Soft Coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) Species Diversity and Chemotypes

Satoe Aratake¹, Tomohiko Tomura¹, Seikoh Saitoh², Ryuoma Yokokura³, Yuichi Kawanishi², Ryuichi Shinjo⁴, James Davis Reimer⁵, Junichi Tanaka³, Hideaki Maekawa²

¹Graduate School of Science and Engineering, University of the Ryukyus, Nishihara, Okinawa, Japan, ²Center of Molecular Biosciences, Tropical Biosphere Research Center, University of the Ryukyus, Nishihara, Okinawa, Japan, ³Department of Chemistry, Biology, and Marine Science, University of the Ryukyus, Nishihara, Okinawa, Japan, ⁴Department of Physics and Earth Sciences, University of the Ryukyus, Nishihara, Okinawa, Japan, ⁵Rising Star Program, TRO-SIS, University of the Ryukyus, Nishihara, Okinawa, Japan

**Abstract**

Research on the soft coral genus *Sarcophyton* extends over a wide range of fields, including marine natural products and the isolation of a number of cembranoid diterpenes. However, it is still unknown how soft corals produce this diverse array of metabolites, and the relationship between soft coral diversity and cembranoid diterpene production is not clear. In order to understand this relationship, we examined *Sarcophyton* specimens from Okinawa, Japan, by utilizing three methods: morphological examination of sclerites, chemotype identification, and phylogenetic examination of both *Sarcophyton* (utilizing mitochondrial protein-coding genes MutS homolog: *msh1*) and their endosymbiotic *Symbiodinium* spp. (utilizing nuclear internal transcribed spacer of ribosomal DNA: ITS-rDNA). Chemotypes, molecular phylogenetic clades, and sclerites of *Sarcophyton trocheliophorum* specimens formed a clear and distinct group, but the relationships between chemotypes, molecular phylogenetic clade types and sclerites of the most common species, *Sarcophyton glaucum*, was not clear. *S. glaucum* was divided into four clades. A characteristic chemotype was observed within one phylogenetic clade of *S. glaucum*. Identities of symbiotic algae *Symbiodinium* spp. had no apparent relation to chemotypes of *Sarcophyton* spp. This study demonstrates that the complex results observed for *S. glaucum* are due to the incomplete and complex taxonomy of this species group. Our novel method of identification should help contribute to classification and taxonomic reassessment of this diverse soft coral genus.

**Citation:** Aratake S, Tomura T, Saitoh S, Yokokura R, Kawanishi Y, et al. (2012) Soft Coral *Sarcophyton* (Cnidaria: Anthozoa: Octocorallia) Species Diversity and Chemotypes. PLoS ONE 7(1): e30410. doi:10.1371/journal.pone.0030410

**Editor:** Dirk Steinke, Biodiversity Institute of Ontario - University of Guelph, Canada

**Received** September 8, 2011; **Accepted** December 15, 2011; **Published** January 17, 2012

**Copyright:** © 2012 Aratake et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** The project of “Research of insertion mechanism into the genome of movable gene with its transporter and development of the general gene introduction system” was supported by Ministry of Education, Culture, Sports, Science and Technology Japan. International Research Hub Project for Climate Change and Coral Reef/Island Dynamics, University of the Ryukyus. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: hidemae@comb.u-ryukyu.ac.jp

**Introduction**

Soft corals (Cnidaria: Anthozoa: Octocorallia) often equal or exceed the total coverage of scleractinian corals in coral reef ecosystems [1–4], and as dominant space-occupiers, important structural components of coral reef communities, and contributors to coral reef biomass [4,3], have been the subjects of biological studies since the nineteenth century.

The subclass Octocorallia includes soft corals, gorgonians, and sea pens. Most soft corals belong to the order Alcyonacea, which is comprised of the families Xenidiidae, Nephtheidae, and Alcyoniidae. The family Alcyoniidae contains the genera *Sinularia*, *Lobophytum* and *Sarcophyton*, and members of this group are among the dominant benthic organisms in the coral reefs in Okinawa and other Pacific Ocean areas [1,2,4,6]. *Sarcophyton* species are very hardy and are dominant in many coral reef areas. *Sarcophyton* species are characterized by a distinct sterile stalk, a broad, flared, smooth, mushroom-shaped top called a capitulum, and by the shape of their sclerites, which are found in the interior coenenchymal tissue of the colony.

