**PHYLOGENETIC RELATIONSHIPS AMONG THE CARIBBEAN MEMBERS
OF THE *CLIONA VIRIDIS* COMPLEX (PORÍFERA, DEMOSPONGIAE,
HADROMERIDA) USING NUCLEAR AND MITOCHONDRIAL DNA
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**Trabajo de Grado para Aspirar al Título de
Magister en Ciencias Biológicas**

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Phylogenetic relationships among the Caribbean members of the *Cliona viridis* complex (Porifera, Demospongiae, Hadromerida) using nuclear and mitochondrial DNA sequences

Abstract

A *species complex* is a group of closely related species, where intraspecific variability overlaps with interspecific variation. Frequently, members of a species complex do not have complete reproductive isolation, therefore, the complex may go through extensive gene flow. In the Caribbean Sea, some encrusting and excavating sponges of the genus *Cliona* (Porifera, Hadromerida, Clionaide) are grouped within the great *Cliona viridis* complex because of its morphological similarities. This study examined the evolutionary relationships of the Caribbean members of the “*Cliona viridis* complex” (*C. caribbaea*, *C. tenuis*, *C. aprica*, and *C. varians*) and related taxa based on nuclear (ITS1 and ITS2) and mitochondrial (3' end of ND6) sequences. The intragenomic ITS variation and its secondary structures were evaluated using a mixed approach of Denaturing Gradient Gel Electrophoresis (DGGE), DNA sequencing and secondary structure prediction. Considerable intragenomic variation was found in all the species, with apparently functional ITS1 and ITS2 secondary structures. Despite the subtle morphological differentiation in these excavating sponges, the intragenomic copies of *C. caribbaea*, *C. tenuis* and *C. aprica* had a polyphyletic placement in the ITS1 and ITS2 genealogies and very low divergence. Therefore, it is clear that these species constitute a species complex (Ct-complex). The only exception was *C. varians*, which presented a monophyletic clade and a group of putative specimens of *Pione*. These species were the closest sister groups to the Ct-complex. Genetic distances revealed that part of the interspecific variation overlapped with intraespecific variation, which suggest either incomplete lineage sorting or extensive gene flow within the Ct-complex. Additionally our results support a monophyletic group with *C. laticavicola* and *C. delitrix*, but absence of reciprocal monophyly in the species, suggests their recent divergence. Our data shows that the 3' end of ND6, mitochondrial gene was highly conserved and not suitable for phylogenetic analysis at the interspecific level.

Key words: Species complex; Porifera; boring sponges; Caribbean; *Cliona*; ITS; ND6; DGGE; Intragenomic variation.

Relaciones filogenéticas entre miembros del Caribe del Complejo *Cliona viridis* (Porífera, Demospongiae, Hadromerida), usando secuencias nucleares y mitocondriales

Resumen

Un *complejo de especies* es un grupo de especies estrechamente relacionadas, donde la variabilidad intraespecífica se solapa con la variación interespecífica. Frecuentemente, miembros de un complejo de especies no presentan un aislamiento reproductivo completo, por lo tanto, pueden experimentar un amplio flujo genético. En el Mar Caribe, algunas esponjas excavadoras incrustantes del género *Cliona* (Porifera, Hadromerida, Clionidae) son agrupadas dentro del gran complejo *Cliona viridis* debido a sus similitudes morfológicas. Este estudio examinó las relaciones evolutivas de los miembros del Caribe del complejo *C. viridis* (*C. caribbaea*, *C. tenuis*, *C. aprica* y *C. varians*) y taxa relacionados, basados en secuencias nucleares (ITS1 e ITS2) y mitocondriales (extremo 3' del ND6). La variación intragenómica del ITS y sus estructuras secundarias fueron evaluadas usando una combinación de Electroforesis en geles de gradiente denaturante (DGGE), secuenciación de ADN y predicción de estructuras secundarias. Variación intragenómica considerable se encontró en todas las especies, que presentan una estructura secundaria del ITS1 e ITS2 aparentemente funcional. A pesar de las sutiles diferencias morfológicas en estas esponjas excavadoras, las copias intragenómicas de *C. caribbaea*, *C. tenuis* y *C. aprica* presentan una distribución polifilética en las genealogías del ITS1 e ITS2, con distancias de ramas muy cortas entre ellas. Por lo tanto, demuestra que estas especies constituyen un complejo de especies (complejo-Ct). Las únicas excepciones fueron *C. varians*, que presenta un clado monofilético, y un grupo de especímenes descritos como *Pione*. Estas especies fueron grupos hermanos estrechamente relacionados al complejo-Ct. Distancias genéticas mostraron que parte de la variación interespecífica se solapa con la variación intraespecífica, sugiriendo una purificación incompleta de linajes o amplio flujo genético entre las especies del complejo-Ct. Adicionalmente, nuestros resultados proveen un grupo monofilético compuesto por *C. laticavicola* y *C. delitrix*, pero con sus relaciones internas completamente polifiléticas, sugiriendo una divergencia reciente entre ellas o que son probablemente una sola especie. El extremo 3' del ND6, un gen mitocondrial, fue altamente conservado en nuestros datos, haciendo esta región poco variable para análisis filogenéticos a un nivel interespecífico.

1. Introduction

Species complex is commonly used to refer to a group of closely related species either on a morphological or molecular level, where intraspecific variability overlaps with interspecific variation (Mayr and Ashlock, 1991; Meyer and Paulay, 2005). Frequently, members or populations of a species complex may go through extensive gene flow (introgression) minimizing speciation rates and therefore preventing a complete reproductive isolation, affecting species integrity (Petit and Excoffier, 2009). Such complexes are very common in marine environments, where great abundance of organisms have few morphological differentiations, but in some cases are genetically different, therefore generating cryptic species (Knowlton, 1993).

For a long time, taxonomists have used a morphological classification system to recognize and delineate species boundaries, which have been shown to be limited for the understanding of the great richness of species in the marine environment. Therefore, the incorporation of molecular methods in the systematics of complex groups is essential to understand and clarify the origin and the evolutionary history of organisms (Erpenbeck and Wörheide, 2007; Xavier et al., 2010). In Porifera the phylogenetic relationships are enigmatic and controversial due to a simple bauplan, limiting the selection of conspicuous morphological characters for a robust phylogenetic reconstruction. The classification system is based fundamentally on the form, type, size, combination and arrangement of the constitutive elements that form the skeleton, which comprise spongin fibers and calcareous (class Calcarea) and siliceous spicules (class Demospongiae and Hexactinellida) (Hooper and van Soest, 2002). Due to the paucity of morphological characters, genetics studies in taxonomically problematic groups have increased exponentially over the last decade, taking into account electrophoresis patterns (e.g., *Petrosia*, Bavestrello and Sarà, 1992; *Thetya*, Sarà et al., 1993; *Cliona*, Barbieri et al., 1995; Bavestrello et al., 1996), isoenzymes and alloenzymes genotyping (e.g., *Clathrina*, Klautau et al., 1994, *Chondrilla*, Klautau et al., 1999; *Plakina*, Muricy et al., 1996; *Latrunculia*, Miller et al., 2001), and mitochondrial (CO1) and ribosomal gene sequences (18S, 28S, ITS1 e ITS2) (e.g., *Crambe*, Duran et al., 2004; *Axinella*, Lopez et al., 2002; Aplysiniidae, Schmitt et al., 2005; *Astrosclera*, Wörheide et al., 2000; Hadromerida and Clathrinida, Wörheide et al., 2004), all demonstrating significant levels of

interspecific genetic divergence and arguing that the biodiversity of sponges at the species level has been greatly underestimated.

