A STUDY OF THE IGS/ETS REGION IN OCTOCORALS AND BLACK CORALS

SONIA ELEONORA HERNÁNDEZ DURÁN

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SONIA ELEONORA HERNÁNDEZ DURÁN

MICROBIOLOGY UNDERGRADUATE THESIS

Director
JUAN ARMANDO SÁNCHEZ
Marine Biologist Ph.D

Co-Director
GLORIA INÉS URIBE
Bacteriologist M. Sc

UNIVERSIDAD DE LOS ANDES
SCIENCE FACULTY
BIOLOGICAL SCIENCE DEPARTMENT
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ACKNOWLEDGMENTS

First of all, I would like to thank my family for being the incredible support that they are and for always keeping me grounded. I know that no matter what I can always count on you and that nothing of this would ever be possible without you.

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SUMMARY

The IGS/ETS region has been virtually unexplored in cnidarians, which makes for an exciting study alternative for developing new molecular markers in these organisms. In this study, the size and repetitive nature of the IGS/ETS of several octorals and black corals were analyzed for interspecific variation using a RFLP approach. All the samples included in this study presented a multicopy region with different number and sizes of copies as well as different numbers of *Mse*I restriction sites at different positions which implies a high variability between coral species. All this makes for an ideal molecular marker in future studies involving corals. However, future work should continue standarization of these methods and include a more diverse group of coral species so as to be able to generalize these findings and construct a more specific picture for the IGS/ETS region in corals.

INTRODUCTION

Very little is known about the intergenic spacer and external transcribed spacer (IGS/ETS) region in corals. The study of this region is particularly complicated and tedious due to its large size and high number of tandem repeats in higher organisms (Coté et al, 2001), and it is not clear whether the existing models for more advanced organisms apply for lower organisms such as cnidarians (Chen et al, 2000).

In general, ribosomal DNA (rDNA) is arranged in transcription units which are separated by an intergenic spacer (IGS) ranging from 2.5 to 30 Kb in animals (Sollner-Webb and Mougey 1991). Each transcription unit consists of 3 ribosomal subunits (18s, 5.8s and 28s) separated by an internal transcribed spacer, ITS1 or ITS2, which contain the information necessary for processing (Harris et al, 2000). Furthermore, this arrangement is flanked at each end by an external transcribed spacer (ETS) (Coté et al, 2001)

Fig. 1 General structure of IGS/ETS region in eukaryotes

It is known that rDNA is prone to concerted evolution, which over time results in a homogenization of all multigene families present in the genome (Harris et al, 2000). However, the IGS/ETS region has been reported to vary significantly within individuals and populations, with polymorphisms that vary in the order of kilobase pairs (Coté et al, 2001) due to a very low concerted evolution rate (Coté et al, 2001, Chen et al, 2000, Harris et al, 2000).

Previous studies have reported IGS/ETS regions of 1.8 kb for the octocoral *Junceella fragilis* (Chen et al 2000) and 2.5 kb for the *Pavona cactus* coral (Smith et al, 1997), where the regions of both organisms present a pattern consistent with a tandem arrangement as previously mentioned. It was the aim of this study to determine the size and repetitive nature of the IGS/ETS of several octocorals and black corals, as well as determining the effectiveness of this region as a molecular marker for interspecific variation using a RFLP technique. Also, an alternative protocol for sequencing large fragments (such as IGS/ETS) was explored so as to omit cloning methods.

Multiple copies of this region between 600 and 1700 bp were observed in both groups and a great deal of interspecific variation was assessed, resulting in what appears to be a very promising molecular marker for further studies regarding corals. However, a much larger sample number must be included in studies similar to this so as to elaborate a more exhaustive model for the IGS/ETS regions in corals and although interspecific variation was determined, various samples of a same species must be included in order to assess intraspecific variation of this region. Finally, it is imperative that standarization of the alternative sequencing protocol be continued so that its effectiveness can be increased and it can be employed in the future with a much higher efficiency.

