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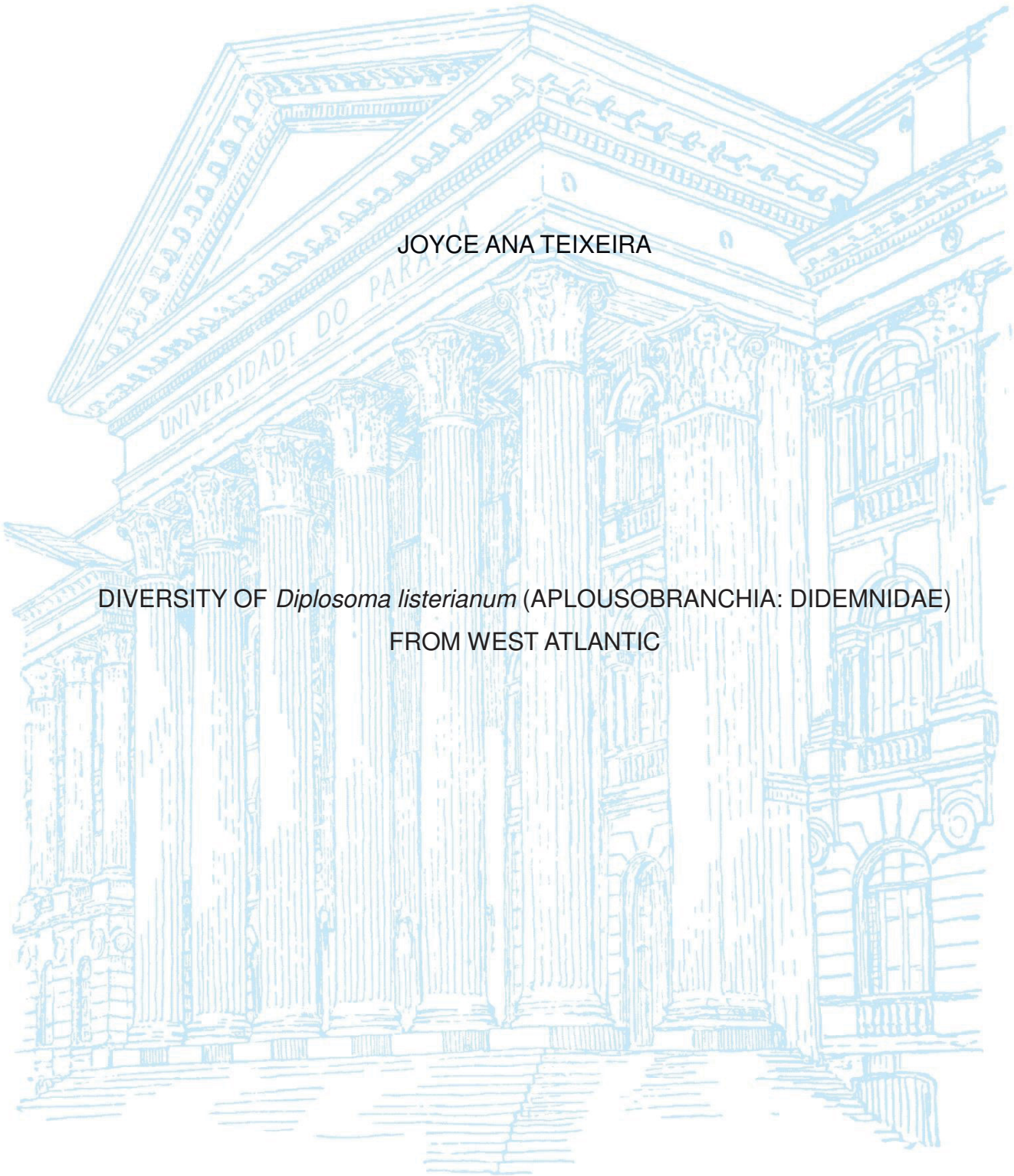

JOYCE ANA TEIXEIRA

DIVERSITY OF *Diplosoma listerianum* (APLOUSOBRANCHIA: DIDEMNIDAE) FROM WEST ATLANTIC

CURITIBA

2018

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DIVERSITY OF *Diplosoma listerianum* (APLOUSOBRANCHIA: DIDEMNIDAE) FROM WEST ATLANTIC

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Orientadora: Profa. Dra. Rosana Moreira da Rocha

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **JOYCE ANA TEIXEIRA** intitulada: **DIVERSITY OF Diplosoma listerianum (APLOUSOBRANCHIA: DIDEMNIDAE) FROM WEST ATLANTIC**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 28 de Setembro de 2018.

ROSANA MOREIRA DA ROCHA
Presidente da Banca Examinadora (UFPR)

MARCIO ROBERTO PIE
Avaliador Interno (UFPR)

RAFAEL ANTUNES BAGGIO
Avaliador Externo (FIES)

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“Fall in love with some activity, and do it! Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough. Work as hard and as much as you want to on the things you like to do the best. **Don't think about what you want to be, but what you want to do**.”

Richard Feynman
(1918-1988)

RESUMO

Diplosoma listerianum é uma das espécies ascídias distribuídas mundialmente. Estudos recentes descobriram que esta espécie pode ser um complexo de espécies formado por, pelo menos, quatro clados muito distintos descobertos por análise molecular usando fragmentos de DNA mitocondrial (mtDNA), mas este estudo não incluiu nenhuma amostra da costa brasileira e muito poucos da Atlântico Oeste. Além disso, a análise morfológica não foi realizada até agora. O objetivo deste estudo foi compreender a diversidade de *D. listerianum* na costa oeste do Oceano Atlântico, utilizando abordagens morfológicas e moleculares. Utilizamos amostras de *D. listerianum* do Brasil, Panamá, México e Estados Unidos para realizar análises moleculares e morfológicas com o objetivo de testar se as populações locais fazem parte de clados espalhados pelo mundo ou espécies nativas escondidas no complexo. O estudo morfológico focado em caracteres larvais e medidas de larvas mostrou uma faixa de tamanho maior do que a relatada anteriormente para este complexo. A análise filogenética resultou em 7 clados, três deles semelhantes aos recuperados anteriormente. Análises de delimitação de espécies (ABGD, GMYC e bPTP) corroboraram os clados obtidos por meio de abordagem filogenética, separando indivíduos do Clado A em dois clados diferentes. O Clado A1 é exclusivamente do Atlântico Oeste, com uma população no lado do Pacífico do Panamá e outra na África do Sul, enquanto o A2 do clado é distribuído em todo o mundo, com representantes em todos os continentes. A relação entre os clados permanece incerta (apoios de baixo valor), o que pode ser explicado por atrações longas, baixo número de amostras e porque um único gene mitocondrial está sendo usado. Este estudo sugere que D. listerianum é uma espécie de evolução rápida, com sinais de especiação contínua e uma grande variabilidade de caracteres fenotípicos.