Most soft coral classification and identification has traditionally been carried out by sclerite characterization. Verseveldt [7] revised the classification of *Sarcophyton* after gross morphological and microscopic examination of *Sarcophyton* species’ type specimens. Since the taxonomic revision by Verseveldt [7], who considered *Sarcophyton* to contain 35 valid species, an additional six species of *Sarcophyton* have been described [8–13].

Recently, McFadden et al. [14] reported on the utility of mitochondrial protein-coding gene MutS homolog (*msh1*) sequences for *Sarcophyton* and *Lobophytum* species identification. The study showed that within *Sarcophyton*, specimens initially identified as *Sarcophyton glaucum* by morphology could be divided into six very distinct genetic clades, suggesting that this morphologically heterogeneous species is actually a complex of cryptic species [14].

The soft coral genera *Lobophytum* and *Sarcophyton* are known to have many secondary metabolites [15–17]. Secondary metabolites in soft corals of *Sarcophyton* have been well characterized with the advancement of instrumental analyses over the past four decades. The soft coral egg-specific secondary metabolite PGA₂ and some diterpenes have been shown to cause contractions of soft coral polyps and the expulsion of eggs during spawning [18], and similar phenomenon by a secondary metabolite (sarcophytoxide) has been reported from *Sarcophyton glaucum* [19]. These examples indicate...
one reproductive isolation factor may be due to chemical signals, and that secondary metabolites may have important function. In addition, some metabolites are toxic and used in competition for space with scleractinian corals [20], and it is believed that octocorals release chemical substances into the water as a commonly used strategy to inhibit growth and survival of their neighbors [21]. Furthermore, it is known in Sarcophyton glaucum that secondary metabolites such as sarcophytol A cause allelopathic effects [19]. Thus, by focusing attention on secondary metabolites it may be possible to better understand the environmental role of soft corals in tropical waters.

One molecule, sarcophytol A, has attracted attention due to its antitumor promoting activity [22]. As sarcophytol A was discovered from Sarcophyton collected at Ishigaki Island, Okinawa, southern Japan, researchers have investigated the chemical activity and three-dimensional structure of the chemical [23–25]. Additionally, Koh et al. [26] investigated the distribution of sarcophytol A in Okinawa, and their study indicated that composition of cembranoids in Sarcophytol A in Okinawa, and their study indicated that Sarcophyton sarcophytol A is not related with morphologically identified species. Subsequently, it was found that two species, Sarcophyton trocheliophorum and Sarcophyton crassicaule, appeared to be the source organisms of sarcophytol A [27], and not only Sarcophyton glaucum as originally reported. During this study, it was also noted that Sarcophyton glaucum’s chemical content varied to a large degree and it was concluded there are at least nine chemotypes within S. glaucum [27].

Thus, it is difficult to conclusively identify the source Sarcophyton species of secondary metabolites from past studies’ data. Furthermore, secondary metabolites obtained from marine organisms are often derived from symbiotic algae and/or symbiotic bacteria [28,29]. Sarcophyton spp. contain endosymbiotic dinoflagellate zooxanthellae (Symbiodinium spp.), but no study has yet examined whether there are any relations between soft coral chemotype, genotype (molecular phylogenetic clade), and their Symbiodinium, despite many studies demonstrating the diversity of Symbiodinium spp. found within different coral reef invertebrate hosts [30,31].

In order to more fully understand the relationship between secondary metabolites and Sarcophyton species, in this study we examined specimens from Okinawa, Japan utilizing three methods; 1) novel morphological examination of sclerites, 2) chemotype identification, and 3) phylogenetic examination of both Sarcophyton (utilizing msh1 sequences) and their endosymbiotic Symbiodinium spp. (ITS-rDNA sequences). From our results, we examine the production pattern of secondary metabolites by Sarcophyton species, and theorize on the mechanism behind such varied secondary metabolite production in this soft coral genus.

Results

Molecular phylogeny using mitochondrial msh1 sequences

Most specimens’ sequences (n = 31) were found to clearly belong to a genus Sarcophyton clade, while three specimens (Sunabe 1, 10, and Mizugama 4) were classified into a mixed clade consisting of previously reported sequences from both Sarcophyton and Lobophytum specimens (Fig. 1). All sequences could be aligned unambiguously. Of the 31 “clear” Sarcophyton sequences, 13 sequences were identified as being from Sarcophyton trocheliophorum, 16 from Sarcophyton glaucum, one from Sarcophyton elegans, with the remaining one sequence not assignable to any previously reported species group. All putative Sarcophyton trocheliophorum specimens had exactly the same sequence regardless of sampling location. Sarcophyton glaucum has previously been divided into six phylogenetic clades A–F [14], and specimens from this study belonged to four of these clades: four sequences within clade B sensu McFadden et al. [14], one within C, five within D, and six within F.