MATERIALS AND METHODS

Sample collection

Black coral samples were collected on September of 2005 at The Flower Garden Banks National Marine Sanctuary in the Gulf of Mexico by NURC phantom S2, a remotely operated vehicle. Octocoral samples were collected on September of 2005 at different locations of Cartagena, Colombia and on September of 2007 at the island of Gorgona, Colombia by members of the BIOMMAR lab from the Universidad de los Andes by scuba diving (Annex 1). A total of 40 samples were collected and preserved in 95% ethanol.

DNA Extraction

DNA from all black coral samples was extracted using the extraction kit DNeasy by Qiagen® under conditions recommended by the manufacturer.

DNA from all the octocoral samples was extracted using a modified version of the protocol by Coffroth et al, 1992.

Approximately 2 mm cube fragments of each sample were used for DNA extraction. Each fragment was ground using sterile pestles in 300 µL of CTAB 2X (Cetyltrimethyl ammonium bromide) at 65 C, and 2 µL of proteinase K were added in order to lyse any proteins present, including DNAses. This lysate was incubated at 65 C overnight. After this, 300 µL of PCIA (Phenol/chloroform – isoamylalcohol 25:24:1) were added to the lysate, agitated and spun in a centrifuge during 10 minutes at 12000 rpm. After centrifugation, the aqueous phase was recuperated and 300 µL of CIA (Chloroform isoamylalcohol 24:1) were added to this phase and centrifuged again for 10 minutes at 12000 rpm.

This new aqueous phase was recovered and the DNA was precipitated using 800 µL 95% ethanol. The DNA was left to precipitate overnight, after which it was centrifuged at 12000 rpm during 30 minutes. The supernatant was discarded and 500 µL of 70% ethanol were added to the remaining pellet and centrifuged during 10 minutes at 12000 rpm. The ethanol was discarded and any remaining ethanol was evaporated at room temperature.
Once the pellet was dried completely, 50 µL of TE buffer were added to the pellet and stored at -20 C.

Confirmation of DNA extraction

Presence of DNA was confirmed by visualizing the extractions in a 0.8% agarose gel with 0.5X TBE buffer after a 30 minute run at 110 V using the Chemidoc ™ XRS BioRad gel documentator and Quantity One 4.0 software.

IGS/ETS PCR standardization

Standardization of DNA concentration

In order to optimize DNA concentration for PCR protocol, a series of dilutions were assayed for each sample, according to the band intensity obtained in the previous step of confirmation of DNA extraction. Extractions with a higher band intensity were diluted to 1/50 and 1/20, while samples with a lower band intensity were diluted to 1/10 and 1/5.

Standardization of PCR conditions

The IGS/ETS region of the corals analyzed was amplified by a PCR protocol using the universal primers 28NF: 5' -GATTATGACT GAACGCCTCT AAGTCAGAAT CC- 3' and *18S*-10B: 5'-TTACCATCGACAGTTGATAGGGCA-3' (Smith et al, 1997). The PCR cycle was performed on a MyCycle thermocycler (Bio-Rad) based on previous work for the ETS/IGS region of the octocoral *Junceella fragilis* (Chen et al, 2000). Specific cycle conditions for the octocoral samples and the black coral samples were further standarized as so, for octocorals the thermal cycle consisted of 1 cycle at 95 °C (3 min); 35 cycles at 94 °C (30 s), 57 °C (1 min), and 72 °C (2 min); and 1 cycle at 72 °C (3 min), and for black corals 1 cycle at 95 °C (3 min); 4 cycles at 94 °C (30 s), 50 °C (1 min), and 72 °C (2 min); 30 cycles at 94 °C (30 s), 52 °C (1 min), and 72 °C (2 min) and 1 final cycle at 72 °C (3 min). The annealing temperature of the primers was standardized using a temperature gradient (54 °C, 55.4 °C, 56.4 °C, 57.2 °C and 58 °C for octocorals and 51 °C, 52.1 °C, 53.4 °C, 54 °C for black coral samples). The optimal $MgCl_2$ concentration was chosen based on a previous work done on PCR conditions for black corals (Umaña, unpublished) at the BIOMMAR lab.