The species belonging to the family Clionaidae represent an important group of excavating and boring sponges, due to their capacity for excavating limestone substrata such as scleractinian corals, shells and coralline algae (Rützler, 2002a). Bioerosion by this group of sponges is a destructive process that contributes to the production of reef sediment and affects the integrity of corals (Rützler, 1975). In healthy reef ecosystems bioerosion and accretion rates are approximately equal (Hein and Risk, 1975), making the erosion capability of this group as essential as any other process to the reef dynamics. Therefore, an increase in the population densities of these sponges may lead to an imbalance in the coral reefs. Due to a positive correlation between erosion rates and organic pollution levels, and dissolved nutrients, the imbalance is even more likely (e.g., Rose and Risk, 1985; Ward-Paige et al., 2005).

In the Caribbean Sea, boring sponges of genus *Cliona* Grant, 1826, have been monopolizing the reef substrate during the last decades causing tissue loss in colonies of the most common reef-building corals. Coral tissue loss leads to long-term changes of community structure and threatens the physical stability of the reefs ecosystems (see López-Victoria et al., 2006; Williams et al., 1999). The impact on the community structure is enhanced by a symbiosis that occurs with photosynthetic zooxanthellae (*Symbiodinium*) that improves the sponge growth and its bioerosion rates (Granados et al., 2008; Hill, 1996; Rützler, 2002b). However, the association sponge-zooxanthellae is still poorly understood compared to the association coral-zooxanthellae (Schönberg et al., 2005). These sponges are known as *Cliona caribbaea* Carter, 1882; *C. aprica* Pang, 1973; *C. tenuis* Zea and Weil, 2003; and *C. varians* Duchassaing and Michelotti, 1864, grouped within the great “*Cliona viridis* complex” (later on referred as *Cv*-complex) that also comprises Atlantic, Mediterranean and Indo-Pacific species (*C. viridis*, *C. nigricans*, *C. orientalis*) for its morphological similarities (Schönberg, 2002). Nevertheless, recent morphological studies performed by Zea and Weil (2003) suggest that *Cliona varians* from the Caribbean should not be included as part of this complex, because of significant morphological differences compared to other species. This study focused on the phylogenetic relationships if the

Cv-complex and the sibling species *C. laticavicola* Pang, 1973, and *C. delitrix* Pang, 1973, in the Caribbean Sea (Fig. 1).

Fig 1. Images of six Caribbean boring sponges. **A.** *Cliona aprica*; **B.** *C. tenuis*; **C.** *C. laticavicola*; **D.** *C. varians*; **E.** *C. caribbaea*; **F.** *C. delitrix*. A, B, D y E belong to the Caribbean Sea Cv-complex. C y F are sister species of the Cv-complex.

Since 1882 different specimens belonging to the genus *Cliona* have been described for the Caribbean Sea using morphological characters (see Zea and Weil, 2003 and references therein). As a result, these specimens are being considered in some cases (1) as a single highly variable species (e.g., *C. aprica* and *C. caribbaea* (Rützler, 2002b), or (2) as different species (Acker and Risk, 1985; Pang, 1973; Rützler, 2002b; Zea, 2001; Zea and Weil, 2003), depending on the scale of study and the variables used (morphological and/or ecological). On the other hand, alternative studies to the traditional morphological taxonomy have been rarely applied to these boring sponges. There are only two different studies that have used molecular data to explore the classification, identification and phylogenetic systematics of some species of the genus *Cliona*. Bavestrello et al. (1996) used electrophoresis analysis aiming to find additional characters that

would distinguish some *Cliona* species from the Mediterranean Sea, and Xavier et al. (2010), more recently, investigated the phylogenetic relationships between two forms of *C. celata* using two mitochondrial (CO1, Atp8) and one nuclear-ribosomal (28S) gene. The previous molecular studies are limited to the Mediterranean Sea, therefore studies for the *Cliona* species distributed in the Caribbean Sea are urgently needed. The new studies will allow inferring and understanding the evolutionary history of these boring sponges.

As a result of the previous ideas, the objective of this study is to help clarifying the evolutionary relationships of the Caribbean members of the *Cv*-complex (*C. caribbaea*, *C. tenuis*, *C. aprica*, and *C. varians*) and other related taxa using molecular data. For this, we employed nucleotide sequences of nuclear ribosomal DNA internal transcribed spacers (ITS1 and ITS2) and mitochondrial (end 3' of ND6) regions from several taxa to: (1) evaluate if the *Cv*-complex is really a complex or if there is any sing of cryptic speciation and, (2) define the levels of interspecific and intraspecific variation among the *Cv*-complex and other related taxa. The ITS regions are commonly used as high resolution markers and have been useful in corals and sponges to study intra- and interspecific evolution to date (van Oppen et al., 2000). On the other hand, we evaluated the end 3' of ND6 as a possible alternative to CO1 barcode fragment that present low variability in this organisms at the intraspecific and population level (Duran et al., 2004).

2. Materials and Methods

2.1 Sample Collection

Specimens belonging to the *Cv*-complex and related taxa were collected by SCUBA diving at eight locations throughout the Caribbean Sea, with a greater effort in the Colombian Caribbean (Fig. 2). The sampling effort covered almost all of the species distribution range. Some of the specimens were obtained from previous studies (i.e., Granados et al. (2008) or by Dr. Sven Zea (U. Nacional, Colombia). *Cliona orientalis* sample was collected by Dr. John Hooper (Queensland Museum, Brisbane) at Heron Island (Queensland, Australia) in August 1998. Other sequences from the Mediterranean and Florida Keys (3 specimens) were obtained from GenBank nucleotide sequence database (Accession Nos. AF062606, AF254582 and AF254583) (see

Appendix A). Specimens of *Spirastrella coccinea* were also collected and used as outgroup in the phylogenetic analyses. All specimens were preserved in 96% ethanol, stored at -80 °C for the molecular analyses, and identified by an expert taxonomist (Dr. S. Zea) using morphological characteristics of the spicules (Appendix A).

2.2 DNA extraction, amplification, and sequencing

DNA was extracted from 96% ethanol preserved tissue following a CTAB-FCIA-CIA protocol (Coffroth et al., 1992). In summary, cells were lysed using CTAB and proteins were digested using proteinase K. DNA was purified using a mixture of phenol, chloroform and isoamyl alcohol (FCIA) (24:24:1) and chloroform, isoamyl alcohol (CIA) (24:1), and precipitated with ethanol (96%, 70%). DNA was suspended in TE buffer (30 µl), and diluted (1:50) for PCR use. Polymerase chain reactions were conducted employing two nuclear (for ITS1 and ITS2 regions) and one mitochondrial (for ND6 region) set of primers designed in this study specifically for this group of sponges (Table 1). The PCR reactions were prepared to a final volume of 20 µl (2 µl of template) using the following concentration of reagents: 1x NH₄ buffer, 0,2 mM dNTPs, 3.0 mM MgCl₂, 1,5 u Taq polymerase (BiolaseTM, Bioline) and 0,15 uM primers. The amplification profiles were carried out in MyCyclerTM thermocyclers (Bio-Rad Laboratories Inc.), with an initial denaturation step of 2 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C and a final elongation of 10 min at 72 °C. PCR products were visualized on 1% agarose gels stained with ethidium bromide to check for the positive amplification of the regions. Subsequently, amplified products were cleaned using EDTA-OH precipitation protocol and sequenced at both ends with BigDye Terminator kit (Applied Biosystems Inc.) in the automated sequencer ABI 310 (Applied Biosystems Inc.).

Table 1.

Primers sets and PCR thermal profiles.

Regio	Primer	Sequence (5'-3')	PCR profile
ITS1	D-ITS1-F	AAAAGTCGTAACAAGGTTTCCG	(94 °C:50 s; 59°C:60 s; 72 °C:120 s) x 35
	D-ITS1-R	TTGCGTTCAAAGACTCGATG	
ITS2	D-ITS2-F	CATCGAGTCTTTGAACGCAA	(94 °C:30 s; 57.5°C:60 s; 72 °C:60 s) x 35
	D-ITS2-R	GAGATTCAGAAACAGAGGTAGGC	
ND6	ND6E-F	TGGCTYCTATGGTCTAGKGGTTAAGAC	(94 °C:50 s; 60°C:50 s; 72 °C:600 s) x 35
	ND6E-R	CCCCAATTATAAA YCCSGYTGGC

Fig 2. Sampling sites in the Caribbean Sea. The letters on the map (A-G) indicate the locations. Numbers in parentheses indicate the sample size for each locality. Red points indicate the sampling sites per locality. F and G are not shown in detail. F. Belize (Carrie Bow Cay); G. Bahamas.