Major compound analyses: Cembrene diterpenes

In total eight cembranoid diterpenes were identified (chemotypes 1–8) (Fig. 2). The abundance of each chemotype at each collection site is shown in Table 1. Among the detected chemotypes, 20 specimens of chemotype 1 (2S,7R,8R-sarcophytol A) were most abundant, followed by chemotype 2 (2S,7R,3R-sarcophytol A) and chemotype 3 (2S,7R,3R-isosarcophytol A). The cembranes found from the 34 specimens were as follows: chemotype 1 - 2S,7R,3R-sarcophytol A, 20 specimens (Sunabe 1, 5, 7, 10, 12, 15, 14, 15, 16, 18, 20, 21, 22, 23, Zampa 1, 5, 9, Mizugama 6, 8, 11); chemotype 2 - 2S,7R,3R-sarcophytol A, six specimens (Sunabe 1, 2, 5, 16, 17, Mizugama 7); chemotype 3 - 2S,7R,3R-isosarcophytol A, three specimens (Sunabe 6, 19, Mizugama 4); chemotype 4 - 7,8-epoxy-1,3,11-cembratrien-15-ol, one specimen (Sunabe 1); chemotype 5 - Sarcophytol A, one specimen (Mizugama 4); chemotype 6 - Embilide, two specimens (Zampa 6, 10); chemotype 7 - 7-hydroxy-1,3,11-cembratrien-20,3-olide, two specimens (Zampa 3, 4); chemotype 8 - 7,8,8-epoxy-1,3,11-cembratrien-15-olide (Sunabe 10).

All specimens of Sarcophyton trocheliophorum included the same chemotype, chemotype 1. Specimens of Sarcophyton glaucum clade F included two chemotypes, 6 and 7. Chemotypes 6 and 7 have lactone function and could be easily distinguished from the other chemotypes. Specimens of Sarcophyton glaucum clade B included only chemotype 1 with the exception of Sunabe 6, which had chemotype 3. Specimens of Sarcophyton glaucum clade D included chemotypes 1, 2, and 3. Though clades B and D included different chemotypes, those chemotypes had similar chemical isomerism, containing dihydro furan and epoxy groups. Sarcophyton glaucum clades B and D, and Sarcophyton trocheliophorum included similar chemotypes despite of clearly belonging to different clades.

The phylogenetic group classified to the “mixed” Sarcophyton-Lobophytum clade includes six chemotypes and in this clade no relationship between chemotype and molecular phylogenetic clade was apparent.

Morphological analyses

Sarcophyton spp. were examined morphologically by observing colony growth form and sclerite characters. We examined sclerites with a light microscope for species identification. Sclerite identification followed Verstepeldt [7], with “clubs” being club sclerites in the surface layer of the disc.

Sarcophyton colonies have a mushroom-shaped polypary consisting of a smooth and marginally folded disc, which projects beyond a clearly differentiated base or stalk (Fig. 3). Surface sclerites were usually long-handled clubs with poorly differentiated heads and fairly sparse, simple ornamentation. Sarcophyton glaucum and closely related Sarcophyton cinereum were identified by the presence of moderately ornamented clubs [7] though there was a range of development of the warts and in the sclerites’ length. Sarcophyton glaucum possessed clubs usually 0.10–0.17 mm in length, and rarely more than 0.35 mm in length, with the clubs having low, rounded processes. Sarcophyton cinereum possessed clubs usually 0.15–0.2 mm long, with the longest measuring 0.70 mm, and the clubs had warty heads. However, using existing identification keys [7], sclerite differences between Sarcophyton glaucum and Sarcophyton cinereum could not be determined.

After obtaining a phylogenetic tree based on msh1 sequences, we re-examined the sclerites with a scanning electron microscope (SEM; S-3500N: Hitachi High-Technologies). Sclerites from the capitulum surface of Sarcophyton were usually long-handled clubs
Chemotype Diversity of Sarcophyton
with poorly differentiated heads and fairly sparse and simple ornamentation. This was seen particularly in specimens of the most dominant species, *Sarcophyton glaucum*. According to the molecular phylogeny, *Sarcophyton glaucum* was comprised of four clades and we therefore compared the sclerites of clades B, D, F of *S. glaucum* (Fig. 4).

Clade D sclerites were longer than sclerites of clades B and F (nested ANOVA, length, D>F=B, P<0.05), and the warts were comparatively concentrated on the head. The sclerites of clade F were comparatively short and slim (nested ANOVA, width, D>B>F, P<0.05). These values are summarized in Table 2.

