Internal Transcribed Spacer 2 (ITS2) Variation in the Gorgonian Coral *Pseudopterogorgia bipinnata* in Belize and Panama

Daniel Dorado and Juan A. Sánchez

ABSTRACT. One of the most intriguing aspects of molecular evolution is the concerted evolution of ribosomal genes, yet the presence of intragenomic rDNA variants is still not well understood. We studied the intragenomic variation of the internal transcribed spacer 2 (ITS2, rDNA) in the gorgonian coral *Pseudopterogorgia bipinnata* (Gorgoniidae: Octocorallia) using a combined approach of denaturing gradient gel electrophoresis (DGGE), DNA sequencing, and RNA secondary structure prediction. We examined intragenomic variants of colonies from Carrie Bow Cay (Belize) and Bocas del Toro (Panama). Despite frequent intragenomic ITS2 variation in *P. bipinnata*, predicted RNA secondary structures exhibited no signs of including pseudogenes and comprised functional copies. Given the low divergence among the ITS2 sequences recovered from DGGE gels, intragenomic variation was restricted to a few mutations that did not compromise the functionality of the ITS2 secondary structure. The presence of common ITS2 intragenomic variants at two distant populations raises new questions such as whether sharing similar copies can be the product of gene flow. Regardless of the limited number of individuals analyzed in this study, the method used here, excising bands from DGGE gels for further amplification and sequencing, examined the reliability of the technique to separate intragenomic variants with up to one nucleotide difference. Studying the intragenomic variation of ITS2 has potential to provide us with information on recent population events such as introgressive hybridization.

INTRODUCTION

Ribosomal DNA (rDNA) intragenomic variation has puzzled molecular systematists and ecologists during the past few years. The rDNA is a multigene family arranged in tandem repeats, frequently achieving several hundreds of repetitions per chromosome. Each repetition is composed of three ribosomal subunits (18s, 5.8s, and 28s), separated by two internal transcribed spacers (ITS1 and ITS2, or ITSs), an external transcribed spacer (ETS), and the non-transcribed intergenic spacers, IGS. The ITS1 and ITS2 spacers form secondary structures that are crucial for ribosomal maturation as well as important for the maturation of the rRNA (Coté and Peculis, 2001). The ITSs are known to have conserved core structures throughout the metazoans (see reviews in Coleman, 2003; Schultz et al., 2005). Changes in the ITS secondary structure are known to produce inhibition of the maturation of rRNA as a consequence of

Daniel Dorado and Juan A. Sánchez, Departamento de Ciencias Biológicas–Facultad de Ciencias, Laboratorio de Biología Molecular Marina–BIOMMAR, Universidad de los Andes, P.O. Box 4976, Bogotá, Colombia. Corresponding author: J. Sánchez (juansanc@uniandes.edu.co). *Manuscript received 9 June 2008; accepted 20 April 2009.*

coevolution between RNA secondary structures and the processing molecular machinery responsible for its removal (Van Nues et al., 1995). As multicopy genes, the rDNA is assumed to evolve via concerted evolution, resulting in the homogenization of the sequences throughout the genome (Harris and Crandall, 2000; Hillis and Davis, 1988), that is, homogenization of copies through unequal crossing-over and gene conversion processes (Liao, 2000). However, variations within individuals have been reported primarily as a result of slow concerted evolution (Harris and Crandall, 2000; Coté and Peculis, 2001), hybridization, or the presence of pseudogenes (Marquez et al., 2003; Harpke and Peterson, 2006). The latter can appear because of the presence of highly divergent rDNA types in different chromosomes (Arnheim et al., 1980), which retain ancestral rDNA polymorphisms for long periods of time (Marquez et al., 2003). Hybridization phenomena between species per se could increase the rDNA diversity in an individual, but as an additional consequence could result in silencing some rDNA loci by chromatin modifications in a nucleolar dominance process (Chen et al., 1998; Frieman et al., 1999; Muir et al., 2001), which can drive some rDNA loci by neutral selection toward pseudogenes (Muir et al., 2001). However, the presence of ITS2 intragenomic variants is a phenomenon that we do not clearly understand.