For the two nuclear markers (ITS1 and ITS2), we took into account those individuals who had multiple sequences in the chromatograms. Multiple sequence samples were amplified again, and the different copies were separated using a Denaturing Gradient Gel Electrophoresis (DGGE) with a 45-80% urea-deionized formamide gradient, ran for 14h at 90V, 60 °C for ITS2, and 15h at 120V for ITS1. The gels were stained with ethidium bromide (10mg/ml) for 30 min and visualized in the same way as the agarose gels. The multiple copies, observed as distinctive bands, were cut and suspended in double distilled water. Subsequently, for each band, re-amplification was performed with a temperature of 2 °C higher than the original annealing temperature. PCR products were treated in the same way as described above. ITS1, ITS2 and end 3' of ND6 sequences have been deposited in GenBank under Accession Nos. XXXXXXXX-XXXXXXX, XXXXXXXX-XXXXXXX and XXXXXXXX-XXXXXXX respectively.

2.3. Alignments and Secondary Structure Inference

Complementary chromatograms were assembled and edited using Geneious 4.8.3 software (Biomatters Ltd.). Sequences from each region (ITS1, ITS2, and ND6) were aligned independently using ClustalW (gap opening penalty=15, gap extension penalty= 6.66) (Larkin et al., 2007). To remove redundant sequences, the alignments were revised in MacClade 4.08 (Maddison and Maddison, 2005). Sequence sets of the nuclear regions (ITS1, ITS2) had an additional analysis with the 4SALE software (Seibel et al., 2006), which allows the incorporation of secondary structure information to improve the alignment. The secondary structure were inferred in the MFOLD server v3.2 based on the minimization of the Gibbs free energy (Zuker, 2003) and on structural homology with the typical eukariotic model (four helixes and a central ring) (Coleman and Mai, 1997; Coleman, 2003). The secondary structures were visualized and edited with the VARNA v3.7 software (Darty et al., 2009). Uncorrected *p*-distances were calculated using PAUP* v4.0b10 (Swofford, 2002) and nucleotide diversities were inferred in DNAsp v5.10.01 (Librado and Rozas, 2009).

2.4. Phylogenetic Inferences

The data set of each individual region was analyzed independently to infer its evolutionary history using maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI)

methods. MP was conducted in PAUP* v4.0b10 (Swofford, 2002) under an heuristic search and the statistical confidence on nodes was estimated via 1000 non-parametric bootstrap. ML analyses were performed with the RAxML software v7.2.7 (Stamatakis et al., 2008) in the CIPRES Science Gateway v3.0 (<http://www.phylo.org>), using the GTR+G+I substitution model and 1000 non-parametric bootstrap. BI was carried out in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using GTR+G (ITS1 e ITS2) and HKY+I (ND6) substitution models obtained in MrModelTest 2.3 (Nylander, 2004). The analyses were run applying 1×10^7 Monte Carlo Markov Chain (MCMC) generations (x4 chains), with a sampling frequency of 100 generations (burnin= 2500). To estimate the evolutionary history using ITS regions and end 3' of ND6 we employed *Spirastrella coccinea* and *Axinyssa flaveolivescens* as outgroup taxa, respectively.

3. Results

For almost every specimen we obtained complete sequences for the three regions (ITS1, ITS2 and ND6), but in some cases, sequences from ITS2 and ND6 presented low quality and were excluded (see Table 2). For the nuclear sequences, ITS1 was longer than ITS2 for every individual on the study. In general, the sequences from individuals that presented multiple copies, exhibited similar characteristics for each of the regions such as sequence length, GC content, and nucleotide diversity. ITS1 had an average longitude of 251 bp, with a standard deviation of only 4.44 bp and an average GC content of 53.03% with a standard deviation of 1.45%. On the other hand, ITS2 presented an average longitude of 179 bp with a standard deviation of 4.31 bp, and an average GC content of 66% with a standard deviation of 2.66%. Nucleotide diversity, in average, was higher for ITS1 ($\pi = 0.00974$) than ITS2 ($\pi = 0.00576$), with a maximum observed variation in *C. tenuis* F49 (ITS1 $\pi_{\max} = 0.02767$) and *C. laticavicola* A910 (ITS2 $\pi_{\max} = 0.01949$).

Table 2

Characteristics of the sequences obtained for each region.

Region	No. of sequences	No. of Genotypes	Length (bp)	GC (%)	Variation (%)
ITS1	106	58	237-255	54.0 – 59.1	15.9
ITS2	61	29	170-187	61.1 – 68.9	22.6
ND6	21	8	307-316	28.2 – 30.9	7.2

The 3' end of ND6 had an average length of 313 bp and a standard deviation of 2.15 bp. The GC content was much lower than the one found in nuclear regions, with an average of 29.74% and a standard deviation of 1.05%. This region was highly conserved in the studied sponges, creating high redundancy on the sequences and very few haplotypes. The haplotypes are shared among all the species of the complex and their sister species. Additionally the amplification and sequencing success was low, making this mitochondrial region not trustable for phylogenetic analyses at the interspecific level.

3.1. Intragenomic Variation of ITS1 and ITS2

Intragenomic variation was not observed for all taxa. Only 46% of the individuals had variation for the ITS1 region and 66% for the ITS2 region. Additionally, a small percentage of the copies could not be re-amplified and, for some specimens, the intragenomic divergence between copies was negligible. This divergence is expressed as nucleotide diversities (π) and maximum p -distances (Table 3 and 4).

Intragenomic variation could be further explored by means of transitions (TS), transversions (TV) or indels (IND) (Table 5). For this study, observed intragenomic polymorphisms were generated mainly by transitions and transversions, and not by indels. Variation on ITS1 was generated, mainly by TS with an average of 3.57 bp, followed by TV with an average of 2.04 bp. Variation on ITS2 is also produced by TS with an average of 1.5 bp, followed by TV with an average of 1.0 bp. Sequence variability, as a result of TS and TV, is reflected on a low sequence length polymorphism for each region, with a standard deviation of 3.23 bp for ITS1 and 4.10 bp for ITS2. ITS1 variation, in most of the sequences, is found at a T_n monomer repeat between positions 160-200 which is interrupted by one or two adenines.

3.2 Overlap between Intra- versus Interspecific Variation of ITS1 and ITS2

Considering the uncorrected p -distances, it is clear that there is a similar degree of overlapping between the intra and interspecific variation in both nuclear regions (Fig. 3). As a result, a great redundancy on the sequence set is generated, showing a tight relationship among taxa. For ITS1, uncorrected p -distances values of 0.0 were observed at the interspecific level on many individuals

Table 3

Studied taxa that presented multiple ITS1 copies, showing sequence length of, GC-content, greatest nucleotide diversity (π), maximum p -distance found between different copies of each taxa and numbers of intragenomic polymorphisms (IGPs).