Palavras chave: Ascídia, COI, Espécies crípticas, Morfometria, Didemnidae

ABSTRACT

Diplosoma listerianum is one of the ascidian species that are distributed worldwide. Recent studies found that this species could be a species complex formed by, at least, four very distinct clades discovered by molecular analysis using mitochondrial DNA (mtDNA) fragments, but this study did not include any samples from the Brazilian coast and very few from the west Atlantic. Furthermore, morphological analysis was not performed until now. The aim of this study was to understand the diversity of *D. listerianum* at the west coast of Atlantic Ocean using morphological and molecular approaches. We used samples of *D. listerianum* from Brazil, Panama, Mexico and United States to perform molecular and morphological analysis intending to test if local populations are part of world-spread clades or native species hidden in the complex. The morphological study focused on larval characters and measurements of larvae showed a size range larger than previously reported for this complex. Phylogenetic analysis resulted in 7 clades, three of them similar to the ones recovered before. Species delimitation analyses (ABGD, GMYC and bPTP) corroborated the clades obtained through phylogenetic approach, separating individuals from Clade A in two different clades. Clade A1 is exclusively west Atlantic with one population at the Pacific side of Panama and another in South Africa, while clade A2 is worldwide spread, with representatives in every continent. Relationship between clades remain uncertain (low value supports), what can be explained by long branch attractions, low sample number and because one single mitochondrial gene is being used. This study suggests that *D. listerianum* is a fast-evolving species, with signs of ongoing speciation and a large variability in phenotypical characters.

Keywords: Ascidian, COI, Cryptic species, Morphometry, Didemnidae

SUMMARY

Introduction

There are several species of ascidians distributed worldwide. The plasticity of ascidians in different environments is an important factor that facilitates bioinvasion, and especially when there is anthropogenic transport involved in the dispersion, and subsidy of new substrates to these organisms in the receiving region, situation that have increased in the last years (Lambert, 2007; Rocha *et al.* 2009). A recent study (Kauano *et al.*, 2016) showed that these organisms survive even when subjected to moderate trawling speed and periods of desiccation of six hours, which may further favor bioinvasion.

Diplosoma listerianum (Milne-Edwards, 1841) is one of many species which benefit from human transport and reports that it is common in harbors, especially in pillars, ropes and even on boats hulls, have been known for many years (Lafargue, 1987; Monniot & Monniot, 1997). The species has some physiological and ecological characteristics which may favor bioinvasion. Among these characteristics we can highlight: the ability to retain and select exogenous sperm in the oviduct (Bishop & Ryland 1991; Bishop & Sommerfeldt 1996), the capacity to fuse with nearby colonies, and the great ability of asexual reproduction by budding (Sommerfeldt *et al.* 2003). In addition to that, *D. listerianum* is reported as a pioneer species in community succession (Rocha 1991) with enough plasticity to grow on natural and artificial substrates, and even on other ascidians. Although it is not tolerant to thermal stress (Sorte *et al.*, 2010), the colonies close to the affected sites were able to provide larvae for the recolonization of these areas, and this occurred despite *D. listerianum* has a short larval swimming time, before fixation and metamorphosis, in relation to other species (Lane 1973). Likewise, it did not show enough resistance to hypo-osmotic stress and was completely killed after experimental tests in the native geographical range, in contrast with other didemnid known by its invasive potential, *Didemnum vexillum* Kott, 2002, with 40% survival in the same conditions (Lenz *et al.* 2011).

The above examples show that long since *D. listerianum* has been used as a model organism in studies of physiology, reproduction, bioinvasion and other topics. However, the results of those studies could be questionable if we do not test the possibility of *D. listerianum* being more than a single species with different responses for each clade. Recent studies have shown that some of the species of ascidians

with worldwide distribution are, in fact, complexes of cryptic species, like *Ciona intestinalis* (Linnaeus, 1767) (Zhan *et al.*, 2010) that looked similar at first sight but resulted to be two different species after genetic separation and detailed morphological and physiological studies with congruent evidence (Pennati *et al.* 2015; Brunetti *et al.* 2015; Tarallo *et al.* 2016). Another example of cryptic speciation is the case of *Botryllus schlosseri* (Pallas, 1766) that has been recently treated as a five-clade group not yet morphologically recognized as species, some of them with widespread haplotypes and evidence of ongoing intra-clade speciation (Bock *et al.* 2012, Griggio *et al.* 2014, Nydam *et al.* 2017).

A large-scale study intending to clarify the issue regarding the possible cryptic speciation of *D. listerianum* was performed using mitochondrial DNA sequences (Pérez-Portela, *et al.* 2013). The authors concluded that *D. listerianum* is a complex of cryptic species formed by four monophyletic clades that genetically diverge up to 17% (Fig. 1). Nevertheless, only 29 samples representing 12 haplotypes from the western Atlantic were used in this study, all of them from Panama, what may have caused a bias and underestimation of the diversity of this huge area. The distribution of *D. listerianum* in the West Atlantic extends from Canada (Ma *et al.* 2018) to Santa Catarina in South Brazil (Rocha et al. 2012).