The amplification reaction used 2.25 µL of the diluted template, 0.3 units of Promega *Taq* polymerase, 3 µL of 5X buffer provided with the enzyme, 0.3 µL of 10mM Promega dNTPs, 2.1 µL of 25mM $MgCl_2$ (final concentration of 3.5mM), 0.225 µL each of forward and reverse primer (10µM) and 6.6 µL of ddH_2O for a final volume of 15 µL.

PCR products were verified by electrophoresis in a 1% agarose gel with the conditions previously mentioned in DNA extraction confirmation.

Restriction and ligation assay - RFLPs

Amplified DNA from *Plumapathes pennacea, Tanacetipathes barbadensis, Antipathes atlantica* (black corals), *Leptogorgia sp.* (sample L3), *Pacifigorgia sp.* (samples G93 and G96*), Muricea atlantica* and *Muricea pinnata* (octocorals) was treated with the *Mse*I restriction enzyme from Invitrogen. For the restriction and ligation reaction 10 µL of PCR product were used, as well as 0.5 µL of *Mse*I enzyme, 2.5 µL of 10X buffer provided with the enzyme, 0.33 µL of T4 ligase from Promega, 5 µL of 10X buffer provided with this enzyme, 1.25 µL of DTT (5mM), 0.42 µL of ddH_2O and 2.5 µL each of *Mse*I adaptor I (5'-TACTCAGGACTCAT-3') and *Mse*I adaptor II (5'-GACGATGAGTCCTGAG-3', Zane et al, 2002) for a final volume reaction of 25 µL.

This reaction was incubated at 37 C for 3 hours after which the restriction fragment length polymorphisms (RFLPs) were checked by 7% polyacrylamide gel electrophoresis with 1X TBE buffer at 110 V during 50 minutes. A 100bp benchtop ladder (Promega) was used to visualize the fragments, as well as a 50bp molecular marker from Invitrogen. Each band present was cut from the gel and resuspended in 50 µL of ddH_2O. Each resuspended DNA fragment was left in a shaker overnight, after which the supernatant was passed to another sterile eppendorf and 450 µL of absolute ethanol were added in order to precipitate the DNA at -20 C during the next 2 days. After centrifugation at 12000 rpm during 30 minutes, each pellet was left to dry and resuspended in 15 µL of TE buffer.

Amplification of restriction fragments

*Mse*I-M13 PCR

Reamplification of each resuspended restriction fragment and addition of the M13 tail (5'-TGTAAAACGACGGCCAGT-3') was obtained using the *Mse*I-M13 primer pair, *Mse*I-M13 forward: 5'-GTA AAA CGA CGG CCA GCA TGA GTC CT-3' and *Mse*I-M13 reverse: 5'-GAGAGMTCC TGA GTA ACA GGA AAC AC-3', where an M13 tail was added to the *Mse*I primer pair. For the amplification reaction 5 µL of a 1/10 dilution of previously resuspended DNA were used, as well as 0.1 unit of Promega *Taq* polymerase, 4 µL of buffer provided with the enzyme, 2.8 µL of $MgCl_2$ (3.5 Mm), 0.4 µL of Promega dNTPs, 0.225 µL each of forward and reverse primer (10 uM) and 2.46 µL of ddH_2O for a final volume of 15 µL. The thermal cycle consisted of 26 cycles at 94 °C (30 s), 53 °C (1 min) and 72 °C (1 min) (Zane et al, 2002).

Confirmation of tailed PCR products was carried out using a 2% agarose gel electrophoresis with 0.5X buffer at 110V during 30 minutes. .

M13 PCR

A PCR with a M13 primer pair (M13 Forward: 5'-GTA AAA CGA CGG CCA G-3' and M13 Reverse: 5'-CAG GAA ACA GCT ATG AC-3') complementary to the M13 tail inserted in the *Mse*I-M13 PCR was carried out as a confirmation step prior to sequencing to determine if the M13 tail was indeed being inserted in the amplified restriction fragments. The thermal cycle employed was as follows: 30 cycles at 94 °C (30 s), 53 °C (1 min) and 72 °C (1 min). After this, PCR products were visualized using a 2% agarose gel with conditions previously mentioned in DNA extraction confirmation.

