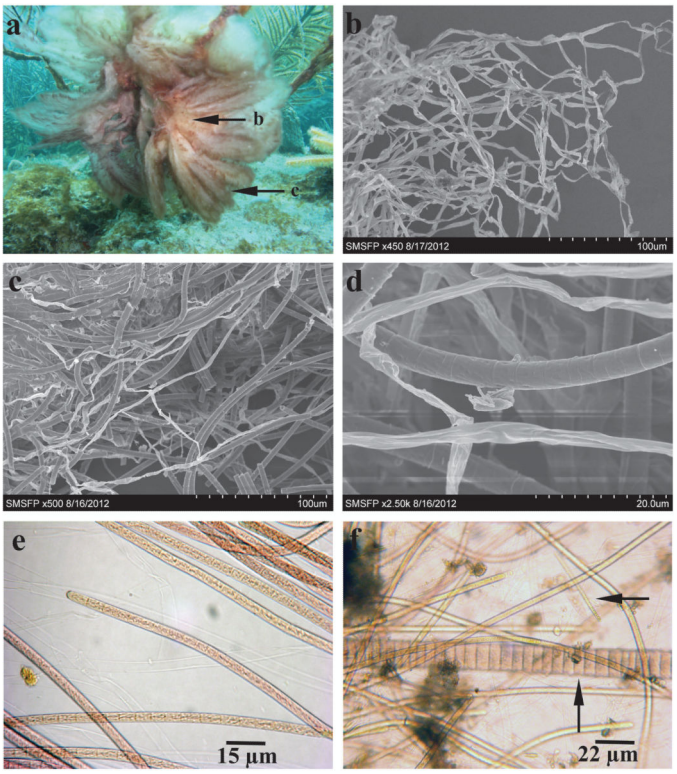

Fig. 2.

Morphological characterization of external and internal sections of *Caldora penicillata* strain FK12-26. (a) Underwater picture of *C. penicilla* attached to gorgonians at depth of 7-8 m at Looe Key, FL (courtesy of B. Lapointe). Arrows highlight sections used for scanning electron microscopy (SEM) imaging in b-d. (b) Whitish, mucilaginous interior bases composed of empty polysaccharide sheath material. (c) Exterior section of cyanobacterial thallus, predominantly containing live, pigmented filaments. (d) Higher resolution SEM image of *C. penicillata* filaments. (e) Light microscopic image of *C. penicillata* filaments. (f) Consortium of *C. penicillata* filaments mixed with other filamentous cyanobacteria. Scale bars: (b) 100 µm, (c) 100 µm, (d) 20 µm, (e) 15 µm, and (f) 22 µm.