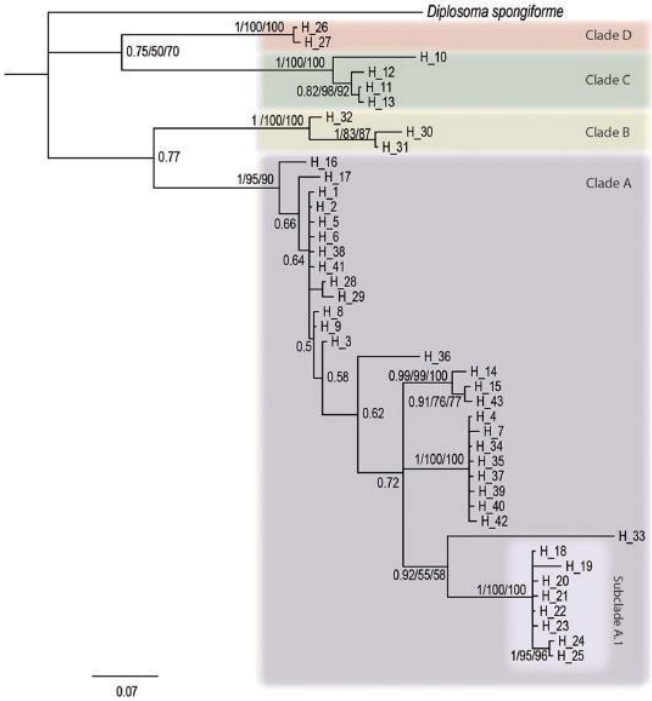

Figure 1: Modified from Pérez-Portela *et al.* (2013), showing the four distinct clades within *D. listerianum*.

While Pérez-Portela *et al.* (2013) identified that *D. listerianum* is a complex of species, they have declared the lack of morphological variation among clades. The original description of *Diplosoma listerianum* (Milne-Edwards, 1841) is made of a few lines and do not even have a type material designed. In addition, three illustrations were made by the author to clarify the external appearance of colonies, but they do not show any internal characteristics of this species.

In 1886, Herdman tried to separate every entity in this genus and reinforced that his new species (*D. macdonaldi*) was a different animal. He made some considerations about siphon lobes, colony color and texture, pigmentation and larval characteristics trying to give a single path leading to his species (Fig. 2). But, in fact, *D. macdonaldi* was another junior synonym of *D. listerianum*, just as *D. rayneri* (Macdonald, 1859), *D. crystallinum* (Giard, 1872) and *D. chamaeleon* (Drasche, 1884). Even with some differences among them, they all became inside the *D. listerianum* complex because the supposed 'unique character' that separate them all were mostly related to tunic color and thickness variation which has been considered intraspecific variation later. From this study, only *D. carnosum* Drasche, 1883 and *D. spongiforme* (Giard, 1872) are considered valid nowadays, due to their larval peculiarities and gonad characteristics that were not described by Herdman (1886).

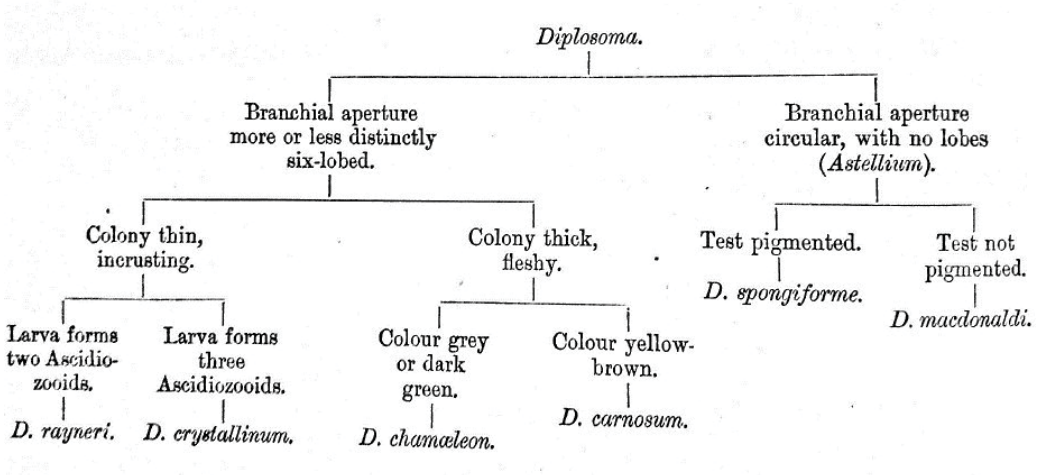

Figure 2: Dichotomous key to *Diplosoma* genus by Herdman (1886).

A century after Milne-Edwards published the first description of the species, Rowe (1966) made a revision of the genus and designed a neotype for this species, including a re-description that brought together most of the well-known

characteristics of the animal, like the pigmented epithelium cells on thorax and abdomen and two testicle follicles. Rowe also tried to study samples from the type-locality in the northeast Atlantic Ocean and he designed a neotype for *D. listerianum* based on them, which unfortunately did not have developed larvae and all the zooids were in the protandric phase.

Monniot (1983) made other considerations about previous studies of *D. listerianum* and about its original description, and she pointed to the lack of zooid and larval characteristics in descriptions until then. Additionally, she remarked the necessity of a better relaxing protocol for this species. But, most important, she noted that differences between samples from distinct localities were observed within the same locality too, reinforcing the great morphological variation in this taxon.

At this point, the animal was already known by its common pigmentation of zooids and colony appearance and Lafargue & Wahl (1987) added a detail about development: eggs starting to divide inside the ovary, different from other species from the same genus in which the eggs start to divide only after expelled to the tunic. Sequential studies that recorded the species occurrence (Lafargue 1983, Millar 1988) did not take any notes about its taxonomical characteristics because it was already considered cosmopolitan and easily recognizable species.

In 2001, Kott published one of the best descriptions for *D. listerianum*, after many years of biodiversity studies that considered it like a species with constant and fixed characteristics (Kott, 1952; Lafargue, 1983). In her monograph, Kott tried to use all the available characteristics, describing carefully the colony, zooids and specially the larvae, including illustrations with scale bars to differentiate *D. listerianum* from the synonyms recorded until then. She specially described the colony attributes that seem to be unique, such as “the extensive common cloacal cavity crossed by thin test connectives in which the zooids are contained, isolated and abdomina are never clumped together in the basal connective”. Kott (2001) also brought a list of previous occurrences and made a good discussion about synonyms and similar species.