Taxa	Location	Length (bp)	GC (%)	π	Max. p -distance	IGPs
<i>C. laticavicola</i> F15	IFT	238	54.2	0.00420	0.00424221	2
<i>C. laticavicola</i> M110	CTG	237	54.4	0.00420	0.24109063	3
<i>C. varians</i> M26	CTG	254	54.7	0	0	2
<i>C. varians</i> M25	CTG	254	54.7	0.00394	0.00393701	2
<i>C. varians</i> M119	SMR	252	55.6	0.00794	0.00797762	2
<i>C. varians</i> F56	IFT	253	54.9	0.01186	0.01181102	2
<i>C. tenuis</i> M05	CTG	251	57.4	0.01116	0.02755906	5
<i>C. tenuis</i> M86	CTG	251	57.5	0	0	2
<i>C. tenuis</i> M67	CTG	251	57.8	0.01328	0.01996928	3
<i>C. tenuis</i> M09	CTG	251	57.4	0.01195	0.01195219	2
<i>C. tenuis</i> M01	CTG	253	58.5	0	0	4
<i>C. tenuis</i> M84	CTG	253	58.5	0.00264	0.00395257	2
<i>C. tenuis</i> F08	IFT	253	58.5	0.01581	0.02367598	4
<i>C. tenuis</i> M53	CTG	254	58.3	0.01859	0.03149606	3
<i>C. tenuis</i> M156	IFT	252	57.5	0.01195	0.01196311	2
<i>C. tenuis</i> F14	IFT	254	58.7	0.01976	0.01982272	2
<i>C. tenuis</i> M32	CTG	254	57.6	0.01200	0.01185491	2
<i>C. tenuis</i> M30	CTG	254	57.9	0.01587	0.01594982	2
<i>C. tenuis</i> M70	CTG	254	57.9	0.00394	0.00787402	3
<i>C. tenuis</i> F49	IFT	253	57.9	0.02767	0.02761299	2
<i>C. tenuis</i> F54	IFT	253	57.9	0.01581	0.01574803	2
<i>C. aprica</i> F10	IFT	253	57.3	0.00397	0.00400771	2
<i>Pione</i> sp. M47	CTG	251	57.0	0.00398	0.00398406	2
<i>Pione</i> sp. M63	CTG	251	56.6	0.01328	0.01992032	2

of the species *C. aprica*, *C. tenuis* and *C. caribbaea* (Fig. 3A, 3B and 3C). There was a particular case where one of the multiple copies of *C. aprica* M11 had an identical distance as some copies of *C. varians*. Likewise, distances up to 0.128 at the intraspecific level were observed between individuals of *C. aprica*, especially in the copy M11A that showed a closer relationship with copies from *C. varians*. If we don't take into account the distances of *C. aprica* M11A, the values at intraespecific level, reach a maximum of 0.035, and at interspecific level of 0.055 mainly between variants of *C. tenuis*. On the other hand, ITS2 showed a similar pattern, with uncorrected p -distance of 0.0 at the intraspecific level between individuals from the species *C. tenuis*, *C. caribbaea*, *C. aprica* and a particular case of a variant of *C. aprica* A752 and all of the variants from *C. varians*. Distances up to 0.168 were observed at the intraspecific level mainly on individuals of *C. aprica*. In general, both groups of data (ITS1 and ITS2) showed that the interspecific variation was higher than the intraspecific variation, which in the ITS1 region had averages of 0.079 and 0.018 and in the ITS2 with averages of 0.085 and 0.013.

Remarkably, the species *C. laticavicola* and *C. delitrix* currently considered distinct species, showed an important overlap between their intra- and interspecific variations. For ITS1, uncorrected *p*-distances values at the intraspecific level (0.017) were higher than interespecific level (0.012), mainly between variants of *C. laticavicola*.

Table 4

Studied taxa that presented multiple ITS2 copies, showing sequence length, GC-content, greatest nucleotide diversity (π), maximum *p*-distance found between different copies of each taxa and numbers of intragenomic polymorphisms (IGPs).

Taxa	Location	Length (bp)	GC (%)	π	Max. <i>p</i> -distance	IGPs
<i>C. laticavicola</i> A909	SMR	173	62.4	0.00578	0.00578035	2
<i>C. laticavicola</i> A910	SMR	173	62.4	0.01949	0.02890173	3
<i>C. tenuis</i> C179	CTG	182	68.1	0	0	2
<i>C. tenuis</i> X2	CTG	182	68.1	0.00549	0.01647624	2
<i>C. tenuis</i> X19	CTG	182	67.6	0.00733	0.01098901	3
<i>C. caribbaea</i> 5	---	182	68.1	0.00221	0.00562061	2
<i>C. varians</i> M25	CTG	181	65.2	0	0	2

3.3 Secondary Structure of ITS1 and ITS2

RNA secondary structures were predicted for all the ITS1 and ITS2 sequences. Each region exhibited consistent homologous structures. The ITS1 region, as well as the ITS2 region, showed a secondary structure made up by a central ring and four helixes (I, II, III, IV) (Fig. 4).

In ITS1 secondary structures, helix I was the most conserved in almost all the studied taxa (7 paired bases), *C. laticavicola* and *C. delitrix* had a shorter helix (4 paired bases). Helix II, III and IV were larger than helix I, and the structure's conservation degree increased in direction 5'→3', being helix IV the most variable and helix I the less variable. Helix IV was where the majority of polymorphisms, and the T_n monomer repeats, were concentrated. On the other hand, some taxa that showed intragenomic variation maintained their structure (e.g., *Pione* sp. M63, *Pione* sp. M47, *C. tenuis* F14, *C. tenuis* M156, *C. tenuis* M84). The most extreme case was *C. tenuis* F14 that presented up to 9 changes between TS and TV, all of them located on helix IV.

The ITS2 region had a much more conserved structure, shared by all taxa. As in the ITS1, the secondary structures from individuals belonging to *C. laticavicola* differ slightly in size and helix

disposition. The secondary structure of ITS2 for *C. delitrix* is not presented here as due the lack of sequencing data for this particular region and taxon. *Cliona laticavicola* A910 was the sample that presented more polymorphisms in their copies, with 12 changes between TS, TV, and IND.

Table 5

Intragenomic variation observed in the studied sponges, for ITS1 and ITS2 regions and number of transitions (TS), transversions (TV) and indels (IND).

Taxa	Location	ITS1			ITS2			Total aligned length (bp)
		TS	TV	IND	TS	TV	IND	
<i>C. laticavicola</i> F15	IFT	0	1	1	--	--	--	239
<i>C. laticavicola</i> M110	CTG	28	28	24	--	--	--	256
<i>C. varians</i> M26	CTG	0	0	0	--	--	--	254
<i>C. varians</i> M25	CTG	1	0	0	0	0	0	254-181
<i>C. varians</i> M119	SMR	2	0	2	--	--	--	254
<i>C. varians</i> F56	IFT	2	1	1	--	--	--	254
<i>C. tenuis</i> M05	CTG	7	0	3	--	--	--	254
<i>C. tenuis</i> M86	CTG	0	0	1	--	--	--	252
<i>C. tenuis</i> M67	CTG	3	2	1	--	--	--	252
<i>C. tenuis</i> M09	CTG	3	0	0	--	--	--	251
<i>C. tenuis</i> M01	CTG	0	0	1	--	--	--	254
<i>C. tenuis</i> M84	CTG	1	0	0	--	--	--	253
<i>C. tenuis</i> F08	IFT	4	0	1	--	--	--	254
<i>C. tenuis</i> M53	CTG	8	1	3	--	--	--	254
<i>C. tenuis</i> M156	IFT	3	0	0	--	--	--	252
<i>C. tenuis</i> F14	IFT	4	5	0	--	--	--	254
<i>C. tenuis</i> M32	CTG	0	0	1	--	--	--	255
<i>C. tenuis</i> M30	CTG	1	7	0	--	--	--	254
<i>C. tenuis</i> M70	CTG	1	1	0	--	--	--	254
<i>C. tenuis</i> F49	IFT	6	0	1	--	--	--	254
<i>C. tenuis</i> F54	IFT	3	0	1	--	--	--	254
<i>C. aprica</i> F10	IFT	1	0	2	--	--	--	255
<i>Pione</i> sp. M47	CTG	0	1	0	--	--	--	251
<i>Pione</i> sp. M63	CTG	4	1	0	--	--	--	251
<i>C. laticavicola</i> A909	SMR	--	--	--	1	0	0	173
<i>C. laticavicola</i> A910	SMR	--	--	--	7	3	2	175
<i>C. tenuis</i> C179	CTG	--	--	--	0	0	0	182
<i>C. tenuis</i> X2	CTG	--	--	--	0	1	1	183
<i>C. tenuis</i> X19	CTG	--	--	--	1	1	1	183
<i>C. caribbaea</i> 5	---	--	--	--	0	1	0	182