Pseudopterogorgia bipinnata Pallas is one of the most abundant shallow-water gorgonian corals in the Caribbean Sea (Bayer, 1961; Sánchez et al., 1997). This species has two particular characteristics: it exhibits large phenotypic plasticity along the depth–wave exposure gradient, and it presents clear intragenomic variation in the ITS2 sequence (Sánchez et al., 2007). Consequently, *P. bipinnata* constitutes an appropriate model species to study the nature and genetics of ribosomal intragenomic variation. In this study we had two main objectives: (1) to isolate sequences of intragenomic ITS2 variants in *P. bipinnata* from populations at Belize and Panama and (2) to examine if intragenomic ITS2 variants were functional copies using predicted RNA secondary structures.

MATERIALS AND METHODS

Samples from *Pseudopterogorgia bipinnata* colonies were obtained by scuba diving at Carrie Bow Cay (n = 27), Belize, and Cristobal Island (n = 11), Bocas del Toro, Panama. A few *P. bipinnata* from the Bahamas (San Salvador) and Colombia (Bancos de Salmedina, Cartagena), as well as a sequence of *Gorgonia mariae*, were chosen as outgroups. However, there was no a priori information on the genetic distance between western and eastern Caribbean populations. Total DNA was extracted using a cetyltrimethylammonium bromide (CTAB), proteinase K, phenol–chloroform–isoamyl alcohol extraction method (Coffroth et al., 1992); DNA was resuspended and conserved in TE buffer at −70°C; DNA quality was checked in agarose (1%) electrophoresis at 80 V for 30 min. Using the best DNA extraction quality, primers 5.8s 5′-AGCATGTCTGTCTGAGTGTTGG-3′ and 28s 5′-GGGTAATCTTGCCTGATCTGAG-3′, designed by Aguilar and Sánchez (2007), were used for the ITS2 amplification. Conditions for polymerase chain reaction (PCR) were as follows: an initial denaturizing step of 2 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 56.8°C, and 1 min at 72°C; and a final extension step of 2 min at 72°C; using 1 unit Taq polymerase (Invitrogen), 3.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs; Biorad Mix), 0.15 μM primers (each), and 4 μL DNA (dilution 1/50) in 20 μL as the final volume. The amplification was standardized with an efficiency of 95%. PCR reactions were screened in denaturing gradient gel electrophoresis (DGGE) containing 8% polyacrylamide, 1 × TAE buffer, and a linear urea-formamide denaturing gradient from 45% to 80%. The gels were pre-run at 60°C and 90 V for 30 min, followed by electrophoresis at 60°C and 90 V for 13 h. Gels were stained with ethidium bromide during 15 min and visualized using a BIORAD Chemidoc system. DGGE separates DNA fragments not only by the fragment size but also by the DNA sequence, where GC-richer sequences migrate further independently of small differences in size (Figure 1). All reactions were conducted without a CG-clamp in the primers, which is a 40 bp GC-rich sequence added before the 5′-primer that adds an additional denaturing domain allowing further migration of the DNA before denaturing (LaJeunesse and Pinzon, 2007). In the case of gorgonian corals, there was no need for the GC-clamp owing to the great migration in the DGGE of gorgonian ITS2 sequences, which avoided the problems involved with PCR reaction tailed primers. Bands visualized in the DGGE gel were excised using sterilized micropipette tips in the Bio-Rad Chemidoc system and placed in 0.5 mL tubes with 100 μL sterilized double distilled water. The tubes were incubated in a shaker at room temperature for 24 h at 150 rpm. Each band extract was collected in a 0.5 mL tube and the DNA was precipitated with 300 μL cold absolute ethanol; tubes were placed at −20°C for 24 h and then centrifuged at 13,000 rpm for 30 min; the supernatant was discarded,