Because of the large occurrence range, differences in the coloration of the living colonies observed and some variation in internal characteristics, 33 different species have been described which were later synonymized with *D. listerianum*. Over the years, the taxon also received species that were moved from another genus in Didemnidae, increasing the synonym list for *D. listerianum* (Shenkar *et al*., 2018). Some species inside the long list of *D. listerianum* synonyms were first described belonging in *Lissoclinum* Verril, 1871 mainly because of the white granular structures present in the tunic that keeps being confused with spicules, in some cases being even quoted as 'mulberry like' structures (Kott, 1952).

Here we complemented Pérez-Portela et al. (2013) study, with samples from west Atlantic coast, and performed a detailed study of the larvae of different populations in pursuit of diagnostic characters to differentiate the clades. Also, new information about morphology, genetics and occurrence of this species complex is given.

Methods

Biological sampling

Biological samples were collected at different sites along the coasts of Brazil, Panama, Mexico and United States (Table 1). Some samples were already available at the scientific collection of Departamento de Zoologia from Universidade Federal do Paraná (DZUP-UFPR). The collection has a large assemblage of tunicates from Brazilian waters, but not all the materials collected were preserved in ethanol 96% – especially the older ones, before the popularization of molecular techniques. For this reason, the present study used a smaller number of samples for molecular analysis.

Samples came from natural and artificial substrates. The material from natural substrates comprise samples from rocky shores and coral reefs collected during low tides or snorkeling and scuba diving. The material that came from artificial substrates were collected on pillars, ropes and settlement plates in marinas. The animals were relaxed using menthol crystals and magnesium chloride for approximate one hour before being fixed in formalin 10% and, when possible, also a little part of the colony was preserved in ethanol 96% for the molecular study purposes.

The settlement plates sets were composed by two black polyethylene plates (12 x 12 cm) with a 2 cm space between them, aiming to create an inside ambient with less predation. Each set of plates were fixed on a rope and every rope was fixed in different floating structures at some marinas, with a bottle filled with sand in the bottom end to create weight and maintain the rope vertical. After one and two months the sets were taken from water and all the tunicate samples were collected, with emphasis on *D. listerianum* colonies.

Molecular approach

For the molecular study,135 samples (Table 1) from different sites along Brazilian coast, Panamá (both sides), Mexico and United States (Florida) (Fig. 3) were submitted to a DNA extraction using the Invitrogen Pure Link Genomic kit according to the manufacturer protocol. Pieces of colony with approximately 1cm² were selected, then cleaned under the stereoscope to remove other animals and excessive sediment. The only adaptation from the manufacturer protocol was an increase of the water-bath time to 12 hours, to improve the initial digestion of tissues. The extracted DNA concentration was measured on a Nanodrop® spectrophotometer and the samples were conditioned at a -20ºC freezer, embedded on Elution Buffer.

After that, the amplification protocol was performed following Pérez-Portela *et al.* (2013). For a total 25 µL reaction we added: 0.5 µL TunF (5' TCGACTAATCATAAAGATATTAG 3'), 0.5 µL TunR (5' AACTTGTATTTAAATTACGATC 3'), 0.5 µL DNTPs (10 mM), 1.25 µL MgCl2 (50 mM), 0.25 µL Taq Polymerase (Phire Green Hot start), 10x 2.5 µL Platinum reaction Buffer, 18.75 µL Ultra-pure water, 2 µL DNA template. Plus, 0.5 µL BSA (Bovine Serum Albumin) was added to the reaction to prevent reaction inhibition by other proteins that may remain from the initial tissue digestion. The Thermocycler Eppendorf Mastercicler Personal® was programmed to one denaturation single step at 94ºC for 2 minutes, followed by 48 cycles 94ºC for 1 minute: denaturation; 40ºC for 1,5 minutes for annealing and 72ºC for 1 minute of extension, and a final extension step at 72ºC for 7 minutes.

The quality of DNA bands was checked in agarose gel and help of a molecular ladder the single bands were recognized around 600bp. With this positive

confirmation, the PCR product was purified and sequenced by a specialized company using the Sanger method with the same primers of the amplification with an adjusted Tm for each primer: 52.4ºC for TunF and 52.2ºC for TunR during the sequencing reaction.

The sequences were edited at BioEdit software (Hall 1999), combining the Forward and Reverse sequences with an additional eye checking of every nucleotide at electropherograms. Then, the sequences were aligned using the progressive algorithm of Clustal W, using default parameters, inside the MEGA software (Tamura *et al.* 2013) and checked and realigned by eye. Sequences were also aligned by an iterative approach at MAFFT (Katoh *et al.* 2017), using FFT-NS-i algorithm, intending to compare both methods. After that, sequences were translated into amino acids to identify stop codons in a search for nuclear sequences (pseudo genes) among the samples, identified by stop codons inside the sequences.

The TCS haplotype network was produced using the PopArt 1.7 software (Clement *et al.* 2002) and the phylogenetic analysis using Bayesian Inference and Maximum likelihood criteria were performed in Mr.Bayes (Huelsenbeck & Ronquist, 2001) and PhyML (Guindon & Gascuel, 2003) after the selection of the evolution model in Jmodeltest2 (Darriba *et al.* 2012). Bayesian Inference was performed in two independent runs with 4 chains each, during 10^6 generations, and trees were retained after the mean of standard deviation reached 0,05. The Maximum likelihood was performed with 500 Bootstrap replications. Bayesian Inference results were also analyzed in TRACER (Rambaut *et al.* 2018) intending to check likelihood values besides effective sample size (ESS) from parameters, and their distributions, in each run.

Diplosoma sequences were searched at GenBank to be used as outgroups in this analysis and only two samples of *Diplosoma spongiforme* were available (GenBank Acess numbers: AY600972.1 and KF309624.1). There are also some COI sequences available from Japanese species, but they do not correspond to the same fragment used here.