3.4 Molecular Phylogenetic Inferences

The phylogenetic hypothesis obtained from nuclear regions, shared four principal clades under the three approaches (MP, ML and BI) with bootstrap values higher than 70% and posterior probabilities higher than 0.80 (Fig. 5 and 6): (1) a polyphyletic clade, uniting the individuals belonging to *C. caribbaea*, *C. tenuis* and *C. aprica* (referred as *Ct*-complex hereafter), with short

branch lengths between them. (2) a monophyletic clade composed by the specimens identified as *Pione* sp., more related to the *Ct*-complex than other sympatric clionids. (3) a monophyletic clade (hereafter referred as “*varians*”) including the individuals belonging to *C. varians* and the closest sister group to *Pione*, and the *Ct*-complex and, (4) a well supported “*laticavicola-delitrix*” clade (*C. laticavicola* + *C. delitrix*), as the sister group of *Pione*, and the *Cv*-complex.

Fig. 3 Frequency histograms of intra- and interspecific variation. The frequencies correspond to the different uncorrected *p*-distances found in ITS1 and ITS2 variants to different combinations from *C. varians*, *C. tenuis*, *C. aprica*, *C. caribbaea* and *Pione* sp. In each box (A – F), histograms of the left correspond to ITS2 distances and histograms of the right to ITS1 distances. For this analysis, *Ct*-complex (in D and E) was assumed as a one species.

The hypothesis obtained from de ND6 region (Fig. 7) lacks of phylogenetic structure and shows all the species to be polyphyletic and certain haplotypes closer to other non clionid families (Spirastrellidae). It was necessary to use a more distant outgroup (i.e., *Axinyssa flaveolivescens* Halichondrida: Halichondriidae) than the one used in the nuclear analyses (*Spirastrella coccinea* Hadromerida: Spirastrellidae) because this region showed a lowest nucleotidic variation percentage (7.2%). The results might been affected by the lack of reliable data for this region.

Fig. 4 Model of the ITS1 and ITS2 transcript secondary structures to *Cliona*. ITS1 in green color, 5.8s in gray color and ITS2 in blue color. Helices are labeled I-IV for each spacer.

Fig 5. Phylogenetic hypothesis of species of the genus *Cliona* belonging to Caribbean Cv-complex and related taxa. The tree was obtained through ML analysis of the ITS1 dataset with non-redundant taxa. Outgroup: *S. coccinea* (Spirastrellidae). Support values $\geq 70\%$ are shown on the branches. Each value over the branches support found with MP, and values below the branches support found with ML/BI. The scale bar represents the number of substitutions per site. The specimens are coded as follows: species, code-intragenomic variant, and locality. Sketches of predicted secondary structures correspond to a model for each clade.

Fig 6. Phylogenetic hypothesis of species of the genus *Cliona* belonging to Caribbean Cv-complex and related taxa. The tree was obtained through ML analysis of the ITS2 dataset with non-redundant taxa. Outgroup: *S. coccinea* (Spirastrelledae). Support values $\geq 70\%$ are shown on the branches. Each value over the branches support found with MP, and values below the branches support found with ML/BI. The scale bar represents the number of substitutions per site. The specimens are coded as follows: specie, code-intragenomic variant, and locality. Sketches of predicted secondary structures correspond to a model for each clade

The internal relationships of the four clades are, in most cases, poorly resolved with low bootstrap and posterior probability supports. This poor topological resolution exemplifies the groups' limited sequence variation and divergence. Additionally, there was not evidence of a geographic pattern. On the other hand, the *Ct*-complex was found to be closely related to the

Mediterranean and Indo-Pacific *C. viridis* and *C. orientalis* than to the coexisting Caribbean species of the “*varians*” clade. This result however, was not consistent in the ITS1 region where *C. orientalis* is the sister taxon of all species in the Caribbean, with the exception of *C. laticavicola* and *C. delitrix*. Finally, some intragenomic copies of several species showed paraphyly, being more closely related to a different species clade. There were three cases of paraphyly for the ITS1: (1) one of the copies of *C. laticavicola* M110, was grouped with the *Ct*-complex clade, (2) one of the copies of *C. aprica* M11, was grouped with the *varians* clade, and (3) one of the specimens of *Pione* sp. was included in the *Ct*-complex instead the clade uniting the other specimens of *Pione* even though its external morphology matched the characteristics of the *Pione* group. On ITS2, paraphyly was also evident in only one of the copies of *C. aprica* A752 associated with the “*varians*” clade.

Fig 7. Phylogenetic hypothesis of species of the genus *Cliona* belonging to Caribbean Cv-complex and related taxa. The tree of the left was obtained through ML analysis of the ND6 dataset. Outgroup: *A. flaveolivescens* (Halichondriidae). Support values $\geq 70\%$ are shown on the cladogram branches of the right. Each value over the branches support found with MP, and values below the branches support found with ML/BI. The scale bar represents the number of substitutions per site. The specimens are coded as follows: specie, code, and locality.

4. Discussion

The lack of reciprocal monophyly for the morphological species of the Ct-complex supports their recent divergence. This pattern is revealed by an overlapping between inter- and intraspecific variations of nuclear regions and well-supported clades in the phylogenetic hypotheses with its internal relationship poorly resolved. Additionally, *C. laticavicola* and *C. delitrix* presented a similar case with its evolutionary relationships. On the other hand, the phylogenetic hypotheses recovered showed other new well-supported clades: *varians* and *Pione*, which were closely related to the Ct-complex.

4.1. Intragenomic variation of ITS1 and ITS2

Intragenomic variation of the rDNA ITS1 and ITS2 was evaluated in 100 specimens of boring sponges belonging to *Cliona*. The intragenomic variation was explored using DGGE to separate effectively the different variants obtained from the PCR products, as well as direct sequencing in some individuals. The DGGE is a fingerprinting technique that has opened new windows for identification, localization, and diagnosis of mutations, sequence polymorphisms in humans, microbial ecology, zooxanthellae diversity, introgressive hybridization in octocorals, among others (e.g., Dorado and Sánchez, 2009; LaJeunesse, 2001; LaJeunesse and Pinzón, 2007; Muyzer, 1999; Muyzer and Smalla, 1998; Myers et al., 1985; Thornhill et al., 2007). This is because in DGGE, DNA fragments of the same length but with different primary sequence can be separated, based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (Muyzer and Smalla, 1998).

The length of each spacers (ITS1, ITS2) in *Cliona* was consistent with previous studies on Porifera (e.g., Lopez et al., 2002; Schmitt et al., 2005; Wörheide et al., 2002), as well as in the pattern where generally ITS1 is longer than ITS2 (e.g., Alvarez et al., 2007; Wörheide et al., 2004). Here, intragenomic variation levels were somewhat similar to most of sponges that showed between two and five copies (e.g., Alvarez et al., 2007). The intragenomic variation of ITS1 and ITS2 could be explained by slow rates of concerted evolution, a process resulting in the homogenization of individuals repeats (Liao, 1999), that would lead to the maintenance of

different numbers of variants with divergent sequences given that the copies within a genome are rarely completely homogenous (LaJeunesse and Pinzón, 2007; Thornhill et al., 2007). However, due to the closely relationship in these Porifera species, additional studies are required to determine which mechanisms generate the observed variation.