RESULTS AND DISCUSSION

Standardization of DNA concentration

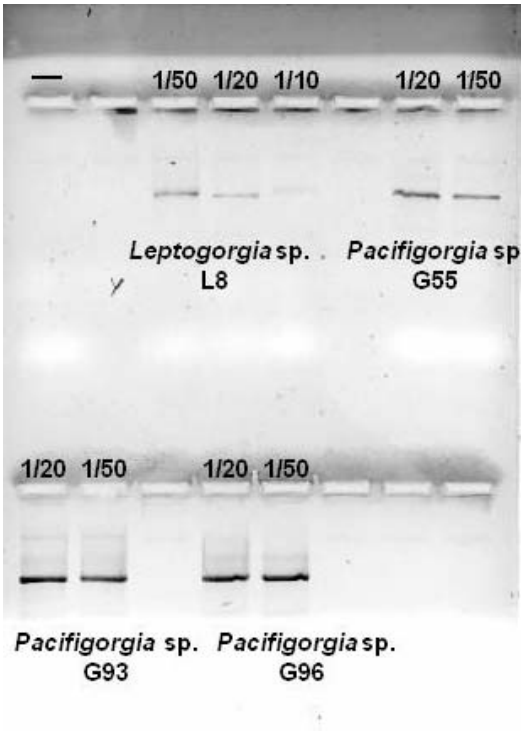

Fig. 2 Standardization of DNA dilutions for *Pacifigorgia* spp. and *Leptogorgia* sp. Lane 1: Negative PCR control (no DNA).

Standardization of template concentration for PCR was critical due to the fact that the DNA was extracted using two different methods (extraction kit and manual extraction). For each sample a series of dilutions were assayed based on the quality of the DNA extracted, the necessary dilutions determined by visualization of the extraction performed.

For most of the samples studied, a dilution of 1/20 was used (black corals and octocorals alike), but in some cases a minor and major dilution was necessary (for example *Plumapathes pennacea* and *Antipathes atlantica* respectively). With some samples the PCR product yield improved with increasing dilutions (as such was the case with *Leptogorgia* sp. sample L8). A possible explanation

for this phenomenon could be the presence of PCR inhibitors in the extracted DNA.