Also, some species delimitation analyzes were performed intending to clarify the putative species inside the complex. For this, we used:

a. **ABGD (Automatic Barcode Gap Discovery):** using divergence between and within species, this method searches the first gap between sequences using the aligned dataset as an input to divide them in groups. The analysis was performed using default parameters at the website (http://wwwabi.snv.jussieu.fr/public/abgd/) (Puillandre *et al.* 2012) using the simple distance model.

b. **bPTP (Bayesian Poisson Tree Process)**: this method comprehends the evolutionary distances between species as a ruler for species delimitation using the substitution number between sequences (Zhang *et al.* 2013). It requires a Bayesian Inference Tree as input file at the website (http://species.h-itsorg/).

c. **GMYC (Generalized Mixed Yule Coalescent)**: Using maximum likelihood ultrametric trees, this method finds the point where there is a transition from populational coalescence to different species (Pons *et al.* 2006). To obtain the trees, BEAST (Suchard *et al.* 2018) software was used under the same parameters as in the phylogenetic analysis and then submitted to the website (http://species.h-itsorg/gmyc).

Sequences corresponding to already known haplotypes of *D. listerianum* were named with same numbers used by Pérez-Portela *et al.* (2013) (Haplotypes 1 to 43) and the new haplotypes received new numbers (Haplotypes 43 to 76) (Table 1).

Divergence, based on p-distances, was also estimated by the Bootstrap method between clades and *D. spongiforme* on software MEGA (Tamura *et al.* 2013)

Table 1: Samples fixed in ethanol 96% used on molecular analysis on this study. Voucher numbers at Scientific collection between parentheses.

Country	State	City	Coordinates		Substrate	Haplotypes (DZUP-DIPL)
Brazil	Santa Catarina	Bombinhas	27°08'43"S	48°28'50"W	Natural	53 (087) , 54 (149), 55 (147), 56, 57
	Santa Catarina	Porto Belo	27° 8'54"S	48°32'7"W	Artificial	57
	Paraná	Pontal do Paraná	25°32'55"S	48°23'17"W	Artificial (plates)	18, 59 (106), 60 (116)
	Espírito Santo	Vitória	20º19'07"S	40º17'56"W	Artificial	18
	Sergipe	São Cristóvão	11° 8'22"S	37° 9'50"W	Artificial (plates)	75
	Alagoas	Maceió	9º40'34"S	35º45'07"W	Artificial	76
	Rio Grande do Norte	Natal	5°45'54"S	35°12'16"W	Artificial	57, 58 (223)
	Ceará	Caucaia	3°41'23"S	38°36'43"W	Natural	73 (133), 74
	Maranhão	São José de Ribamar	2°29'19"S	44°02'7" W	Natural	18 (128, 129, 132), 71 (130)
Panama	Bocas del Toro	Bocas del Toro	9°20'9"N	82°14'46"W	Artificial	18 (240), 24, 64 (241), 65 (233), 66, 67 (233), 68 (233)
			9°20'13"N	82°10'42"W	Artificial	69 (230)
			9°15'18"N	82° 8'29"W	Natural	19
			9°21'3"N	82°15'26"W	Artificial (plates)	19, 62 (225), 63 (224)
	Panama	Isla Taboguilla	8°48'28"N	79°31'24"W	Artificial	19, 61
Mexico	Yucatán	Arrecife Alacranes	22°36' N	89°42' W	Natural	44 (134), 45 (137), 46 (141), 47 (135), 48, 72
		Sisal	21°10'4"N	90° 1'55"W	Artificial	70
USA	Florida	Apollo Beach	27°46'48"N	82°25'26"W	Artificial	18
		Bradenton	27°28'5"N	82°41'46"W	Artificial	50, 51
		St. Petersburg	27°28'5"N	82°41'46"W	Artificial	49, 52

Morphological approach

In the laboratory, all samples were analyzed under a stereoscope microscope (Leica MZ12 5) to confirm the identity based on the characters described in literature. The observation included the colony external appearance (size, color, texture) and the extraction of zooids and larvae for the study of internal morphology and measurement of larval structures. Individuals that were in good condition were stained with Harris Hematoxylin for better visualization and permanent slides with those stained zooids and larvae were confectioned.

Confection of permanent slides started by an alcoholic dehydration series (Ethanol 70, 80%, 90% and Butanol for one minute each) and then embedding the biological samples in Durcupan® epoxy. Then, covered by a coverglass with clay in the corners to prevent crushing the samples. Permanent slides remained at a slide warmer for 15 days, until resin was totally dry.

Morphological analysis was performed under stereoscope microscope (Leica MZ12 5® and Leica DMC 4500®) and some pictures were taken with scale bars for posterior measurement of the larval structures using the ImageJ software (Schneider *et al.* 2012).

Because of the zooid contraction and the little amount of structures inside it, the larval structures were considered more stable to be measured because larvae do not contract during the material collection and fixation. Larvae used in this study were considered mature by the presence of a well-developed oozooid, with well-distinguishable perforated pharynx, inside it. The measurements chosen to this study were named from A to Q (Table 1, Fig. 3). All the distances including circular structures were made from their center, considering that structures change during the development and this procedure could minimize some bias caused by the developmental stage.

Table 2: Measurements taken from larvae

Larvae	A- Trunk length B- Tail length C- Total length
Adhesive Papillae	D- Calyx diameter of the dorsal papillae E- Calyx length of the dorsal papillae F- Peduncle width of the dorsal papillae G- Distance between first (dorsal) and second papillae H- Distance between second and third (ventral) papillae
Ectodermal ampullae	I- Distance between anterior margin of the dorsal ampullae and larvae anterior surface J- Peduncle width of the dorsal ampullae
Statocyte	K- Distance from statocyte and first papillae L- Distance from statocyte until the transition between the trunk and the tail M- Statocyte diameter N- Distance between statocyte and larvae ventral surface
Tail	O- Width at the wider section
Embryos	P- Thorax length Q- Embryo stigmata length – dorsal row

A Pearson's (Pearson, 1987) correlation test was performed on PAST software (Hammer *et al.* 2001) to understand how each variable (measures) were related to each other and to identify patterns.

