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Intragenomic ITS2 variation in Caribbean seafans

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Abstract. The internal transcribed spacer 2 (ITS2) is part of the nuclear ribosomal cistron, whose secondary structure has important functions for ribosome assembling. Contrasting results in terms of inter- and intra-specific ITS2 variation have been found in a number of taxa including reef cnidarians. Different findings such as single or multiple intragenomic variants, even pseudogenes, have brought a great deal of confusion regarding the evolution and usefulness of ITS2 for phylogenetic reconstruction as well as the generality of the nRNA concerted evolution process. We examined intragenomic ITS2 copies in Caribbean seafan octocorals *Gorgonia* and *Pseudopterogorgia* (Gorgoniidae: Octocorallia) using Denaturing Gradient Gel Electrophoresis (DGGE) coupled with DNA sequencing and prediction of RNA secondary structures. Candidate pseudogenes were seldom found but most intragenomic ITS2 variants were functional secondary structures. Intragenomic variants were either dominant or codominant banding patterns in DGGE gels. Preliminary phylogenetic analyses showed that part of the intragenomic variation in *G. mariae* grouped partially with *Pseudopterogorgia* spp. as well as with other *Gorgonia* species. This finding supports the polyphyly of Caribbean sefans as observed with mitochondrial DNA and suggests a likely hybridization origination for *G. mariae*.

Key words: Intragenomic variation, ITS2, rDNA, Caribbean seafans, *Gorgonia*, hybridization, DGGE.

Introduction

The Internal Transcribed Spacer 2 (ITS2) is part of the ribosomal DNA cistron, which is transcribed but do not form part of the functional ribosomal complex. Although ribosomal genes are sequences repeated hundred of times in several chromosomes there are mechanisms that homogenize all the ribosomal genes (Elder and Turner 1995). Concerted evolution in ribosomal genes, although not entirely understood, is supposed to occur in a few generations owing a combination of processes such as intrachromosomal homogenization, gene conversion and unequal crossing over (Liao et al. 2000). Consequently, all copies of ribosomal genes are usually identical and can be considered single-copy genes for phylogenetic purposes. Nonetheless, ribosomal DNA intragenomic variation has puzzled molecular systematists and ecologists in the last few years.

The ITS2 attains higher evolution rates than other ribosomal genes. In addition, ITS2 has exhibited intragenomic variation in organisms ranging from plants to vertebrates. For that range of eukaryotes the intragenomic variation has been related to hybridization events (Coleman 2003). In the particular case of corals, however, it has been very difficult to explain the presence of intragenomic variants including a range of competing explanations such as incomplete lineage sorting, resulting in the preservation of ancestral polymorphisms, and introgressive hybridization (Volmer and Palumbi 2004). Opportunely, ITS2 is gaining credibility very fast. Coleman (2007), Schultz (2005) and Muller et al. (2007) are examples of some recent reviews showing generalities shared by all eukaryotes on several hallmarks of the ITS2 secondary structure, which have provided robustness using this sequence in phylogenetic and evolutionary studies. The function of the ITS2 secondary structure, unknown for many years, has a very important role during the ribosomal assembling. It is known experimentally that certain changes in the secondary structure prevent the formation of ribosomes (Cote and Peculis 2001, Van Nues et al. 1995). In addition, the same ITS2 sequence can turn into two different secondary structures, where the proximal part does not vary and it has an important function such as the C2 processing site and other parts of the molecule have a multifunctional role. Having that in mind, we can rely upon secondary structure to tell apart pseudogenes from functional ITS2 copies.

Seafans are shallow-water octocorals, which are very abundant in Caribbean coral reefs (Bayer 1961). Members of the family Gorgoniidae in this region are very diverse and have a symbiosis with zooxanthellae (Sánchez and Wirshing 2005). A particularity of gorgonians is their phenotypic plasticity (Sánchez et al. 2007, Gutierrez-Rodriguez et al. 2008), which enables them to colonize most reef habitats. However,