Standardization of PCR conditions

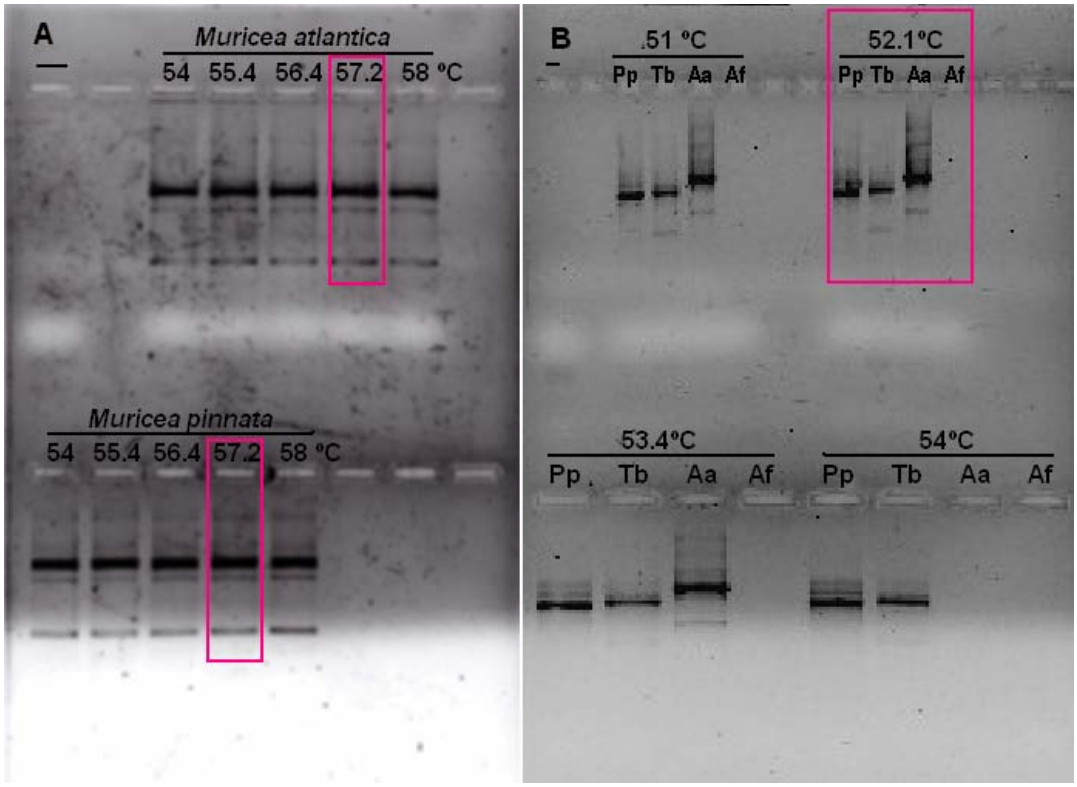

Fig. 3 Temperature gradient for octocoral samples (A), and black coral samples Pp: *Plumapathes pennacea*, Tb: *Tanacetipathes barbadensis*, Aa: *Antipathes atlantica*, Af: *Antipathes furcata* (B). All samples are diluted 1/20. The first lane in each gel is a negative control.

A separate standardization of PCR conditions was necessary for the octocoral group of samples and the black coral samples, the same thermal cycle was not suitable for both groups. Even though the primers used in both PCR cycles

were the same (28NF and *18S*-10B), the optimal annealing temperature varied significantly between the octocorals and black corals (57 ºC and 52 ºC respectively). This temperature was chosen based on the amplification behaviour of all the samples included in the standardization assay and on the intensity of all the bands present in each PCR product. For this reason 54 ºC was the poorest annealing temperature for black corals since *Antipathes atlantica* was no longer amplified and amongst the remaining temperatures from the gradient, 52.1 ºC was the one where all the bands amplified from each sample presented the most intensity. A similar analysis was performed with the octocoral temperature gradient.

The amplification efficiency prior to standardization ranged between 9-11% (least and most efficient amplification assay respectively). After the optimal conditions were established, an efficiency of 88-91% was attained for both sample groups. This efficiency was estimated based on the number of samples amplified in a given PCR, with equal number of samples in each assay (data not shown).