Analyses of covariance (ANCOVA) showed that the slope of regression line between length and width was statistically different between clade B and clade F (P<0.001), and between clade D and clade F (P<0.001), but not between clade B and clade D (P=0.78). However, the adjusted mean significantly differed between clade B and D (P<0.001) (Fig. 5).

*Sarcophyton trocheliophorum* could be easily identified by the presence of torch-shaped small sclerites in the surface of the capitulum (Fig. S1).

**Phylogenetic analysis of Symbiodinium ITS-rDNA**

Most obtained *Symbiodinium* ITS-rDNA sequences were found to match most closely with *Symbiodinium* clade C *sensu* LaJeunesse [30] with 96–100% identity (data from NCBI GenBank) and all novel sequences from this study belonged to clade C, consisting of numerous sequences closely related to type C1 *sensu* LaJeunesse [30]. Chemotypes were graphed onto the resulting *Symbiodinium* ITS-rDNA phylogenetic tree (Fig. S2), but no relation between *Symbiodinium* ITS-rDNA and chemotype was discernable.

**Discussion**

The molecular phylogenetic tree based on *msh1* revealed two large and very well-supported clades; one including only *Sarcophyton* and the other a mix of *Sarcophyton* and *Lobophtum*). Similar to a previous report on intergeneric diversity in *Sarcophyton* [14] clades of *Sarcophyton glaucum* were observed. Uniquely, correlations between *Sarcophyton* chemotypes and molecular phylogenetic clades were observed in this study.

*Sarcophyton glaucum* specimens formed at least four distinct subclades (B, C, D, and F). Clade F consisted of chemotypes 6 and 7, which contain emblide and an analogue encompassing a ε-lactone ring in their structure and therefore clade F likely retains a different set of biosynthetic pathways from the other *Sarcophyton glaucum* clades. Clade B consisted of chemotypes 1 and 3, clade C of chemotype 2, and clade D of chemotypes 1, 2, and 3. By examining the structures of these chemotypes by high performance liquid chromatography (HPLC) and Nuclear Magnetic Resonance...
(NMR), it was determined that the structures of chemotypes 1, 2, and 3 are isomeric. The structures of chemotype 1 and chemotype 2 were diastereomeric, while those of chemotype 3 and chemotypes 1 and 2 were structurally isomeric (or regioisomeric). These results mean that these clades likely share similar biosynthetic or oxidative enzymes involved in the production of cembranoids. Additionally, all three examined specimens (Sunabe 1, 10, Mizugama 4) belonging to the “mixed clade” of Sarcophyton and Lobophytum were also found to have mixed chemotypes (Sunabe 1 - chemotypes 1, 2 and 4; Sunabe 10 - chemotypes 1 and 8; Mizugama 4 - chemotypes 3 and 5). This situation could potentially be caused by interspecific hybridization, as previously suggested by McFadden et al. [14].

The current confused situation of Sarcophyton taxonomy is caused by the combination of three problems; 1) relatively few diagnostic morphological characters available for study in Sarcophyton, 2) our present lack of understanding of intraspecific variation of diagnostic morphological characters within this genus, and 3) a historical lack of taxonomic and ecological work on Sarcophyton [14]. Therefore, molecular phylogenetic analyses alone are not yet sufficient to clearly identify Sarcophyton specimens. However, our results suggest that detailed, morphometric examinations of sclerites may greatly aid in clarifying the meaning of molecular phylogenetic analyses of Sarcophyton species. The outcome of chemotype and statistical analyses of sclerites fully supported the molecular phylogenetic analyses’ results. In this study, sclerite examination detected differences between three Sarcophyton glaucum subclades. Therefore, we expect that further in-depth examinations may yield additional diagnostic morphological characters. Based on the all results of this study, we propose that clades B, D

**Table 1. Summary of field sites and chemotypes.**

| Sampling site | Sample size | Chemotype No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---------------|-------------|--------------|---|---|---|---|---|---|---|---|---|
| Sunabe        | 23          |              | 14| 5 | 2 | 1 | 1 |   |   |   |   |
| Zanpa         | 10          |              | 3 | 1 | 1 | 3 | 2 |   |   |   |   |
| Mizugama      | 11          |              | 3 | 1 | 2 | 1 |   |   |   |   |   |
| Total         | 44          |              | 20| 6 | 5 | 1 | 1 | 3 | 2 | 1 | 1 |