such variability imposes challenges to delineate species boundaries. Caribbean seafans comprise three species, *G. ventalina, G. flabellum*, and *G. mariae,* all of them include particular scaphoid sclerites (Bayer, 1961). Another characteristic of *Gorgonia* is the reticulate branching forming a mesh-like pattern, which is very dense in all species but *G. mariae*. Some of the growth forms in *G. mariae* are pinnate with occasional fused branches, resembling the genus *Pseudopterogorgia*. Of particular interest, molecular phylogenetic analyses among gorgoniids present different results regarding the position of *G. mariae*, mitochondrial DNA place it with *Pseudopterogorgia* spp. (Sánchez et al. 2003) whereas nuclear DNA (ITS2) with the other *Gorgonia* species (Aguilar and Sánchez 2007). Such incongruence between molecular phylogenies together with the intermediate branching characteristics of *G. mariae*, resembling both *Pseudopterogorgia* and *Gorgonia*, suggest a hybrid origin for this species (see reviews in Seehausen 2004and Mallet 2005). To approach the previous problem, we had two major goals in this study: first was to examine the nature of ITS2 intragenomic variation in seafans and sea whips (*Pseudopterogorgia*), and second to reconstruct preliminary phylogenetic hypothesis of ITS2 intragenomic variants.

Material and Methods

We studied the intragenomic variation in *Gorgonia* spp. and a few species of *Pseudopterogorgia*. We had fresh and museum material from Colombia, Panama, Belize, Puerto Rico and Bahamas. Total DNA was extracted using a CTAB, proteinase K, Phenol – Clorophorm-Isoamyl alcohol extraction method (Coffroth et al. 1992). DNA was resuspended and conserved in TE buffer at –70ºC. DNA quality was checked in agarose (1%) electrophoresis at 80V 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 PCR were as follows: an initial denaturizing step: 2 min at 94ºC, followed by 35 cycles: 30 sec. at 94ºC, 30 sec. at 56.8ºC and 1 min at 72ºC, final extension step: 2 min. at 72ºC. Using 1 unit of Taq polymerase (Invitrogen), 3.5 mM MgCl2, 0.2 mM DNTPs (Biorad Mix), 0.15 uM primers (each) and 4µL of DNA (dilution 1/50) in 20µL as final volume. The amplification was standardized with an efficiency of 95%. PCR reactions were screened in a gel containing 8% polyacrilamide, 1X TAE Buffer and linear urea-formamide denaturing gradient from 45% to 80%. The gels were pre-run at 60ºC and 90V for 30 min, followed by the electrophoresis at 60ºC and 90V for 13 h. Gels were stained with ethidium bromide during 15 min and visualized using a BioRad ChemiDoc system. All reactions were conducted without CG-clamp in the primers. Bands visualized in the DGGE gel were excised using sterilized micropipette tips in the BioRad ChemiDoc system and placed in 1,5 mL tubes with 100µL of sterilized double distilled water. The tubes were incubated in a shaker at room temperature for 24 hours at 150 rpm. Each band extract was collected in a 1,5 mL tube and the DNA was precipitated with 300 µL of cold absolute ethanol, tubes were placed at -20ºC for 24 hours and then centrifuged at 13000 rpm for 30 min, supernatant was discarded and pellet dried and resuspended in 15 µL sterilized double distilled water.

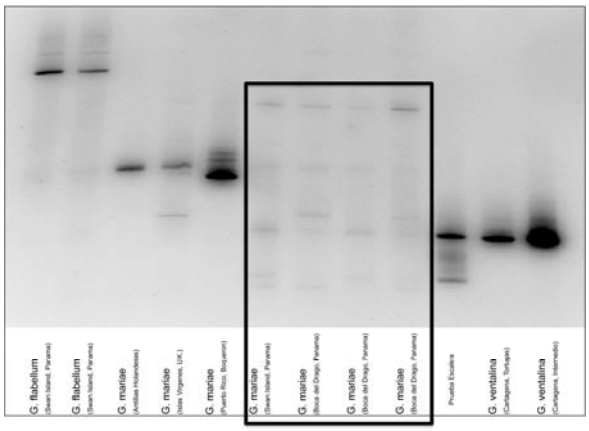

Figure 1: Examples of ITS2 DGGE banding patterns from seafans *Gorgonia* spp. colonies. Inset square: banding patterns of *Gorgonia mariae* from Panama (Bocas del Toro). Linear urea-formamide denaturing gradient of 45% to 80%.

Re-amplification of bands was conducted using PCR as described above except that DNA was used without dilution. Purification of PCR products for sequencing was performed by Exo-Sap method using 1 u of Exonuclease, 0,2 u of Shrimp Alkaline Phosphatase and 2µl SAP buffer 10x per 20 µl in a 0,2 ml tube. Reactions were held at 37ºC for 1 hour and at 80ºC for 15 min. Sequencing reactions were performed with BigDye 3.1 system according to manufactures 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 chromatograms in Sequencer 4.7 software.