IGS/ETS PCR Products

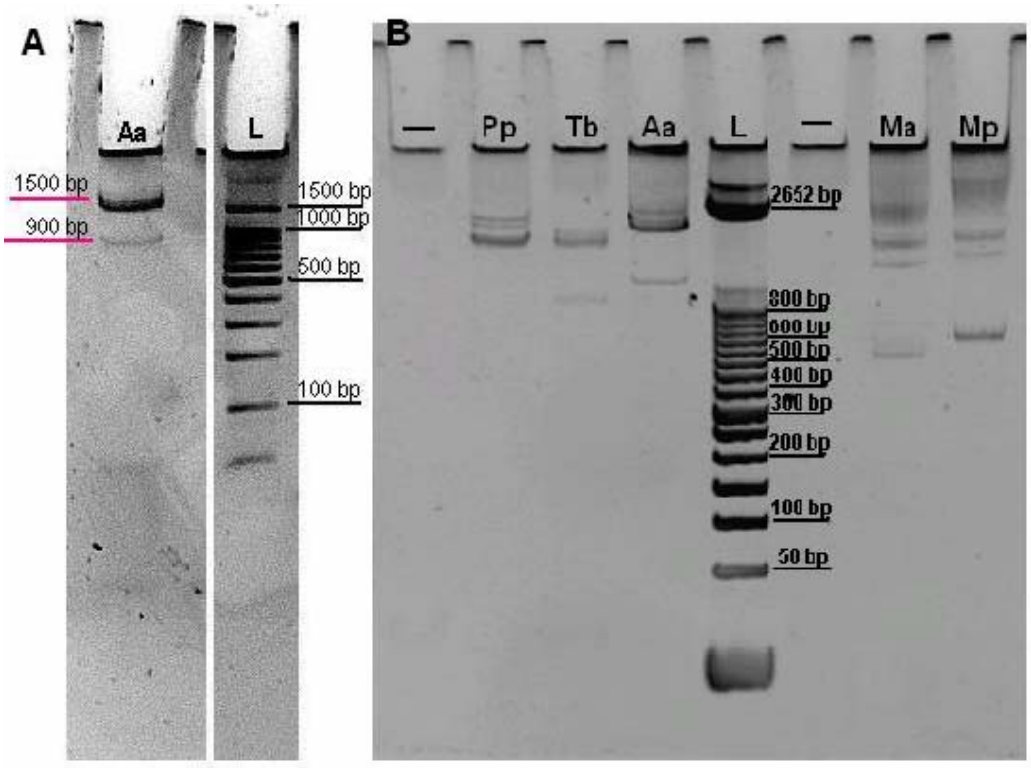

Fig. 4 PCR products of octocorals and black corals. A) Band pattern of Aa: *Antipathes atlantica*, L: 100bp marker. B) PCR products of black corals Pp: *Plumapathes pennacea* (dilution 1/5), Tb: *Tanacetipathes barbadensis* (dilution 1/50), Aa: *Antipathes atlantica* (dilution 1/20) and octocorals Ma: *Muricea atlantica* (dilution 1/20) and Mp: *Muricea pinnata* (dilution 1/20) L: 50 bp marker. Negative controls (-)

The PCR products obtained in this study presented multiple bands, varying in size and intensity. As the annealing temperature was increased for any given sample, all the bands obtained at a lower temperature were still present and even in a more intense fashion. This indicates that the multiple band patterns were not the artefact of an unspecific annealing of the primers. Also, each multiple band pattern was maintained in following replicates. It would appear that the ETS region of the corals analyzed is not evolving in a concerted manner since there doesn't seem to be occurring a homogenization process of

the multiple copies present (Elder and Turner, 1995). However this aspect can only be confirmed analyzing the sequence of each band obtained in the PCR products.

Unfortunately it was not possible to continue working with a 100bp molecular weight marker (only the PCR product of *Antipathes atlantica* was viewed with this marker), therefore the PCR products of the rest of the samples were viewed using a 50bp molecular marker. Since this marker didn't cover the molecular weight range of the ETS amplification, an extrapolation from comparison of each sample with *Antipathes atlantica* was performed. The fragment lengths ranged between 600 bp and 1700 bp approximately (Figure 4, Annex 2).
Although fragment length varied interspecifically, a pattern could be observed between the black coral samples, and another pattern between the octocoral samples. The tendency for the octocoral samples analyzed (*Muricea pinnata*, *Muricea atlantica*, *Leptogorgia* sp. and *Pacifigorgia* spp.) was of four bands and a larger difference between band length, approximately 600-700 bp difference between the last two bands amplified in *Muricea* spp. samples (Figure 4 lanes Ma and Mp). The band pattern between these two species is very similar (Figure 4), as is the band pattern between the samples of *Pacifigorgia* spp. and the *Leptogorgia* sp. sample (Figure 2).

On the contrary, black coral samples presented 3 bands in average and a 300-400 bp difference between the last 2 bands amplified (Figure 4, lanes Tb and Aa).

The most intense PCR product bands throughout the samples studied ranged between 1400 and 1700 bp (Figure 4) which is similar to the fragment length of 1800 bp reported previously for the octocoral *Juniceella fragilis* (Chen et al, 2000).

A much larger set of PCR products from a more extensive sampling are required in order to construct a bigger picture on the given characteristics of the ETS region amongst corals.