Many of the studied specimens that exhibited a single copy were identified by two means: (1) because a single copy was evident in the DGGE, or (2) the direct sequencing was successful and did not require the DGGE step. Intragenomic variation was explained mainly by TS, followed by TV, and not by length polymorphisms. This result is consistent with the probabilities of substitution rates in the evolution of DNA at closely related species, where TS are more frequent than TV (Kristina Strandberg and Salter, 2004). On the other hand, some researchers attribute these substitutions to errors during PCR, mainly to the low efficiency of the polymerase (Kobayashi et al., 1999), although in our study some samples were also amplified using high quality polymerase such as *Pfu*, obtaining exactly the same results.

In spite of the substitutions presented in the different variants, the RNA transcript secondary structures from both regions showed high homology and the typical eukaryotic helicoidal ring model (see Coleman and Mai, 1997; Coleman, 2003). Most changes occurred in the inner ring and loop regions of the structures, which did not generate large variations in the arrangement of the helices. This suggests the functionality of the RNA transcript and the reliability in the obtained sequence variation.

4.2 Overlap of the Inter- and Intraspecific Variation

The uncorrected *p*-distances calculated for the nuclear regions show an overlap between inter- and intraspecific variation among the species belonging to the *Ct*-complex. The splitting point of these variations has been considered a key feature for the delimitation and identification of species (Meyer and Paulay, 2005).

The magnitude of overlapping of the intra- and interspecific variations among the species of the *Ct*-complex and the species' non-monophyly is probably due to three reasons: (1) introgression,

(2) imperfect taxonomy and (3) incomplete sorting of ancestral polymorphism (Funk and Omland, 2003). Introgression is of minor importance due to the inability to evaluate the degree of reproductive isolation among the *Ct*-complex species. On the other hand, imperfect taxonomy can cause non-monophyly when morphotypes are misrecognized as discrete species. Additionally, incomplete lineage sorting can produce overlapping of intra- and interspecific variations when ancestral polymorphisms have not yet coalesced and the species are incipient (Rosenberg, 2003). Therefore, the establishment of limits between species of the *Ct*-complex requires not only robust morphological characters, but also molecular techniques that take into account the genetic differences among species. This approach would fulfill the phylogenetic species concept (Baum, 1992; Knowlton, 2000).

4.3. Phylogenetic Hypotheses

The phylogenetic hypotheses presented in this study, support in some way, what was found by Zea and Weil (2003), whose detailed observations on internal and external morphology (size and shape of spicules) of these sponges in the Caribbean led them to suggest the hypothesis whereby is not appropriated to include *C. varians* in the complex. *C. varians* has a thick external tissue (up to 1 to 2 cm), with an amber coloration and U shaped spirasters (see Zea and Weil, 2003). Additionally, in an ecological context, these sponges grow over the substratum before excavating (Vicente, 1978). These sponges don't use the pioneering tissue to weaken the coral substratum as the other species do (López-Victoria et al., 2003 (2004)). Our results from molecular data support this hypothesis of excluding *C. varians* from the *Cv*-complex, showing a monophyletic clade for this species with high bootstrap and posterior probability values. On the contrary, the subtle morphological differences found by Zea and Weil (2003) between *C. caribbaea*, *C. tenuis* and *C. aprica* (here *Ct*-complex) are not supported by the nuclear data of ITS1 and ITS2. Even though these nuclear regions presented considerable variation, the relationships within the *Ct*-complex are completely polyphyletic. The absence of monophyletic clades within the *Ct*-complex was an expected result taking into account the grade of overlap found between the intra- and interspecific variation (Fig.3), and the polyphyletic distribution of intragenomic variants (Appendix B and C).

The monophyletic clade named “*Pione*” is conformed exclusively by specimens identified as

Pione sp. *Pione* was described as a sister group of the genus *Cliona* given its external morphology, described as always papillated or in alpha state, with little papillae not larger than 1 mm in diameter and a particular composition of spicules (Rosell and Uriz, 1997; Rützler, 2002a). Nevertheless, in our genealogy this group was more closely related to the *Ct*-complex than other species belonging to *Cliona*. It is likely that the individuals labeled as *Pione* sp. were misidentified and they might be better allocated to the genus *Cliona* as species not yet described and with considerable genetic differences comparing to the remaining Caribbean clionids.

The species grouped within the great *Cv*-complex present considerable morphological (Schönberg, 2002) and molecular similarities in spite of their geographic distances. A specimen from *C. orientalis* Thiele, 1990, which is very common in the Australian Great Barrier reef (Schönberg, 2000) and grouped inside the great *Cv*-complex, was included in this study to evaluate the relationship of this species with regard to Caribbean clionids. According to the ITS1, *C. orientalis* is a well-supported sister taxon of the “*varians*”, “*Pione*” and the *Ct*-complex clades (Fig. 5), and according to the ITS2, it is a sister taxon of the *Ct*-complex (Fig. 6). On the other hand, *C. viridis* from the Mediterranean was also included and according to the ITS2, is more closely related to the Caribbean *Ct*-complex than *C. orientalis*. Additionally, an individual collected in Santa Marta that couldn't be identified to species level (*Cliona* n. sp. A919), shows similar morphological features to *C. orientalis* and is well supported as its sister taxon in both ITS regions. In turn, *C. orientalis*, *C. viridis*, and *Cliona* n. sp. are sister taxa of *Ct*-complex supporting that species of the cosmopolitan *Cv*-complex are closely related. However, only a proper biogeographic study including the appropriate sample size of individuals of *Cv*-complex will allow us to support this statement.

The 3' end of the ND6 region showed little resolution at the species level (Fig. 7). Additionally, the small number of sequences obtained for this region is due to the low amplification and sequencing success. These problems depended on the quality of tissue, free of other associated species, associated to specificity of primers set, because only those species with a thick external tissue were successfully amplified and sequenced. Finally, this region was not taken into consideration to formulate any hypothesis about the evolutionary relationships of the complex,

because of the low phylogenetic signal and number of sequences.

4.4. *Phylogenetic Relationship between Cliona laticavicola and C. delitrix*

Taxa belonging to the species *C. laticavicola* and *C. delitrix* from different Caribbean locations, formed a single monophyletic group in the genealogy, but its relationships were completely polyphyletic. In other words, the intra- and intrespecific variations among these taxa are widely overlapping (data not shown here). *C. delitrix* is considered one of the most destructive and common bioroders in Caribbean reef corals, growing mainly on massive corals colonies such as *Montastrea cavernosa* (Rose and Risk, 1985). *Cliona laticavicola* is restricted to the Caribbean sea, presents a papillate aspect of orange color (Schönberg et al., 2006), very similar to early growth stages of *C. delitrix*, but it is symbiotic with zooxanthellae (Granados et al., 2008). Recent morphological studies of these species, suggest that there are no differences between them and that they are probably a single species (Chaves-Fonnegra and Zea, 2010 com. pers.), and this is corroborated by our molecular data (Fig. 5 and 6). A more exhaustive sampling, to obtain morphological and ecological data is urgently needed to evaluate this hypothesis. Finally, there is strong evidence of ancestral polymorphisms between this clade and the Ct-complex, where one of the four copies of the taxon *C. laticavicola* M110, presents a closer relationship with specimens of *C. tenuis*, which are distributed in the same locality (Cartagena).