For chemotype information see Figure 2. Specimens obtained from three field sites (April 2007-November 2007) were collected at depths of 5–20 m (see Materials and Methods).

doi:10.1371/journal.pone.0030410.t001

Figure 3. In situ photographs of colonies of Sarcophyton. A. Sarcophyton trocheliophorum, Sunabe 12. B. Sarcophyton glaucum clade B, Sunabe 13. C. Sarcophyton glaucum clade C, Mizugama 7. D. Sarcophyton glaucum clade D, Sunabe 17. E. Sarcophyton glaucum clade F, Zanpa 3. F. Sarcophyton ehrenbergii mixed clade, Sunabe 1.
doi:10.1371/journal.pone.0030410.g003
and F of *Sarcophyton glaucum* should be formally classified into independent species in the future.

All *Sarcophyton* specimens contained *Symbiodinium* clade C sensu LaJeunesse [30], belonging to closely related ITS-rDNA types. However, in total eight chemotypes were found within *Sarcophyton* specimens, and there was no meaningful correlation between *Symbiodinium* and chemotype. Further support can be found from the azooxanthellate soft coral genus *Dendronephthya* soft coral, in which several types of diterpenes are found [32] despite the lack of *Symbiodinium*. Thus, we believe it is unlikely *Symbiodinium* is involved in the synthesis of the chemical examined in this study.

It is noteworthy that some interspecific, different clades have similar secondary metabolites. We suspect that the secondary metabolites of *Sarcophyton* may have some kind of relationship with their environment although this was not examined in this study. By focusing on the relationship between chemotype and sampling site, some indicative patterns are apparent. Chemotypes 6 and 7 were only found at Zampa regardless of *Sarcophyton* clade. Clades B, C, D of *Sarcophyton glaucum* were not collected in Zampa, and clade F was not obtained in Sunabe. At Mizugama, located between Sunabe and Zampa on the west coast of Okinawa Island in 2007 (Fig. 6). No specific permits were required for the described field studies. The three locations examined in this study are popular public diving spots and are not privately owned, and are not in a protected area. This study did not involve any endangered or protected species. The numbers of specimens from each collection site were: 23 from Sunabe, 10 from Zampa, and 11 from Mizugama, respectively. Specimens were designated Sunabe 1 to Sunabe 23, Zampa 1 to Zampa 10, Mizugama 1 to Mizugama 11. Specimens were separated into subsamples for chemical analyses, morphological analyses, and genetic analyses. Genetic subsamples were fixed in 70–99% cold ethanol and kept at −30°C until DNA extraction.

**DNA extraction**

Each genetic subsample was cut into small pieces of approximately 20 mg, and treated with 20 mL proteinase K in 180 mL 100% cold ethanol and kept at −30°C until DNA extraction.

**PCR analyses of mitochondrial msh1: Sarcophyton**

The 5′ end of the mitochondrial *msh1* gene was amplified by PCR using the primers ND4F599F (5′-GCGATTATGCTTACGTTTTAG-3′) and Mat-3458R (5′-TSGAGCCTAAAAAGG-CACCTCC-3′) [14]. The PCR reaction used 20 pmol of each primer, 4 mL of dNTP mix, 0.25 mL of Taq polymerase, 5 mL of Taq Buffer, and 1 mL of raw genomic DNA. Several samples were cloned into the pCR2.1 vector of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). All primers were anchored in an adjacent mitochondrial gene to prevent amplification of genes from nuclear or symbiont (*Symbiodinium* spp.) genomes. PCR products were sequenced using an ABI PRISM Big Dye Terminator cycle sequencing kit Ver. 3.1 (Applied Biosystems, Foster City, CA) with a DNA sequences system (Model 3100 or 3130, Applied Biosystems).

**Materials and Methods**

**Collection of specimens**

*Sarcophyton* specimens were collected from a depth range of 5–20 m by SCUBA at three locations (Sunabe 26′19′N; 127°44′E, Zampa 26′26′N; 127°42′E, and Mizugama 26′21′N; 127°44′E) on the west coast of Okinawa Island in 2007 (Fig. 6). No specific permits were required for the described field studies. The three locations examined in this study are popular public diving spots and are not privately owned, and are not in a protected area. This study did not involve any endangered or protected species. The numbers of specimens from each collection site were: 23 from Sunabe, 10 from Zampa, and 11 from Mizugama, respectively. Specimens were designated Sunabe 1 to Sunabe 23, Zampa 1 to Zampa 10, Mizugama 1 to Mizugama 11. Specimens were separated into subsamples for chemical analyses, morphological analyses, and genetic analyses. Genetic subsamples were fixed in 70–99% cold ethanol and kept at −30°C until DNA extraction.