RFLPs (Restriction Fragment Length Polymorphisms)

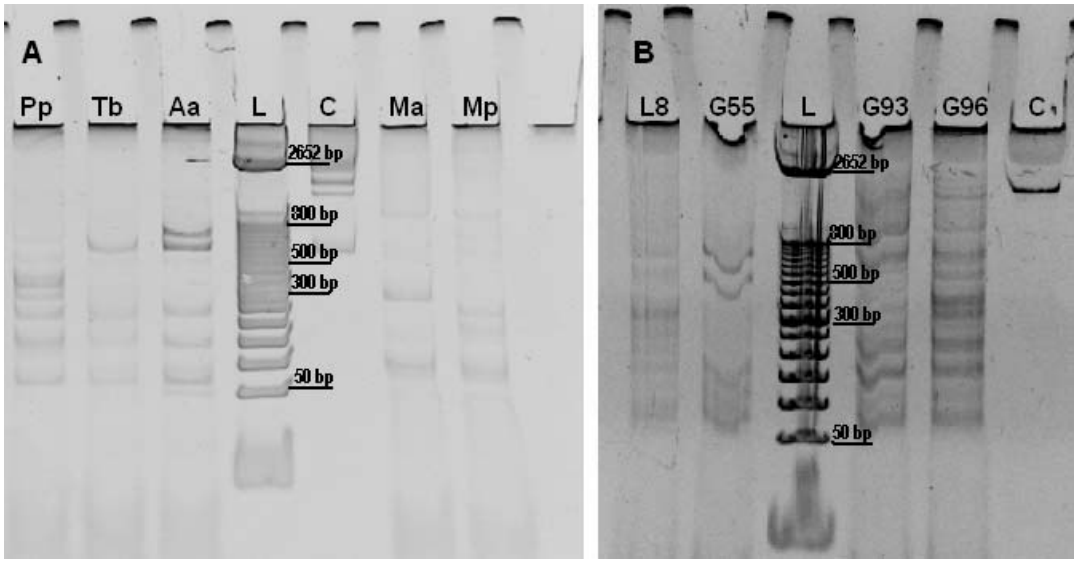

Fig. 5 RFLPs of black corals and octocorals A) Pp: *Plumapathes pennacea*, Tb: *Tanacetipathes barbadensis*, Aa: *Antipathes atlantica*, L: 50 bp marker, C: *Antipathes atlantica* PCR Product, Ma: *Muricea atlantica*, Mp: *Muricea pinnata*. B) L8: *Leptogorgia* sp. (L8), G55: *Pacifigorgia* sp. (G55), L: 50 bp marker, G93: *Pacifigorgia* sp. (G93), G96: *Pacifigorgia* sp. (G96), C: *Pacifigorgia* sp. (G55) PCR product.

All samples assayed were cut by the restriction enzyme *Mse*I yielding a series of different length fragments (Figure 5), this implies that these corals have a different number of *Mse*I restriction sites at different positions. Again, these

restriction sites are not necessarily conserved between species, although similar band patterns were observed between *Tanacetipathes barbadensis* and *Antipathes atlantica* (Figure 5, lanes Tb and Aa), between *Muricea atlantica* and *Muricea pinnata* (Figure 5 lanes Ma and Mp) and between Pacifigorgia sp. Samples (Figure 5, lanes G93 and G96). No general band pattern was observed amongst all the samples analyzed, suggesting that these sequences are not conserved between groups or species. However a much larger sample number including other coral species must be studied in order to confirm these generalized affirmations.

Amplification of restriction fragments

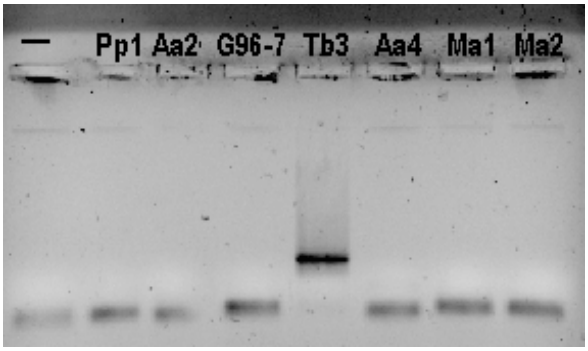

Fig. 6 Amplification of restriction fragments using M13 tailed primers.

Although a high intensity band was obtained with the restriction fragments cut from the gel, the efficiency for the amplification of each fragment was dramatically reduced seeing that only one band is being amplified (Figure 6 lane Tb3). However, the M13 tail is being inserted as can be appreciated by the reamplified fragment in figure 6.