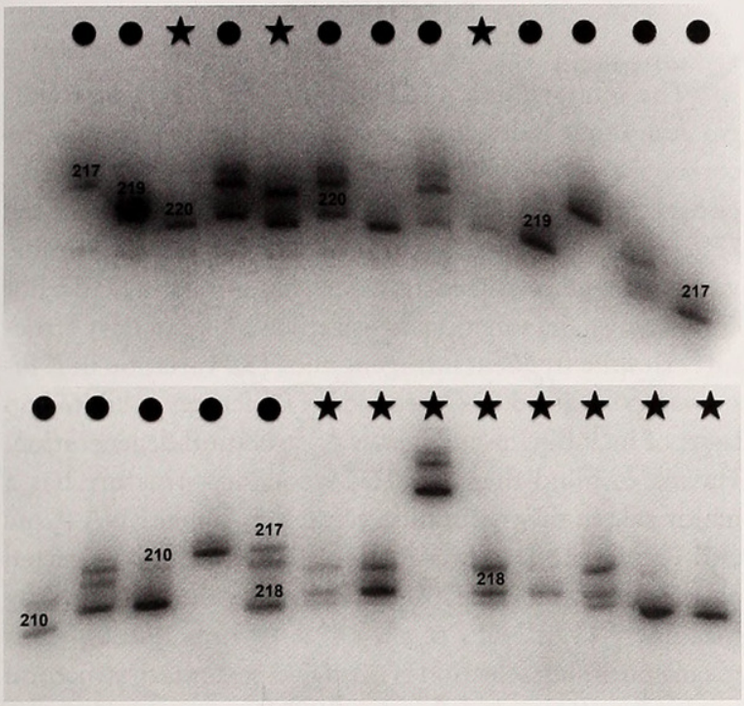

FIGURE 1. Runs (2) of internal transcribed spacer 2 (ITS2) denaturing gradient gel electrophoresis (DGGE) banding patterns from *Pseudopterogorgia bipinnata* colonies from Panama (Bocas del Toro; stars) and Belize (Carrie Bow Cay; circles). Numbers correspond to the sequence size when available. The gels have a common artifact in the form of a "smile" (more accentuated in the upper gel), where lateral wells tend to migrate slightly further because of the pressure acting on the gel edges.

and the pellet was dried and resuspended in 15 μL sterilized double distilled water.

Reamplification of bands was conducted using PCR as just described, using the same set of primers, except that DNA was used without dilution and the annealing temperature was raised a few degrees to increase specificity. Purification of PCR products for sequencing was performed by the Exo-SAP (Exonuclease 1 and shrimp alkaline phosphatase) method using 1 unit Exonuclease, 0.2 units shrimp alkaline phosphatase, and 2 μL SAP buffer 10× per 20 μL in a 0.2 mL tube. Reactions were held at 37°C for 1 h and at 80°C for 15 min. Sequencing reactions were performed with the BigDye 3.1 system according to the manufacturer's instructions (Applied Biosystems) and sequenced in a capillary electrophoresis automated sequencer (ABI310). Each sample was sequenced with forward and reverse primers. The consensus sequences were obtained by assembling the two complementary electrophenograms in Sequencer 4.7 software.

Secondary structures of all sequences were obtained by reconstructing by comparison via Pairwise Alignment (Bioedit) with previously reported structures in octocorals (Aguilar and Sánchez, 2007). The sequences were then submitted with a few constraints and restrictions in MFOLD at a default temperature of 37°C (Zuker, 2003). Constraints force bases to be double stranded whereas restrictions cause them to be single stranded, which are chosen depending on the sequence homology between the sequences with known structure against each problem sequence without known structure. A good example for a constraint are the two complementary sequences that make a stem; an example of a restriction is a string of free nucleotides between helices or any kind of loop. The structure chosen was the one with the greater negative free energy but conserving the ring model known for ITS2. The obtained secondary structures were used to construct a matrix for cladistic analysis as described by Aguilar and Sánchez (2007). Phylogenetic analyses included maximum parsimony and maximum likelihood as well a Bayesian inference for a combined sequence-molecular morphometric analysis (see details in Grajales et al., 2007).