Secondary structures of all sequences were obtained 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 constrains and restrictions in MFOLD (Zuker 2003) at

a default temperature of 37ºC. 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 correct the alignment using the program 4SALE (Seibel et al. 2006). Phylogenetic analyses included maximum parsimony, maximum likelihood and Bayesian inference (see details in Grajales et al. 2007).

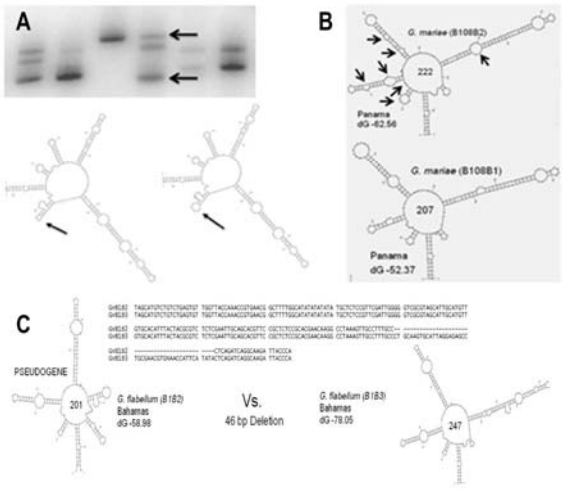

Figure 2: ITS2 predicted secondary structures from the intragenomic variants. A. Two different intragenomic ITS2 variants from an individual colony of *Pseudopterogorgia bipinnata* from Belize, corresponding to one mutation only. B. Two out of five different intragenomic ITS2 variants from an individual colony of *Gorgonia mariae*, including multiple differences. C. Diagnostic DGGE band (left) and a pseudogene (right) lacking most of the proximal helix in *G. flabellum*. Numbers refer to the nucleotide length and arrows point differences among structures.

Results

Gorgonia seafans exhibited a great deal of intragenomic variation as observed with multiple bands in DGGE (e.g., Fig. 1). There were different intragenomic patterns including species with a main "diagnostic" band, or dominant. Some individuals, mainly from *Gorgonia mariae,* did not have a dominant diagnostic band but up to five codominant bands (Fig. 1 inset). The intragenomic variation detected in DGGE gels, after reamplifying and sequencing, included the less amount of variation, a single nucleotide polymorphism (SNP), very common in colonies of *Psedopterogorgia bipinnata* (e.g., Fig. 2A), which was a good indicator that the method was reliable to separate all possible intragenomic variation. In addition, differences among intragenomic variants from the same individual could be highly significant with more than 15 INDELS and substitutions as in the case of *G. mariae* (e.g., Fig. 2B). The extreme case was observed in *G. flabellum* with more than 40 changes between two variants where one of them lacked the proximal helix (Fig. 2C), which should correspond to a pseudogene because it does not have the C2 processing site.

Preliminary phylogenetic analyses among the recovered intragenomic variants from DGGE gels were overall well supported by the three different phylogenetic approaches (Fig. 3). Paralyphyletic intragenomic variants were found in *Gorgonia mariae, P. bipinnata* and *G. flabellum*, although most *P. bipinnata* variants formed a monophyletic group. In contrast, some species such as *G. ventalina*, *P. acerosa,* and *P. rigida* had not variants or exhibited little divergence among intragenomic variants. The only pseudogene found in *G. flabellum* was located completely off *Gorgonia* and *Pseudopterogorgia* species near the outgroup. Although not all the intragenomic variants were successfully recovered from DGGE gels it was clear for *G. mariae* that its intragenomic variants were grouped with both *Pseudopterogorgia* and *Gorgonia* species within well supported nodes (Fig. 3).

Discussion

The study of ITS2 intragenomic variation provides additional compelling evidence for a likely hybrid origin of *Gorgonia mariae*, involving other *Gorgonia* and *Pseudoterogorgia* species as parental sources. Nonetheless, intragenomic variants from other *Gorgonia* and *Psedopterogorgia* species suggest that gene flow among seafans and sea whips might be more common than previously thought. Swarms of interbreeding species, known as a syngameon, have been proposed as a mechanism to quickly promote sympatric speciation via hybridization under adaptive radiation conditions (Seehausen 2004). The suggestive idea of the syngameon has been also proposed for scleractinian corals (Veron 1995, Kenyon 1997) and could explain the great diversity and phenotypic plasticity found in gorgonian corals. The alternative hypothesis about ITS2 intragenomic variation as incomplete lineage sorting of ancestral polymorphisms, might be rejected because ancestral polymorphisms, which should be older, might degenerate and turn into pseudogenes as seen with a corrupted RNA secondary structure. As mentioned before, pseudogenes were found only in *G. flabellum* but were infrequent and easy to detect. Therefore, functional ITS2 copies should be more recent as to prevent complete concerted evolution.