Measurement data was also divided into two groups: seven samples with mtDNA sequences belonging in clade A1 (A) and the rest of Brazilian samples available (B) and supposedly also belonging to clade A1. A PCoA was conducted to visually explore the distribution of our samples in the multidimensional space, and this analysis was performed in R using Vegan package (Oksanen *et al.* 2018).

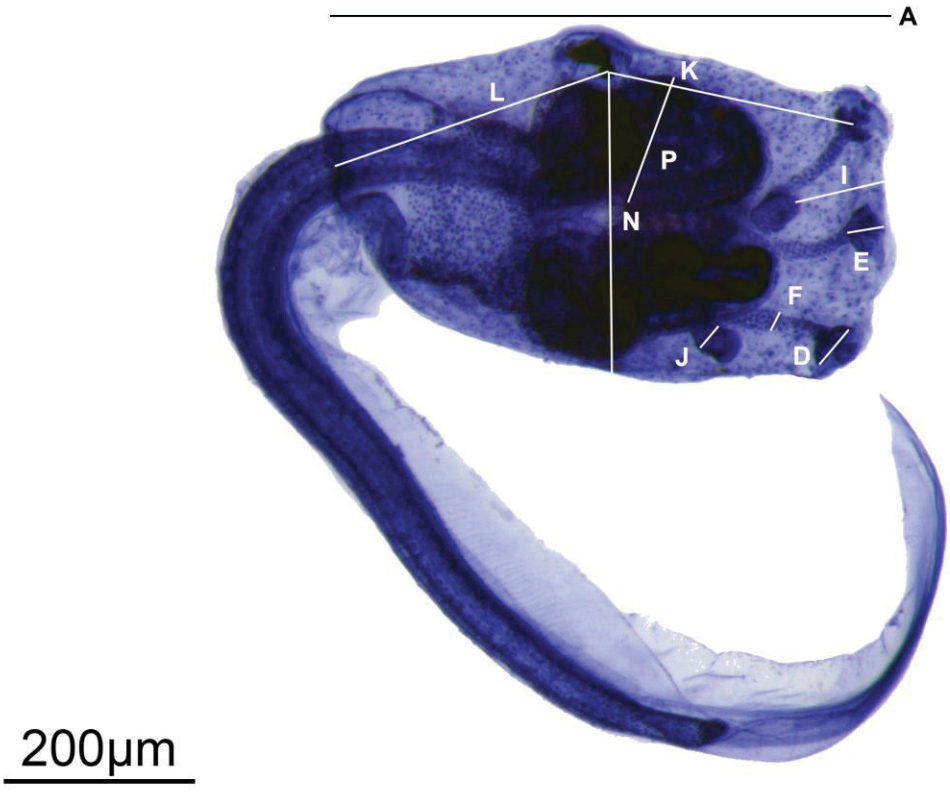

Figure 3: Some of the measures that were taken from larvae, exemplifying how distances and sizes were measured, letters according to Table 2. Although measurements were taken from the dorsal papillae and ampullae, they were represented in other structures for clarity.

Also, intending to better understand the distribution of this complex, the areas of occurrence were plottled in a map using Photoshop CS6. Areas of occurrence were found in taxonomy and bioinvasion literature searching for *D. listerianum* occurrences and for every synonym that are included in this name.

Results

Molecular Study

The ethanol samples gave us 55 sequences, with a length of 531bp and a nucleotide diversity of π=0.038453, 202 segregating sites, and Tajima's D equal to -1,89099. We found 33 new haplotypes for *Diplosoma listerianum*, and 22 sequences that corresponded to already known haplotypes (Table 1). Even with changes in the Tm temperature at sequencing protocol, a lot of samples had failed during the

sequencing procedure probably because of a slippery homopolymeric A/T region found inside the fragment that we amplified.

Best value of model fitting was given by TPM1uf+I+G under the AIC. Then, phylogenetic analyzes resulted on a different topology from the one obtained by Pérez-Portela *et al.* (2013), although it conserved the main clades proposed by them (A to D), with the addiction of three new clades (E, F and G) (Fig. 4). Clade A has become two highly supported units: Clade A1 corresponds to awest Atlantic group mainly, while A2 includes haplotypes from all around the world, being the most widespread clade. No new haplotypes from Clade A2 were found. Clade B composition was not altered, and it is still composed by exclusively Japanese haplotypes, but it was highly distanced from Clade A2 in the new topology. Also, Clades C and D maintained the same composition, but their relationship was affected by the insertion of Clades F and G, Panamanian and Mexican clades, respectively.