CONCLUSIONS

The IGS/ETS in octocorals and black corals is a multicopy region with different number and sizes of copies for the octocorals and black corals studied. Also, all the samples analyzed presented different number of *Mse*I restriction sites and at different positions. This translates into a high interspecific variability for this region due to a low concerted evolution rate which makes it an ideal molecular marker for future study.

A much larger number of samples must be analyzed in order to obtain a vaster picture of the disposition of the IGS/ETS region in corals, as well as various samples of the same species for the assessment of the intraspecific variation of this region.

Finally, an efficiency of approximately 90% was achieved for the IGS/ETS PCR. However, in subsequent PCR cycles the efficiency of the products obtained decreased significantly. Further standarization work is required to optimize these protocols so as to facilitate the study of this region in corals and be able to continue with the alternative method for sequencing with the *Mse*I-M13 PCR.

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ANNEX 1 – SAMPLE COLLECTION

CODE	SPECIES	GROUP	LOCATION	DATE
DFH11-1A	*Plumapathes pennacea*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-2A	*Plumapathes pennacea*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-3A	*Elatopathes abietina*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-3B	*Elatopathes abietina*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-4B	*Tanacetipathes barbadensis*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-5A	*Tanacetipathes barbadensis*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-6A	*Elatopathes abietina*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-6B	*Elatopathes abietina*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-7A	*Tanacetipathes barbadensis*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-8A	*Aphanipathes pedata*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-10A	*Antipathes atlantica*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-11A	*Antipathes furcata*	Black Coral	EFGBNMS, Gulf of Mexico	September 2005
DFH11-12C	*Antipathes furcata*	Black Coral	Volcano Banks, Gulf of Mexico	September 2005
DFH11-13B	*Acanthopathes thyoides*	Black Coral	WFGBNMS, Gulf of Mexico	September 2005
DFH11-19C	*Sticopathes* sp.	Black Coral	WFGBNMS, Gulf of Mexico	September 2005
DFH11-20A	*Antipathes atlantica*	Black Coral	WFGBNMS, Gulf of Mexico	September 2005
DFH11-20B	*Tanacetipathes tanacetum*	Black Coral	WFGBNMS, Gulf of Mexico	September 2005

C7	*Antipathes caribbeana*	Black Coral	WFGBNMS, Gulf of Mexico	September 2005
C12	*Antipathes caribbeana*	Black Coral	WFGBNMS, Gulf of Mexico	September 2005
C13	*Plumapathes pennacea*	Black Coral	WFGBNMS, Gulf of Mexico	September 2005
A363	*Muricea atlantica*	Octocoral	Isla Tesoro, Cartagena	September 2005
A486	*Muricea pinnata*	Octocoral	Salmedina, Cartagena	September 2005
A526	*Muriceopsis flavida*	Octocoral	Burbujas, Cartagena	September 2005
A545	*Eunicea flexuosa*	Octocoral	Burbujas, Cartagena	September 2005
L3	*Leptogorgia* sp.	Octocoral	Juan Chincho, Gorgona	September 2005
L5	*Leptogorgia* sp.	Octocoral	Juan Chincho, Gorgona	September 2005
L8	*Leptogorgia* sp.	Octocoral	Juan Chincho, Gorgona	September 2007
G55	*Pacifigorgia* sp.	Octocoral	Montañitas, Gorgona	September 2007
G93	*Pacifigorgia* sp.	Octocoral	Montañitas, Gorgona	September 2007
G96	*Pacifigorgia* sp.	Octocoral	Montañitas, Gorgona	September 2007

EFGBNMS: East Flower Garden Bank National Marine Sanctuary

WFGBNMS: West Flower Garden Bank National Marine Sanctuary

ANNEX 2 – IGS/ETS LENGTH

SPECIES	BAND LENGTH
Plumapathes pennacea	1000 bp
	1200 bp
	1300 bp
Tanacetipathes barbadensis	850 bp
	1000 bp
	1100 bp
Antipathes atlantica	900 bp
	1500 bp
	1600 bp
	1800 bp
Muricea pinnata	500 bp
	1000 bp
	1200 bp
	1250 bp
Muricea atlantica	600 bp
	1100 bp
	1250 bp