RESULTS

Denaturing gradient gel electrophoresis (DGGE) analysis revealed that most individuals from Belize and Panama contained intragenomic variants of ITS2 (see Figure 1). There were as many as three different bands per individual that were similar or nearly equal in length because of their closeness in the DGGE gel (Figure 2). Some banding patterns were identical for individuals from both Belize and Panama, which indicated exact ITS2 copies, although some patterns unique to each population were also observed (see Figure 1). Great effort was made to obtain sequences from most bands, but not all of them were successfully recovered. The sequences had an average GC content of 55.6%, which afforded the great migration of intragenomic ITS2 variants in the DGGE. The sequences from *P. bipinnata* had more than 85.6% of sequence similarity, contrasting with just 48% with respect to *G. mariae*.

Predicted secondary structures from all excised bands exhibited functional structures with the conserved six helicoidal ring model previously reported for octocorals (Aguilar and Sánchez, 2007), but great variability was observed in the length and complexity of each stem and spacer (Figures 2, 3). Intragenomic differences were frequently discrete changes that did not affect the predicted secondary

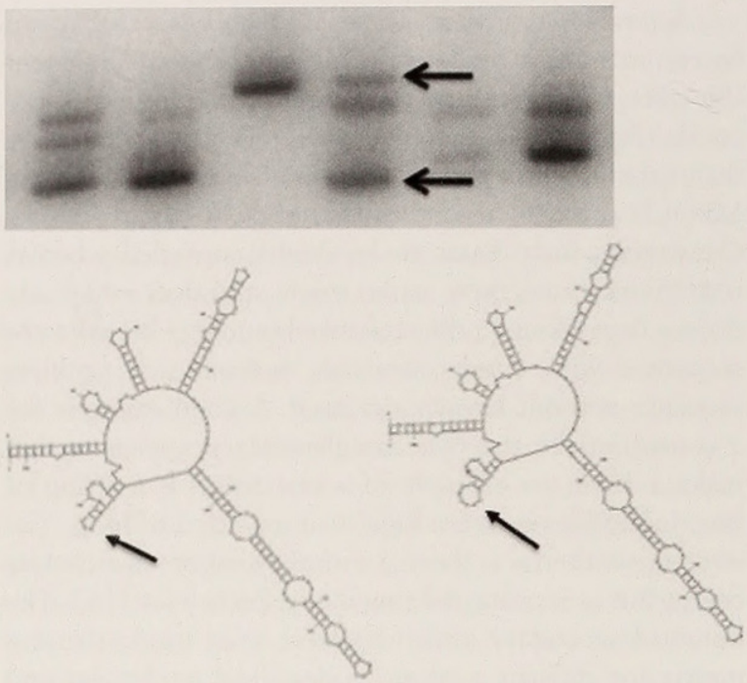

FIGURE 2. Two different intragenomic ITS2 variants from an individual colony of *Pseudopterogorgia bipinnata* from Belize. The variants were excised from the two bands indicated by arrows in the DGGE gel above, reamplified, and sequenced. The arrows below show the differences between the two predicted RNA secondary structures corresponding to one INDEL (insertion or deletion) only.