**Table 2. Mean and standard deviation (SD) of length and width (mm) from sclerites of each phylogenetic clade.**

| Clade | n  | Length     | Width      |
|-------|----|------------|------------|
|       |    | mean±SD*   | mean±SD*   |
| Clade B | 4  | 0.2289±0.0602*a | 0.0282±0.0042*a |
| Clade D | 5  | 0.3146±0.0820*b | 0.0348±0.0068*b |
| Clade F | 5  | 0.2096±0.0739*a | 0.0238±0.0051*a |

*Values were calculated based on pooled data.

Letters following SD values indicate different statistical significances in nested ANOVA.

Each specimen had 100 sclerites examined.

doi:10.1371/journal.pone.0030410.g004

---

*Figure 4. Sclerites of *Sarcophyton glaucum* clades B, D and F, and their averages length and width. All sclerites shown are surface sclerites; Clade B obtained from specimens Sunabe 6 and Sunabe 13; Clade D from Sunabe 2, Sunabe 19; Clade F from Zampa 3, Mizugama 5, Mizugama 9. Images were taken using a scanning electron microscope.*

doi:10.1371/journal.pone.0030410.t002

---
PCR analyses of ITS-rDNA: *Symbiodinium*

The internal transcribed spacer of ribosomal DNA (ITS-rDNA) was amplified using primers ITS-4 (5'-TCCTCCGCTTATGATATGC-3') [37] and zooxanthellae-specific zITSf (5'-CCGCTTAATTACGGACTGACGATG-3') [38,39]. The purified PCR-amplified DNA fragments were cloned into the pCR2.1 vector of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Several clones of ITS-1 - 5.8S rDNA - ITS-2 from each site were sequenced using an ABI PRISM Big Dye Terminator cycle sequencing kit Ver. 3.1 (Applied Biosystems, Foster City, CA) with a DNA sequencing system (Model 3100 or 3130, Applied Biosystems).

Novel sequences from this study are available at GenBank under the accession numbers AB665446-AB665479 (*msh1*) and AB665603-AB665723 (ITS-rDNA) (Table S3).

Phylogenetic analyses

Nucleotide sequences were assembled and proofread using Sequence Scanner v1.0 software, and aligned using MEGA 4 [40]. Members of the genera *Sinularia* and *Dampia* were included as outgroup taxa of the *msh1* alignment (Table S1). For the *Symbiodinium* ITS-rDNA alignment, *Symbiodinium* sp. 1591 type C91 (GenBank accession number AJ291519) [41] was included as the outgroup (Table S2). Consequently, two alignments were generated, one of soft coral *msh1* sequences (34 taxa; 735 base pairs) and one of *Symbiodinium* ITS-rDNA sequences (121 taxa; 704 base pairs). Both alignments are available upon request from the corresponding author. The datasets of *msh1* alignments and ITS-rDNA alignments were separately subjected to maximum-likelihood (ML) and neighbor-joining (NJ) [42] analyses. In addition, phylogenetic trees of *msh1* were obtained using MrBayes and maximum parsimony method (MP) analyses. ML analyses were performed using PhyML online web server [43]. PhyML was performed using an input tree generated by BIONJ with the general time-reversible model [44] of nucleotide substitution incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR+I+Γ). The proportion of invariable sites, a discrete gamma distribution and base frequencies of the model were estimated from the dataset. PhyML bootstrap trees (1000 replicates) were constructed using the same parameters as the individual ML trees. The NJ tree was constructed using maximum composite likelihood model. Support for NJ branches was tested by bootstrap analysis of 1000 replicates. The NJ and MP methods were conducted using MEGA 4. Bayesian phylogenetic analyses were conducted using MrBayes 3.1.2 [45] with a GTR+I+Γ model run for 10,000,000 generations with sampling of trees at 100-generation intervals (burn-in = 1500 generations).

Major compound identification

Specimens for chemical analyses were extracted with acetone two times, and the acetone solution was then filtered and concentrated under vacuum, with the residual material was

---

**Figure 5. Scatter plot and regression line of length and width of sclerites of each *Sarcophyton glaucum* clade.** Horizontal axis: width of sclerites, vertical axis: length of sclerites. doi:10.1371/journal.pone.0030410.g005
partitioned between CH$_2$Cl$_2$ and water. The lipophilic portion was subjected for chemical analyses. Each CH$_2$Cl$_2$ extract was analyzed first with thin layer chromatography (TLC) and $^1$H NMR (Nuclear Magnetic Resonance) to examine whether a dominant marker cembrane existed or not. Then, the presence of major cembranol was confirmed qualitatively by high performance liquid chromatography (HPLC) equipped with a photodiode array detector using an ODS column with linear gradient elution profile. $^1$H and $^{13}$C NMR spectra were taken on a Jeol A-500 by dissolving extracts or pure compound in CDCl$_3$ using tetramethylsilane as an internal standard.