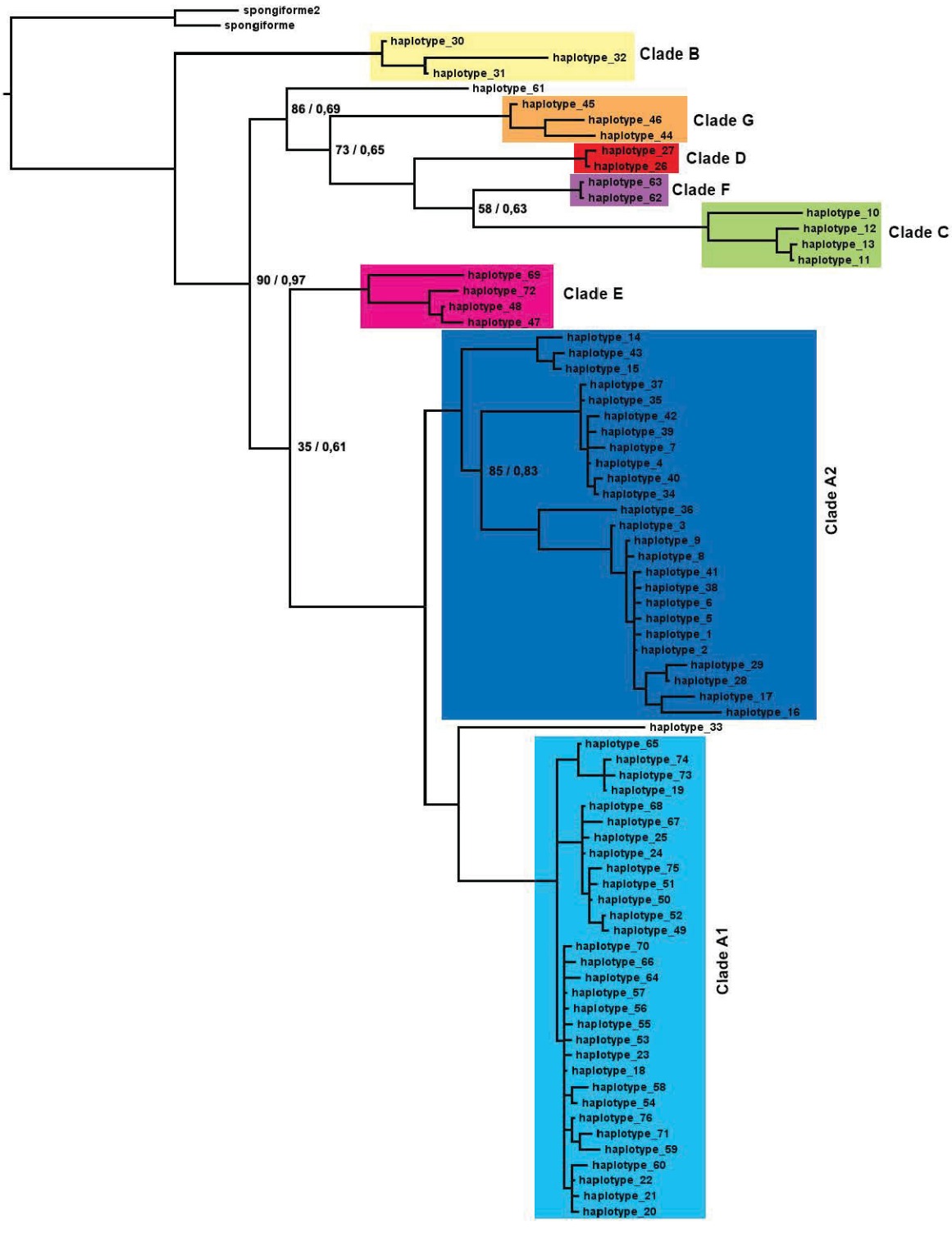

Figure 4: Consensus topology of the phylogenetic analysis under the Bayesian Inference and Maximum Likelihood criterion. The colors in Pérez-Portela et al. (2013) were maintained: Blue- former Clade A and subclade A1, now A1 and A2; Green- Clade C; Red- Clade D. The other clades were retrieved in this study. Bootstrap and Posterior Probability values are represented where they were smaller than 95 and 0,95 respectively.

The TCS Haplotype Network shows the relationship between haplotypes and the number of mutations between them (Fig. 5). Black dots between known haplotypes suggest unknown/unsampled haplotypes. The most abundant haplotypes (2, 4, 18) were the most widespread, highly connected with less abundant haplotypes around them. The Atlantic Clade A1 is the most central group, being connected at two points to other clades, distant from Clade A2 by 41 mutations, Clade C by 96 mutations, Clade D by 112 mutations, Clade E by 52 mutations, Clade F by 93 mutations and Clade G by 85 mutations. The most isolated group was Clade B from Japan, being connect with Clade A2 only, from which it is 66 mutations distant.

Also, we observed a great divergence among clades and between them and the outgroup (Table 3). Largest interclade divergence was between clades B and C (20.2%) and the lowest was found between clades A1 and A2 (10.9%). Largest genetic divergence between clades and *D. spongiforme* was 22.2% (Clades A2 and C).

Table 3: Percentage of genetic divergence (based on p-distances) between clades from the *D. listerianum* complex and *D. spongiforme* for COI fragment.

	A1	**A2**	**B**	**C**	**D**	**E**	**F**	**G**
A2	10.9							
B	17.3	17.3						
C	20.0	20.1	20.2					
D	16.9	17.1	19.3	17.7				
E	13.7	15.0	16.1	17.7	15.3			
F	16.3	16.3	16.5	14.0	11.2	14.4		
G	18.6	19.2	19.1	17.4	15.7	15.2	15.1	
Diplosoma spongiforme	19.5	22.2	20.1	22.2	20.3	18.2	20.1	19.7