structures (see Figure 2). The ITS2 in *P. bipinnata* from Panama and Belize varied from 212 to 224 nucleotides (Figure 3). In general, the stems 2, 3, and 6 were shorter than the stems 4 and 5, with stem 5 being the longest. Multiple internal loops were frequent in stems 3, 4, and 5, with more nucleotides (nt) on stem 5, where up to six internal loops were observed (Figure 3). The spacers were often short, ranging from 1 to 4 nt. Spacer '1i' showed a conserved sequence, UG, with little variation across individuals, while spacer '4i' was the longest, with 4 to 12 nt and a conserved core sequence (AGUNCAGC) observed in most of individuals (Figure 3). Phylogenetic results from sequence and alignments or combined data sets, including 11 excised bands from individuals from Belize and 3 from Panama, showed little divergence between Panama and Belize despite the long distance with respect to a few individuals from Bahamas and Colombia (Figure 4). Very modest support was found within individuals from Panama or Belize, and no particular grouping could be discerned (data not shown). In addition, no particular features of the ITS2 secondary structure as seen with helix 5, which showed the largest number of characters, were supporting any particular clade or group of individuals (Figure 4).

DISCUSSION

The intragenomic ITS2 variation in *Pseudopterogorgia bipinnata* individuals involved functional copies, as corroborated by reconstructing their predicted RNA secondary structures. Given the low divergence among the ITS2 sequences recovered from DGGE gels, intragenomic variation was restricted to a few mutations that did not compromise the functionality of the ITS2 secondary structure. Despite frequent intragenomic ITS2 variation in *P. bipinnata*, predicted RNA secondary structures exhibited no signs of including pseudogenes or structural degeneration. Having in mind that the ITS2 secondary structure has a major role in the maturation of the ribosomal RNA (Coté and Peculis, 2001), little tolerance of changes is expected as a result of the restrictions imposed by the ITS2 splicing machinery (Van Nues et al., 1995; Coleman, 2003); this means purifying selection is acting on secondary structural constraints (Coté and Peculis, 2001) or concerted evolution mechanisms are acting similarly (Liao, 2000; but see Nei and Rooney, 2005; Harpke and Peterson, 2006). Similarly, compensatory base changes (CBC), occurring at the stem regions, are very unlikely to occur at the intraspecific level (Müller et al., 2007). Thus, it is expected that only variants or alleles carrying only minor changes occur, which was evident with the functionality of co-occurring secondary structures found at the intraspecific level.

ITS intragenomic variation has been also observed in scleractinian corals. Van Oppen et al. (2001) examined diverse nuclear and mitochondrial DNA sequences, concluding that paraphyly from intragenomic ITS copies could be explained by extensive introgressive hybridization and reticulate evolution. Similarly, Marquez et al. (2003) found the presence of ribosomal pseudogenes as a possible consequence of multiple hybridization events. However, Vollmer and Palumbi (2004) examined the multiple copies of the Caribbean *Acropora* species and concluded that there is no proper way to evaluate if the intragenomic shared variation of genes such as ITS1 and ITS2 was the result of incomplete lineage sorting or recent hybridization processes. Nonetheless, all the studies mentioned studied the intragenomic variation of ITS using the DGGE technique, and it is clear that traditional cloning methods overestimate the intragenomic diversity (LaJeunesse and Pinzon, 2007).

Regardless of the limited number of individuals analyzed in this study, the method used here, excising bands from DGGE gels for further amplification and sequencing, probed its reliability to separate intragenomic variants up to one nucleotide difference (see Figure 2). DGGE is a