Structure identification of cembrane diterpenes

Observed compounds (designated compounds 1–8) were identified by comparing NMR spectral data with those previously published after obtaining nearly pure material with separation on column, TLC, or HPLC.

Analysis of sclerites

From the capitulum of each specimen, a small portion (0.4 cm$^2$) was removed and treated with 10% sodium hypochlorite. After removal of excess hypochlorite with water, sclerites were observed under light microscope at $\times$400 magnification. Subsequently, spicules for Sarcophyton glaucum and Sarcophyton trocheliophorum were observed with a scanning electron microscope (SEM; S-3500N: Hitachi High-Technologies) to examine sclerite size and potential morphological differences between different specimens. For each specimen, morphological traits (length and center width) of sclerites (n = 100) were measured with ImageJ 1.44 software (NIH).

A nested ANOVA was used to examine the effect of genetic clade on morphological traits (length or width of sclerites). Firstly, a nested ANOVA was conducted using data from all clades. Secondary, if the effect of clade was significant ($P<0.05$), nested ANOVA was performed for each of all possible pairs of clades (i.e. clade F vs B, B vs D, or D vs F). P-values from the analyses were adjusted with Bonferroni correction. Analyses of covariance (ANCOVA) were performed to examine the difference in ratio of length and width of sclerites among clades (Sarcophyton glaucum B, D, F). We evaluated discrepancies in P values for each data set, considering significant differences at $P$ values of 0.001. Statistical analysis was performed using R software (version 2.12.0; R Foundation for Statistical Computing, Vienna, Austria).

Supporting Information

Figure S1 Sclerites of Sarcophyton trocheliophorum. Surface sclerites of Sunabe 7 are shown. Images were taken using a scanning electron microscope.

(TIF)

Figure S2 Phylogenetic analyses of Symbiodinium spp. Neighbor-joining (NJ) tree of an alignment of nuclear internal transcribed spacer of ribosomal DNA (ITS rDNA) sequences of
symbiotic *Symbiodinium* dinoflagellates (clade C) associated with genus *Sarcophyton*. Values at branches represent NJ and maximum likelihood (ML) bootstrap values, respectively. (~) indicates bootstrap values <50%. Sequences in bold without GenBank accession numbers are ITS-rDNA sequences obtained in this study. Colored dots indicate chemotypes as in Figure 2. (TIF)

**Table S1** List of mitochondrial protein-coding gene MutS homolog *msh1* sequences from previous studies used in phylogenetic analyses in the present study. Species, GenBank accession numbers, geographic origin, latitude and longitude, and collection date are also shown. (DOC)

**Table S2** List of internal transcribed spacer of ribosomal DNA (ITS-rDNA) sequences from previous studies used in phylogenetic analyses in the present study. Species, GenBank accession numbers, geographic origin, and host species are shown. (DOC)

**References**

1. Dimen ZD (1983) Patterns in the distribution of soft corals across the central Great Barrier Reef. Coral Reefs 1: 229-236.

2. Fabricius KE (1997) Soft coral abundance on the central Great Barrier Reef: effects of Acanthaster planci, space availability, and aspects of the physical environment. Coral Reefs 16: 159-167.

3. Riegler B, Schleyer MH, Cook PJ, Branch GM (1995) Structure of Africa’s southernmost coral communities. Bull Mar Sci 56: 676-691.

4. Tursch B, Tursch A (1982) The soft coral community on a sheltered reef quadrat at Laing Island (Papua New Guinea). Mar Biol 68: 321-332.

5. McFadden CR, Sanchez JA, France SG (2010) Molecular phylogenetic insights into the evolution of Octocorallia: a review. Int Comp Biol 50: 389-410.

6. Channathakul T, Chansang H, Watanasit S (2010) Soft coral (Gnidaria: Alcyonacea) distribution patterns in Thai water. Zool Stud 49: 72-84.

7. Versavele J (1982) A revision of the genus *Sarcophyton* Lesse (Octocorallia, Alcyonacea). Zool Verhanden (Leiden) 192: 1-91, 24pl.