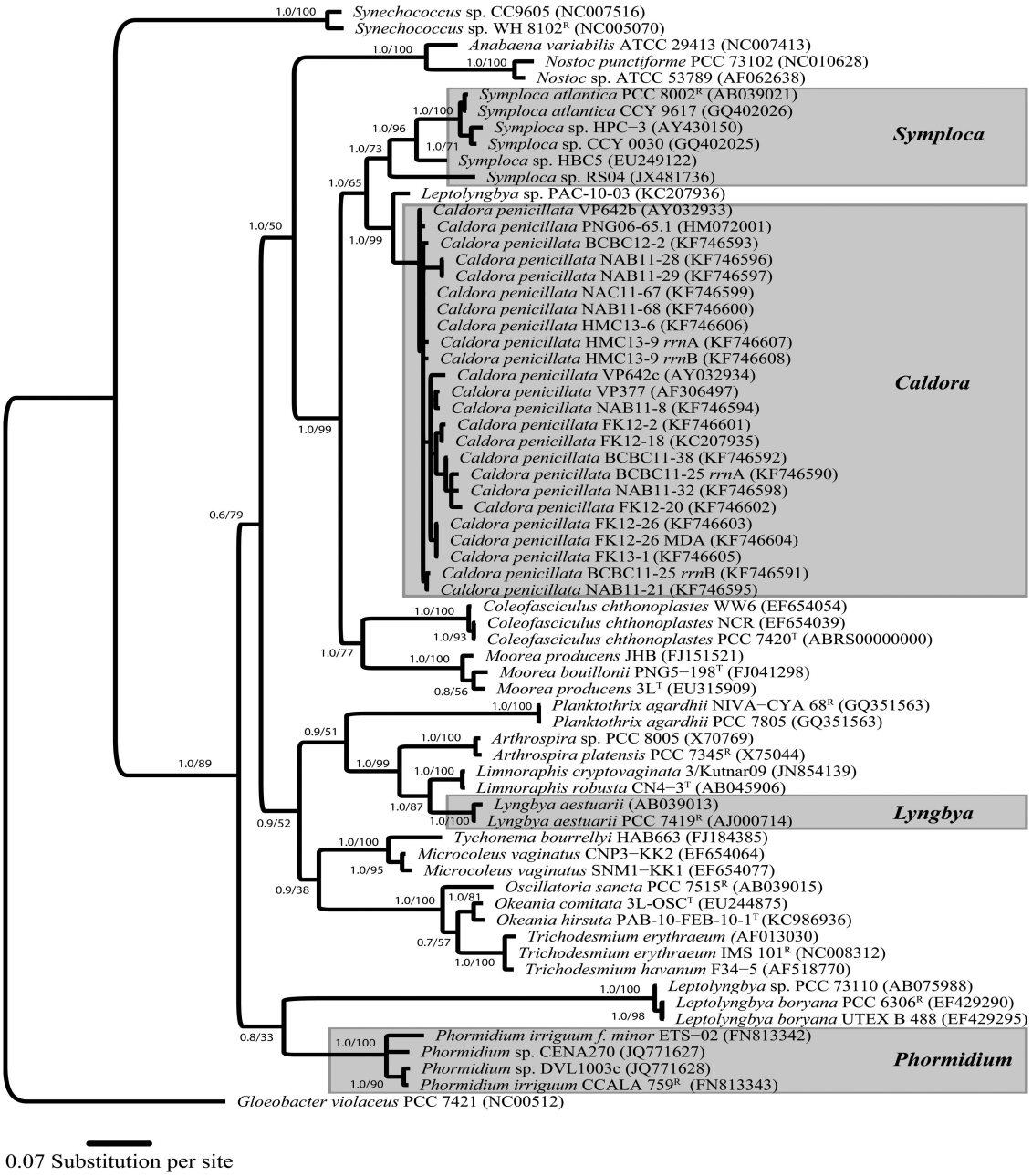

Fig. 3.

Evolutionary tree based on SSU (16S) rRNA gene sequences with *Caldora penicillata* as well as *Symploca*, *Lyngbya*, and *Phormidium* clades highlighted with gray boxes. Phylogenetic inferences were performed using Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. Support values are indicated at the nodes as posterior probability and bootstrap support. Reference strains are designated as (^{R}) and type strains as (^{T}). Unicellular *Gloeobacter violaceus* PCC 7421^{R} strain (GenBank acc. nr. NC005125) was outgroup. Specimens indicated as species or strain; GenBank accession numbers in brackets. Scale bar depicts 0.04 expected nucleotide substitutions per site using GTR+I+G substitution model.