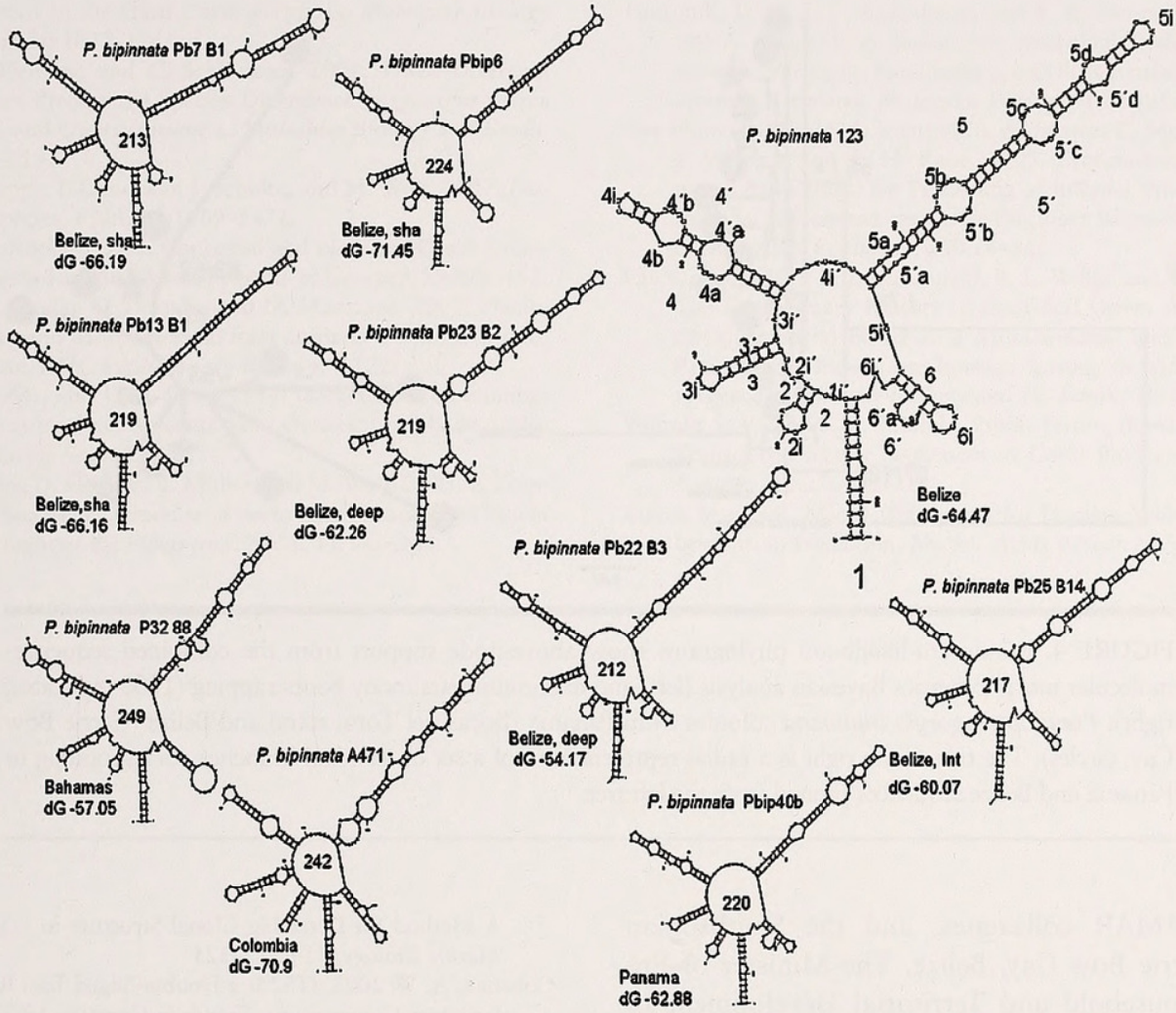

FIGURE 3. Predicted ITS2 RNA secondary structures in *Pseudopterogorgia (P.) bipinnata*. The upper right structure shows the characters for the molecular morphometrics analysis used in the combined Bayesian inference analysis. The number within the ring structure refers to the total number of nucleotides at each structure.

method useful to detect the most prevalent intragenomic variants of ribosomal genes, whereas traditional methods to screen intragenomic variation such as cloning miscalculate the codominance of the different copies (LaJeunesse and Pinzon, 2007; Thornhill et al., 2007). There are two main approaches for depicting the nature of intragenomic ITS2 variants in octocorals. One method is to study in detail the genetics of the different ITS2 variants by crossing individuals with different intragenomic patterns, which can provide inheritance information and linkage disequilibrium configurations. An alternative method includes techniques such as reverse transcription (RT)-PCR and quantitative real-time PCR, which can offer more accurate information on the functionality of the different intragenomic ITS2 copies. The RT-PCR technique can filter copies that are not expressed in the cell, and quantitative PCR can quantify the amount of ITS transcripts from each particular copy. These methods could also test if the intensity of bands in DGGE actually corresponds to the number of copies of a particular intragenomic variant.