The preliminary phylogenetic hypothesis showed well-supported nodes suggesting multiple origins of *G. mariae* ITS2 intragenomic variants. *G. mariae* could acquire different ITS2 copies through the process known as introgressive hybridization (see review in Mallet 2005). Consequently, unless concerted evolution had occurred, hybridization processes leave

a footprint in the ITS2 generating mosaic copies from diverse parental genomes in *G. mariae*.

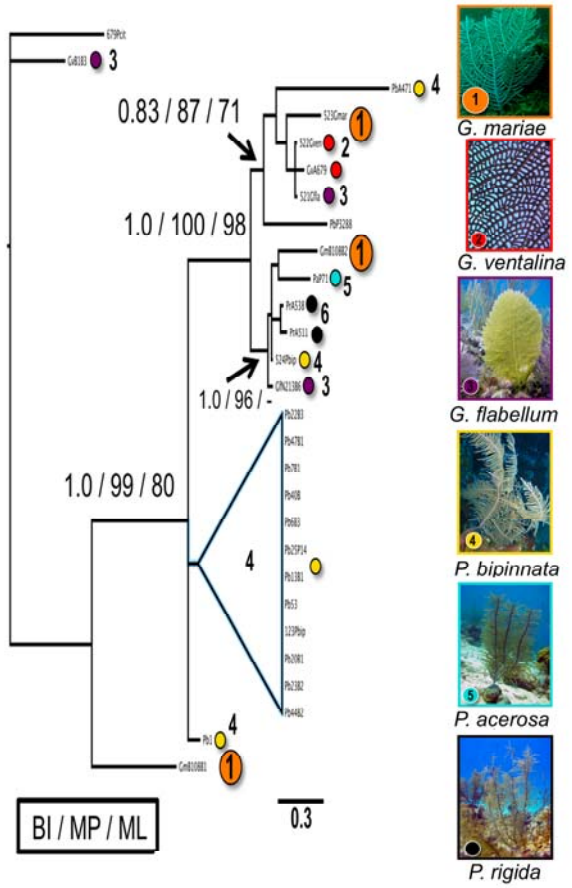

Figure 3: Preliminary phylogenetic results among ITS2 intragenomic variants in Caribbean *Gorgonia* and *Pseudopterogorgia* species. The tree is an optimum Maximum likelihood phylogram showing above node support from Bayesian Inference-BI / Maximum Parsimony-MP / Maximum Likelihood-ML. Scale bar 0.3 substitutions per site. Outgroup: *Pterogorgia citrina*.

Opportunely, scleractinian corals have provided straightforward evidence of introgressive hybridization particularly within species of *Acropora*, which have enlightened on the great potential for introgressive hybridization in sessile marine organisms with broadcast spawning. For instance, Odorico and Miller (1997) found consistent information in *Acropora's* ITSs with a reticulate evolution scenario. Van Oppen et al. (2001) examined diverse nuclear and mitochondrial DNA sequences concluding that paraphylly from most species could be explained by extensive introgressive hybridization and reticulate evolution. Likewise, Marquez et al. (2003) found the presence of ribosomal pseudogenes as a possible consequence of multiple hybridization events. Hybrid origin has also been proposed for soft corals species using ITS (McFadden and Hutchinson 2004). Nonetheless, Vollmer and Palumbi (2004) examined the multiple copies of the Caribbean *Acropora* species and concluded that there is no a 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. Yet ancestral polymorphisms should certainly retain more substitutions per site when compared with functional genes and for a ribosomal gene such as ITS2 that means purifying selection acting on secondary structural constraints (Cote and Peculis 2001) or concerted evolution mechanisms acting similarly (Liao et al. 2000, but see Nei and Rooney 2005 and Harpke et al. 2006). It is clear that the ITS2 is not a standard gene for reconstructing phylogenetic hypothesis (e.g., Harris and Crandall 2000) but it seems to retain clues from introgressive hybridization events, which should be examined in detail before reaching a robust conclusion. In conclusion, ITS2 is unfolding a different story of the diversification in octocorals thanks to the aid of techniques such as DGGE (see technological advantages over cloning in: Lajeunesse and Pinzon 2007) and RNA secondary structure prediction, which are techniques strongly recommended for the analysis of this kind of sequences.

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