8. Alderslade P (1993) A redescription of *Alcyonium agaricoides* Simpson with a generic placement in *Sarcophyton* (Coelenterata: Octocorallia). Precious Corals Octocorals Reefs 1: 20-29.

9. Alderslade P, Shirwaiker R (1991) New species of soft corals (Coelenterata: Octocorallia) from the Laccadive Archipelago. Beagle Rec North Territory Mus Sci 18: 189-233.

10. Benayahu Y, Perkol-Finkel S (2004) Soft corals (Octocorallia: Alcyonacea) from southern Taiwan. Zool Stud 43: 537-543.

11. Benayahu Y, van Ofwegen LP (2009) New species of *Sarcophyton* and *Labophyton* (Octocorallia: Alcyonacea) from Hong Kong. Zool Med Leiden 83: 853-876.

12. Li C (1984) Studies on the *Alcyonium* agaricoides complex. Zool Verhandel (Leiden) 192: 1-91, 24pl.

13. Versavele J, Benayahu Y (1983) On two old and fourteen new species of Alcyonacea (Coelenterata, Octocorallia) from the Red Sea. Zool Verhanden (Leiden) 208: 3-33, 7pl.

14. McFadden CS, Alderslade P, van Ofwegen LP, Johnsen H, Rusmevichientong A (2006) Phylogenetic relationships within the tropical soft coral genera *Sarcophyton* and *Labophyton* (Anthozoa, Octocorallia). Invertebr Biol 125: 298-305.

15. Blunt JW, Copp BR, Munro MHG, Northcote PT, Primper MR (2004) Marine natural products. Nat Prod Rep 21: 1-49.

16. Gross H, Kinug GM (2006) Terpenoids from marine organisms: unique structures and their pharmaceutical potential. Phytochemistry Rev 5: 115-141.

17. Hegay MEF, Su-J H, Sung P-J, Shyu S-H, H-J (2011) Cembraneoids with 3,14-Ether Linkage and a secocebrain with bistearinhydroxy from the Dongsha Atoll soft coral *Labophyton* sp. Mar Drugs 9: 1243-1253.

18. Pap MA, Czapka MF, Carlile CH, Lawn I, Coll JC (1989) Stimulation of the conformations in the polyps of the soft coral *Xenia elongata* by compounds extracted from other Alcyonacea soft corals. Comp Biochem Physiol 94C: 677-681.

19. Fleury BG, Coll JC, Sammarco PW (2006) Competition (secondary) metabolites in a soft coral: sex-specific variability, inter-clonal variability, and competition. Mar Ecol 27: 204-218.

20. Sammarco PW, Coll JC, Barre S, Willis B (1983) Competitive strategies of soft corals (Coelenterata: Octocorallia): Allopathic effects on selected scleractinian corals. Coral Reefs 2: 173-178.

21. Coll JC, Bowden BF, Tapiaios DM, Dunlap WC (1982) In situ isolation of allelochemicals released from soft corals (Coelenterata: Octocorallia): a totally submersible sampling apparatus. J Exp Mar Biol Ecol 60: 293-299.

**Table S3** Collection information for specimens included in molecular phylogenetic clade. (DOC)

**Acknowledgments**

We thank Drs. H. Oku, Ph. D. Yehuda Benayahu, A. Yamada, M. Hojo, Y. Nakajima, G. Tokuda and Ms. K. Yamada for their help and useful comments. The University of the Ryukyus approved this study. Two anonymous reviewers greatly improved the manuscript.

**Author Contributions**

Conceived and designed the experiments: SA SS JDR JT HM. Performed the experiments: SA TT RY. Analyzed the data: SA SS YK JDR. Contributed reagents/materials/analysis tools: JT RS HM. Wrote the paper: SA JDR HM.
40. Takuma K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.
41. Pawlowski J, Holzmann M, Fahrni JF, Pochon X, Lee JJ (2001) Molecular identification of algal endosymbionts in large miliolid Foraminifera: 2. dinoflagellates. J Eukaryot Microbiol 48: 368–73.
42. Saitou N, Nei M (1987) The Neighbor-Joining method - a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425.
43. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, et al. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. System Biol 59: 307–21.
44. Rodriguez F, Oliver JL, Marin A, Medina JR (1990) The general stochastic model of nucleotide substitution. J Theor Biol 142: 485–501.
45. Ronquist F, Huelsenbeck JP (2003) Bayesian phylogenetic inference under mixed models. Bioinformatics (Oxford) 19: 1572–1574.