In conclusion, this study shows that sponges belonging to the genus *Cliona*, with a sympatric distribution in the Caribbean, have some degree of intragenomic heterogeneity in the internal transcribed spacers 1 and 2. Polytomic intragenomic variants could be evidence of processes of incomplete lineage sorting of ancestral polymorphisms that are still present in the Caribbean clionids. At the intraspecific level, this pattern is also evident with some genetic distances equal to those shown to interspecific level and vice versa, generating a polyphyletic clade with very short divergence formed by *C. caribbaea*, *C. tenuis* and *C. aprica*. It is very probable that these sibling species don't have yet reproductive isolation barriers or that they are in the first stages of ecological speciation. On the other hand, the monophyletic clades of *C. varians* and *Pione* sp. recovered suggest that these species probably do present reproductive isolation in regards to the other species from the Ct-complex. These phylogenetic relationships are consistent with

differences found in the internal and external morphology (Zea and Weil, 2003), and suggest that the *Ct*-complex is conformed by *C. caribbaea*, *C. tenuis* and *C. aprica* and is the sister group of *C. varians* and *Pione* sp. Our data also suggest that *C. varians* should no longer be included in either the great *Cv*-complex or the *Ct*-complex. We suggest that (1) *Pione* sp. in this study should be reallocated to *Cliona* with additional morphological studies and (2) *Cliona* n. sp. A919 might be an introduced individual of *C. orientalis* in the Caribbean Sea. Morphological and ecological studies on the *laticavicola-delitrix* group are greatly needed to understand better ecological differences currently observed. Finally, to corroborate the species limits in the Caribbean clionids, as well as to sort out the nature of the intragenomic variation, an analysis of ITS2 nucleolar dominance is needed in order to tell which of the copies are actually expressed and functional. Furthermore, to understand the complete evolutionary relationship of the *Cv*-complex, is necessary to design a biogeographic study included all the species from the complex and employing variable mitochondrial regions in order to explore the history of the colonization processes.

Appendix A. List of investigated sponge samples with systematic position of sample (genus, species), sample number, the sample site and location, depth, and the indication if the PCR product has been sequenced directly.

#	Genus	Specie	Code	Site	Location	Depth (m)	Direct seq of PCR		
							ITS1	ITS2	ND6
1	<i>Cliona</i>	<i>aprica</i>	177		Bahamas 2004		Y		
2	<i>Cliona</i>	<i>aprica</i>	9					N	
3	<i>Cliona</i>	<i>aprica</i>	AC32	Los Venados	Isla Fuerte	6		Y	
4	<i>Cliona</i>	<i>aprica</i>	AC38	Los Venados	Isla Fuerte	6	N		
5	<i>Cliona</i>	<i>aprica</i>	A752	Punta Betin	Santa Marta			N	
6	<i>Cliona</i>	<i>aprica</i>	A793	Bahia Chengue	Santa Marta			N	
7	<i>Cliona</i>	<i>aprica</i>	A911	Bahia Chengue	Santa Marta			N	
8	<i>Cliona</i>	<i>aprica</i>	CL-1 S	Bajo Bonito	San Andrés Isla	12	Y		
9	<i>Cliona</i>	<i>aprica</i>	F10	Canal del Inglés	Isla Fuerte	7	N		
10	<i>Cliona</i>	<i>aprica</i>	F34	Canal del Inglés-Backreef	Isla Fuerte	0.5	Y		
11	<i>Cliona</i>	<i>aprica</i>	F35	Canal del Inglés-Backreef	Isla Fuerte	0.5	Y		
12	<i>Cliona</i>	<i>aprica</i>	M11	Caño Ratón, Sotavento	Isla Grande, Islas del Rosario, Cartagena	4	N	Y	
13	<i>Cliona</i>	<i>aprica</i>	M13	Caño Ratón, Sotavento	Isla Grande, Islas del Rosario, Cartagena	2.5	N		
14	<i>Cliona</i>	<i>aprica</i>	M37	Isla Pavitos	Islas del Rosario, Cartagena	7		Y	
15	<i>Cliona</i>	<i>aprica</i>	M50	Luis Guerra	Isla Grande, Islas del Rosario, Cartagena	14	Y		
16	<i>Cliona</i>	<i>aprica</i>	M69	Arrecife Nico	Isla Barú, Cartagena	6		Y	
17	<i>Cliona</i>	<i>aprica</i>	M72	Arrecife Nico	Isla Barú, Cartagena	6	N	Y	
18	<i>Cliona</i>	<i>aprica</i>	M97	Banco de Salmedina	Bancos de Salmedina, Cartagena	5			Y
19	<i>Cliona</i>	<i>aprica</i>	N8					N	
20	<i>Cliona</i>	<i>caribbaea</i>	104		Bahamas 2003		Y		
21	<i>Cliona</i>	<i>caribbaea</i>	150		Bahamas 2004		Y		
22	<i>Cliona</i>	<i>caribbaea</i>	5					N	
23	<i>Cliona</i>	<i>caribbaea</i>	M61	Banco Montañita	Isla Barú, Cartagena	25	N	Y	Y
24	<i>Cliona</i>	<i>caribbaea</i>	PSA199		San Andrés Isla		Y		
25	<i>Cliona</i>	<i>delitrix</i>	242		Bahamas 2007		Y		
26	<i>Cliona</i>	<i>delitrix</i>	287		Bahamas 2007		Y		
27	<i>Cliona</i>	<i>delitrix</i>	51		Bahamas 2003		Y		Y
28	<i>Cliona</i>	<i>delitrix</i>	B01	Carrie Bow Cay	Belize	8.5	Y		Y
29	<i>Cliona</i>	<i>delitrix</i>	PSA208		San Andrés Isla		N		
30	<i>Cliona</i>	<i>laticavicola</i>	A909	Bahia Chengue	Santa Marta			N	
31	<i>Cliona</i>	<i>laticavicola</i>	A910	Bahia Chengue	Santa Marta			N	
32	<i>Cliona</i>	<i>laticavicola</i>	F15	Canal del Inglés	Isla Fuerte	7	Y		Y
33	<i>Cliona</i>	<i>laticavicola</i>	F50	Bajo Bushnell	Isla Fuerte	15			Y
34	<i>Cliona</i>	<i>laticavicola</i>	F53	Bajo Bushnell	Isla Fuerte	15			Y