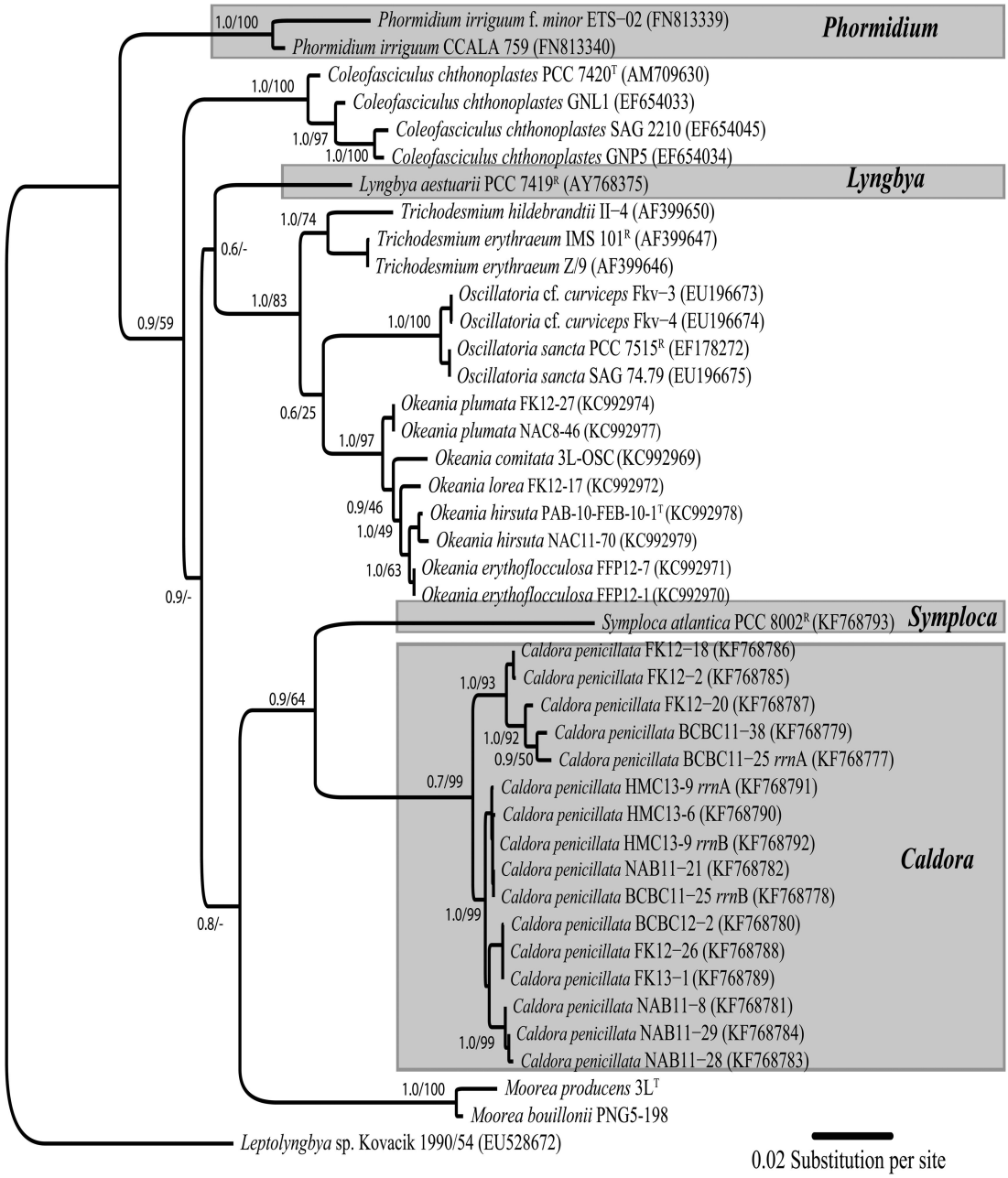

Fig. 4.

Phylogenetic inference of *Caldora penicillata* specimens based on 16S-23S internal transcribed spacer (ITS) regions highlighted in gray box, as well as *Symploca*, *Lyngbya*, and *Phormidium* clades. Support values indicated as posterior probability (BI) and bootstrap support (ML). Scale bar indicates 0.02 expected nucleotide substitutions per site using GTR +G substitution model.