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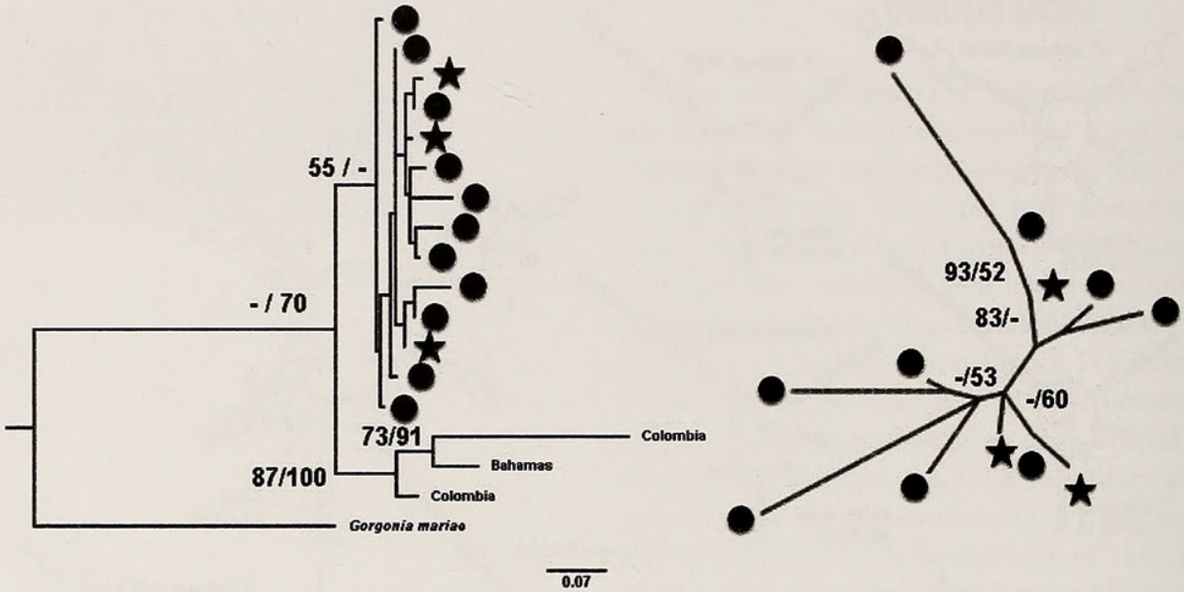

FIGURE 4. Maximum-likelihood phylograms show above-node support from the combined sequence-molecular morphometrics Bayesian analysis (left) and maximum-parsimony bootstrapping (1000 replicates, right): *Pseudopterogorgia bipinnata* colonies from Panama (Bocas del Toro; stars) and Belize (Carrie Bow Cay; circles). The tree at the right is a radial representation of a set of terminal branches corresponding to Panama and Belize sequences pruned from the left tree.

Cairns, BIOMMAR colleagues, and the Smithsonian Station at Carrie Bow Cay, Belize. The Minister of Environment, Household and Territorial Development of Colombia granted access to genetic resources to JAS for the DNA analyses included in this paper (Contract 007, resolution 634; 14 March 2007). This work is contribution number 841 of the Caribbean Coral Reef Ecosystems Program (CCRE), Smithsonian Institution, supported in part by the Hunterdon Oceanographic Research Fund.

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