35	<i>Cliona</i>	<i>laticavicola</i>	M110	Arrecife la Playita	Isla Barú, Cartagena	10	N	Y	Y
36	<i>Cliona</i>	<i>laticavicola</i>	M48	Luis Guerra	Isla Grande, Islas del Rosario, Cartagena	14		Y	Y
37	<i>Cliona</i>	<i>laticavicola</i>	M51	Luis Guerra	Isla Grande, Islas del Rosario, Cartagena	10	Y	Y	
38	<i>Cliona</i>	<i>laticavicola</i>	M52	Luis Guerra	Isla Grande, Islas del Rosario, Cartagena	10			Y
39	<i>Cliona</i>	<i>laticavicola</i>	U02	Snake Bay	Curaçao	16	Y		Y
40	<i>Cliona</i>	<i>oculta</i>	272		Cartagena		Y		
41	<i>Cliona</i>	<i>orientalis</i>	CO	Heron Island	Queensland, Australia	18	Y	Y	
42	<i>Cliona</i>	<i>tenuis</i>	AC26	Los Venados	Isla Fuerte	3	N		
43	<i>Cliona</i>	<i>tenuis</i>	AC39	Los Venados	Isla Fuerte	6	Y		
44	<i>Cliona</i>	<i>tenuis</i>	B506		Cartagena			N	
45	<i>Cliona</i>	<i>tenuis</i>	B516		Cartagena			N	
46	<i>Cliona</i>	<i>tenuis</i>	C179	Las Palmas	Cartagena			N	
47	<i>Cliona</i>	<i>tenuis</i>	F06	Canal del Inglés	Isla Fuerte	7	Y		
48	<i>Cliona</i>	<i>tenuis</i>	F08	Canal del Inglés	Isla Fuerte	7	N		
49	<i>Cliona</i>	<i>tenuis</i>	F14	Canal del Inglés	Isla Fuerte	7	N		
50	<i>Cliona</i>	<i>tenuis</i>	F38	Bajo Bushnell	Isla Fuerte	15	Y		
51	<i>Cliona</i>	<i>tenuis</i>	F41	Bajo Bushnell	Isla Fuerte	15	Y		
52	<i>Cliona</i>	<i>tenuis</i>	F45	Bajo Bushnell	Isla Fuerte	15	Y		
53	<i>Cliona</i>	<i>tenuis</i>	F48	Bajo Bushnell	Isla Fuerte	15	Y		
54	<i>Cliona</i>	<i>tenuis</i>	F49	Bajo Bushnell	Isla Fuerte	15	N		
55	<i>Cliona</i>	<i>tenuis</i>	F54	Bajo Bushnell	Isla Fuerte	15	N		
56	<i>Cliona</i>	<i>tenuis</i>	M01	Punta Brava	Isla Grande, Islas del Rosario, Cartagena	8	N	Y	
57	<i>Cliona</i>	<i>tenuis</i>	M05	Boya Spawning	Isla Grande, Islas del Rosario, Cartagena	5	N	Y	
58	<i>Cliona</i>	<i>tenuis</i>	M09	Boya Spawning	Isla Grande, Islas del Rosario, Cartagena	3	N	Y	
59	<i>Cliona</i>	<i>tenuis</i>	M102	Banco de Salmedina	Bancos de Salmedina, Cartagena	12	Y	Y	
60	<i>Cliona</i>	<i>tenuis</i>	M108	Arrecife la Playita	Isla Barú, Cartagena	10	N	Y	
61	<i>Cliona</i>	<i>tenuis</i>	M139	Bajo Alicia	Isla Fuerte	11	N	Y	
62	<i>Cliona</i>	<i>tenuis</i>	M156	Bajo Alicia	Isla Fuerte	4.4	N		
63	<i>Cliona</i>	<i>tenuis</i>	M162	Bajo Alicia	Isla Fuerte	4.4	N		
64	<i>Cliona</i>	<i>tenuis</i>	M29	Isla Tesoro, Barlovento	Islas del Rosario, Cartagena	11	N	Y	
65	<i>Cliona</i>	<i>tenuis</i>	M30	Isla Tesoro, Barlovento	Islas del Rosario, Cartagena	11	N	Y	
66	<i>Cliona</i>	<i>tenuis</i>	M32	La Coca	Islas del Rosario, Cartagena	15	N	Y	
67	<i>Cliona</i>	<i>tenuis</i>	M53	Latifundio	Isla Grande, Islas del Rosario, Cartagena	8	N	Y	Y
68	<i>Cliona</i>	<i>tenuis</i>	M62	Banco Montañita	Isla Barú, Cartagena	16	N	Y	
69	<i>Cliona</i>	<i>tenuis</i>	M67	Banco Montañita	Isla Barú, Cartagena	15	N	Y	
70	<i>Cliona</i>	<i>tenuis</i>	M70	Arrecife Nico	Isla Barú, Cartagena	6	N	Y	Y
71	<i>Cliona</i>	<i>tenuis</i>	M84	Banco Burbujas	Bancos de Salmedina, Cartagena	11	N	Y	
72	<i>Cliona</i>	<i>tenuis</i>	M86	Banco Burbujas	Bancos de Salmedina, Cartagena	11	N	Y	

73	<i>Cliona</i>	<i>tenuis</i>	M95	Banco de Salmedina	Bancos de Salmedina, Cartagena	5	Y	Y	
74	<i>Cliona</i>	<i>tenuis</i>	N35					N	
75	<i>Cliona</i>	<i>tenuis</i>	PSA193		San Andrés Isla		Y		
76	<i>Cliona</i>	<i>tenuis</i>	U01	Porto Marie	Curaçao		Y		
77	<i>Cliona</i>	<i>tenuis</i>	X19	Tesoro Noroeste	Cartagena			N	
78	<i>Cliona</i>	<i>tenuis</i>	X2	Tesoro Noroeste	Cartagena			N	
79	<i>Cliona</i>	<i>varians</i>	A786	Bahia Chengue	Santa Marta			N	
80	<i>Cliona</i>	<i>varians</i>	A919	Bahia Granate	Santa Marta			Y	
81	<i>Cliona</i>	<i>varians</i>	C02	El Morro	Santa Marta	10	Y		Y
82	<i>Cliona</i>	<i>varians</i>	C03	El Morro	Santa Marta	10	Y		Y
83	<i>Cliona</i>	<i>varians</i>	F56	Latal	Isla Fuerte	0.5	N		Y
84	<i>Cliona</i>	<i>varians</i>	F58	Latal	Isla Fuerte	0.5	Y		Y
85	<i>Cliona</i>	<i>varians</i>	M107	Arrecife la Playita	Isla Barú, Cartagena	10	Y	Y	Y
86	<i>Cliona</i>	<i>varians</i>	M119	Calichan	Isla Aguja, Santa Marta	10	N		Y
87	<i>Cliona</i>	<i>varians</i>	M111	Cinto	PNN Tayrona, Santa Marta	2	Y	Y	Y
88	<i>Cliona</i>	<i>varians</i>	M125	Bahia Granate	Santa Marta	12	N	Y	
89	<i>Cliona</i>	<i>varians</i>	M128	Punta Aguja	Isla Aguja, Santa Marta	13		Y	Y
90	<i>Cliona</i>	<i>varians</i>	M25	Isla Gloria, Sotavento	Isla Grande, Islas del Rosario, Cartagena	5	N	N	Y
91	<i>Cliona</i>	<i>varians</i>	M26	Isla Gloria, Sotavento	Isla Grande, Islas del Rosario, Cartagena	5	N		Y
92	<i>Cliona</i>	<i>viridis</i>	AF062606	La Vesse	Mar Mediterraneo, Francia			N	
93	<i>Cliona</i>	<i>sp.</i>	57*		Bahamas 2003		Y		
94	<i>Anthosigmella</i>	<i>varians</i>	AF254582	Tennessee Reef	Florida Bay, Florida Keys			Y	
95	<i>Anthosigmella</i>	<i>varians</i>	AF254583	Tennessee Reef	Florida Bay, Florida Keys			Y	
96	<i>Pione</i>	<i>sp.?</i>	F36	Canal del Inglés	Isla Fuerte	0.5	Y		
97	<i>Pione</i>	<i>sp.?</i>	M38	Isla Pavitos	Islas del Rosario, Cartagena	7		Y	Y
98	<i>Pione</i>	<i>sp.?</i>	M47	Luis Guerra	Isla Grande, Islas del Rosario, Cartagena	10	N	Y	
99	<i>Pione</i>	<i>sp.?</i>	M49	Luis Guerra	Isla Grande, Islas del Rosario, Cartagena	14	Y		
100	<i>Pione</i>	<i>sp.?</i>	M63	Banco Montañita	Isla Barú, Cartagena	16	N		
101	<i>Spirastrella</i>	<i>coccinea</i>	C04	El Morro	Santa Marta	10	N		Y
102	<i>Spirastrella</i>	<i>coccinea</i>	M120	Calichan	Santa Marta	10	N	Y	Y
103	<i>Axinyssa</i>	<i>flaveolivescens</i>	C01	El Morro	Santa Marta	10			Y

Appendix B. Phylogenetic hypothesis of species of the genus *Cliona* belonging to Caribbean Cv-complex and related taxa. The tree was obtained through ML analysis of the ITS1 dataset with redundant taxa. Outgroup: *S. coccinea* (Spirastrelledae). Support values $\geq 70\%$ are shown on the branches. Each value over the branches support found with MP, and values below the branches support found with ML/BI. The scale bar represents the number of substitutions per site. The specimens are coded as follows: specie, code-intragenomic variant, and locality.

Appendix C. Phylogenetic hypothesis of species of the genus *Cliona* belonging to Caribbean Cv-complex and related taxa. The tree was obtained through ML analysis of the ITS2 dataset with redundant taxa. Outgroup: *S. coccinea* (Spirastrelledae). Support values $\geq 70\%$ are shown on the branches. Each value over the branches support found with MP, and values below the branches support found with ML/BI. The scale bar represents the number of substitutions per site. The specimens are coded as follows: specie, code-intragenomic variant, and locality.

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