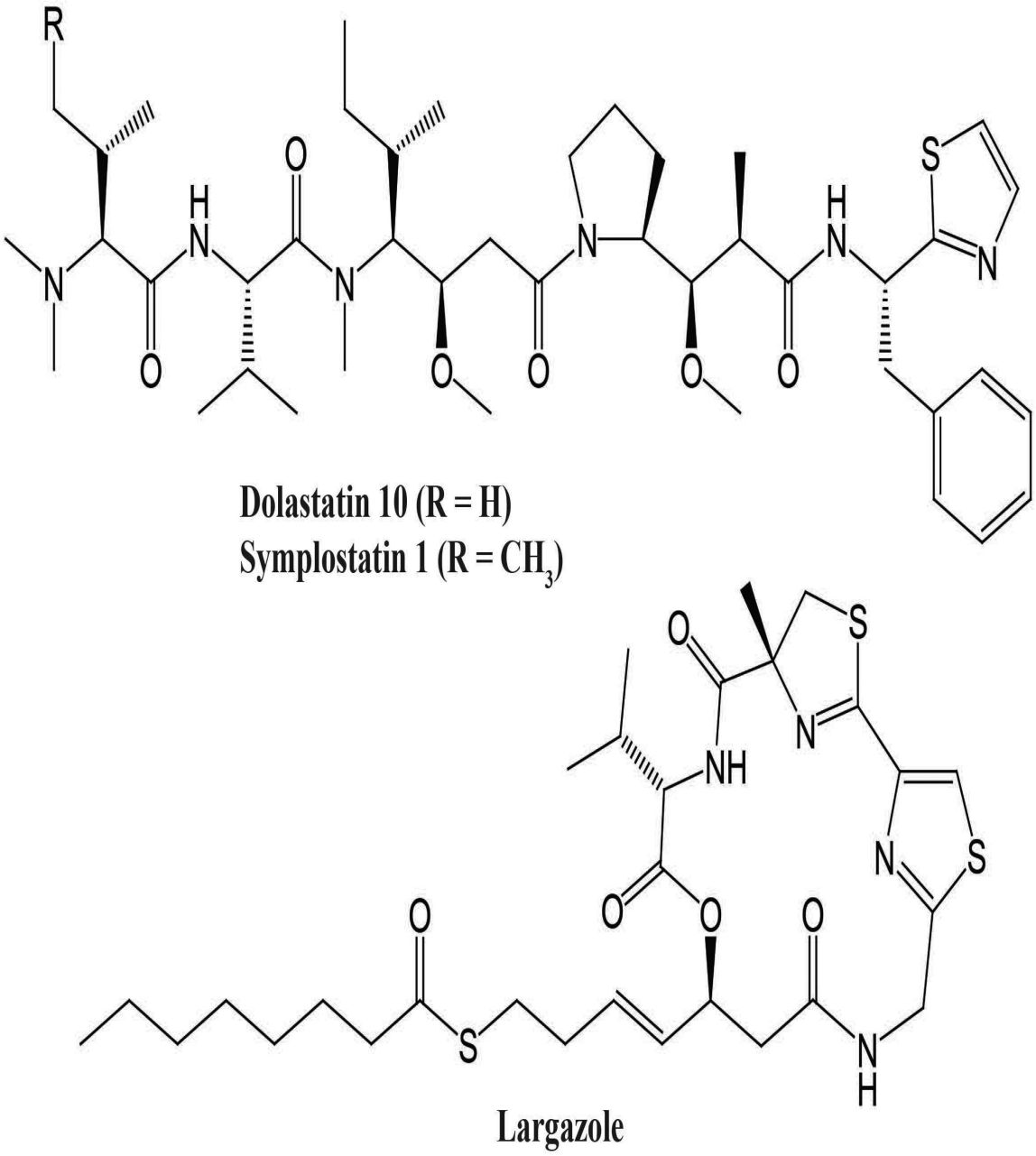

Fig. 5.

Molecular structures of bioactive secondary metabolites dolastatin 10, symplostatin 1, and largazole; distribution of compounds among *Caldora penicillata* specimens shown in Table 1.

Table 1

Production of bioactive secondary metabolites in *Caldora penicillata* specimens.

Strain	Geographic location	Dolastatin 10	Symplostatin 1	Largazole
BCBC11-25	Raph's wall, Belize	+	++	−
BCBC11-38	South Water Cay, Belize	++	+++	+
BCBC12-2	Carrie Bow Cay, Belize	+++	+	++
NAB11-8	Lac Bay, Bonaire	+++	−	+
NAB11-21	Playa Franz, Bonaire	++	+++	−
NAB11-28	Lac Bay, Bonaire	+++	+	++
NAB11-29	Lac Bay, Bonaire	+++	+	++
NAB11-32	Cali reef, Bonaire	+	−	−
NAC11-67	Porto Marie, Curaçao	++	+	−
NAC11-68	Porto Marie, Curaçao	++	+	−
FK12-2	Wonderland, Florida Keys	++	+	+
FK12-18	Big Pine Ledge, Florida Keys	+++	+	++
FK12-20	Wonderland, Florida Keys	++	++	+
FK12-26	Looe Key, Florida Keys	++	++	+
FK13-1	Looe Key, Florida Keys	++	++	+
HMC13-6	Becerros, Honduras	++	−	−
HMC13-9	Caratasca, Honduras	++	−	+

(+++) main secondary metabolite, (++) major secondary metabolite (+) minor secondary metabolite or trace compound estimated based on liquid chromatographic electrospray ionization mass spectrometer (LC-ESI-MS) abundance, (−) not detected.