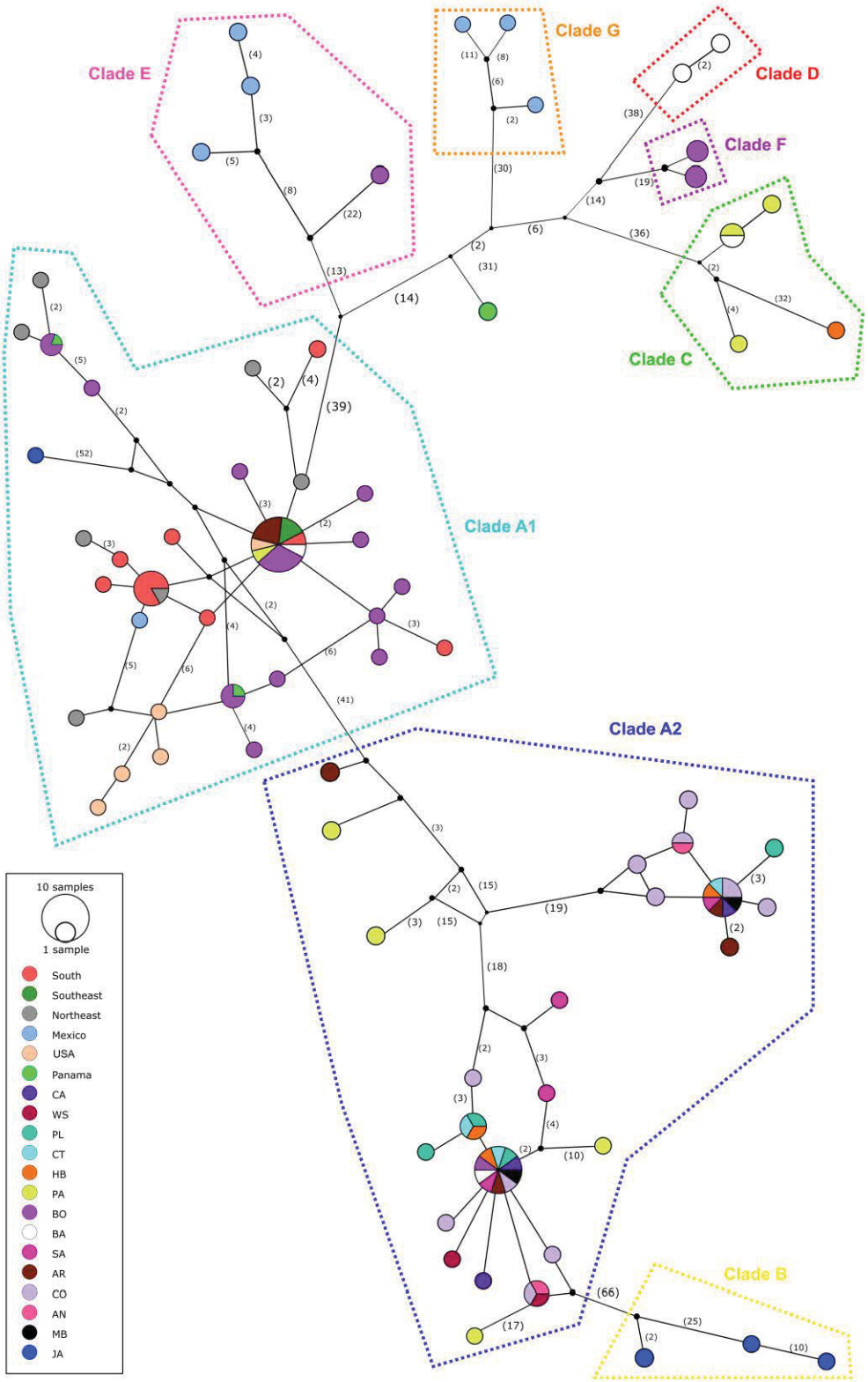

Figure 5: Haplotype Network for of *Diplosoma listerianum.* Numbers indicate the number of mutations between haplotypes when >1 and black dots represent unsampled haplotypes. Clades identified in Fig. 4 are represented. Colors indicate the localities and circle size indicates the number of samples found in each haplotype.

The Atlantic Ocean correspond to the area of greatest diversity within this species complex, with six of the seven clades found in the tropical west Atlantic (Fig. 6). Panama alone concentrated 30% off all haplotypes, 18 of them exclusive of this country. Clade A1 spreads in the West Atlantic from Florida to South Brazil, with one population in the Pacific side of Panama, and another in the Indian Ocean side of South Africa.

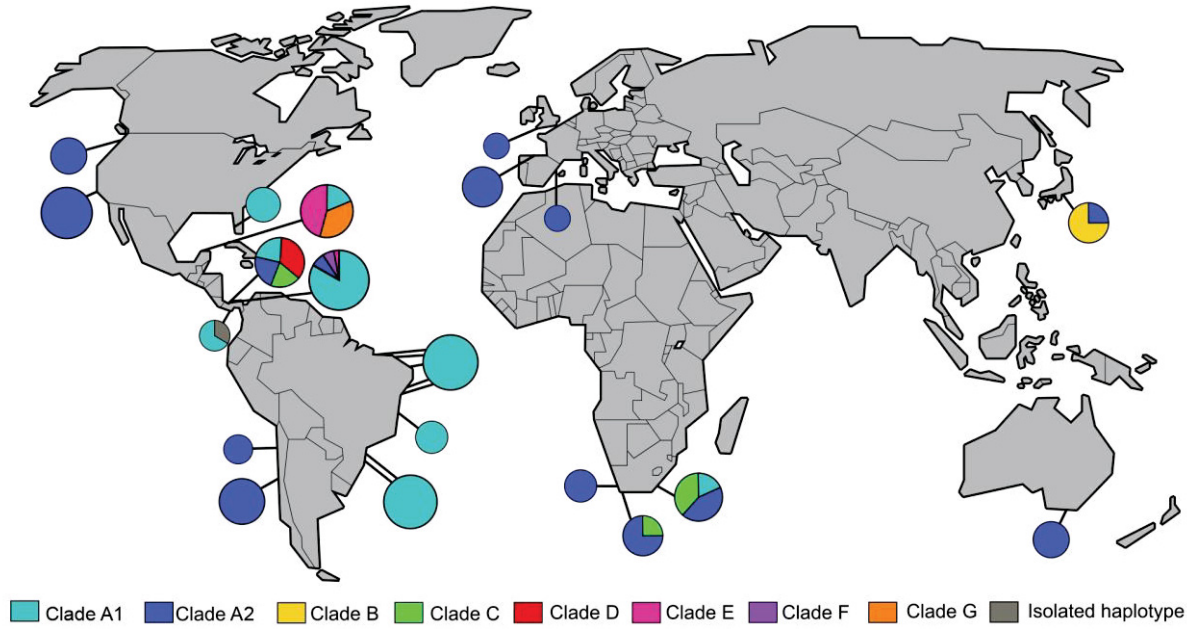

Figure 6: Sampling locations of *D. listerianum* and the clades frequencies. Pie size is proportional to total number of haplotypes (1 to 17).

Species delimitation analyses resulted in different number of units: 19 from ABGD, 15 from bPTP and 21 from GMYC, and the phylogeny returned 17 different units. Consensus was deducted through the concordance between the methods and the clades obtained by phylogenetic analyses. Thus, 17 different identities are proposed for this species complex. Some clades were formed by more than just a single species, Clade A1 is composed by three entities while Clade A2 is formed by four and Clade C and E are composed by two.

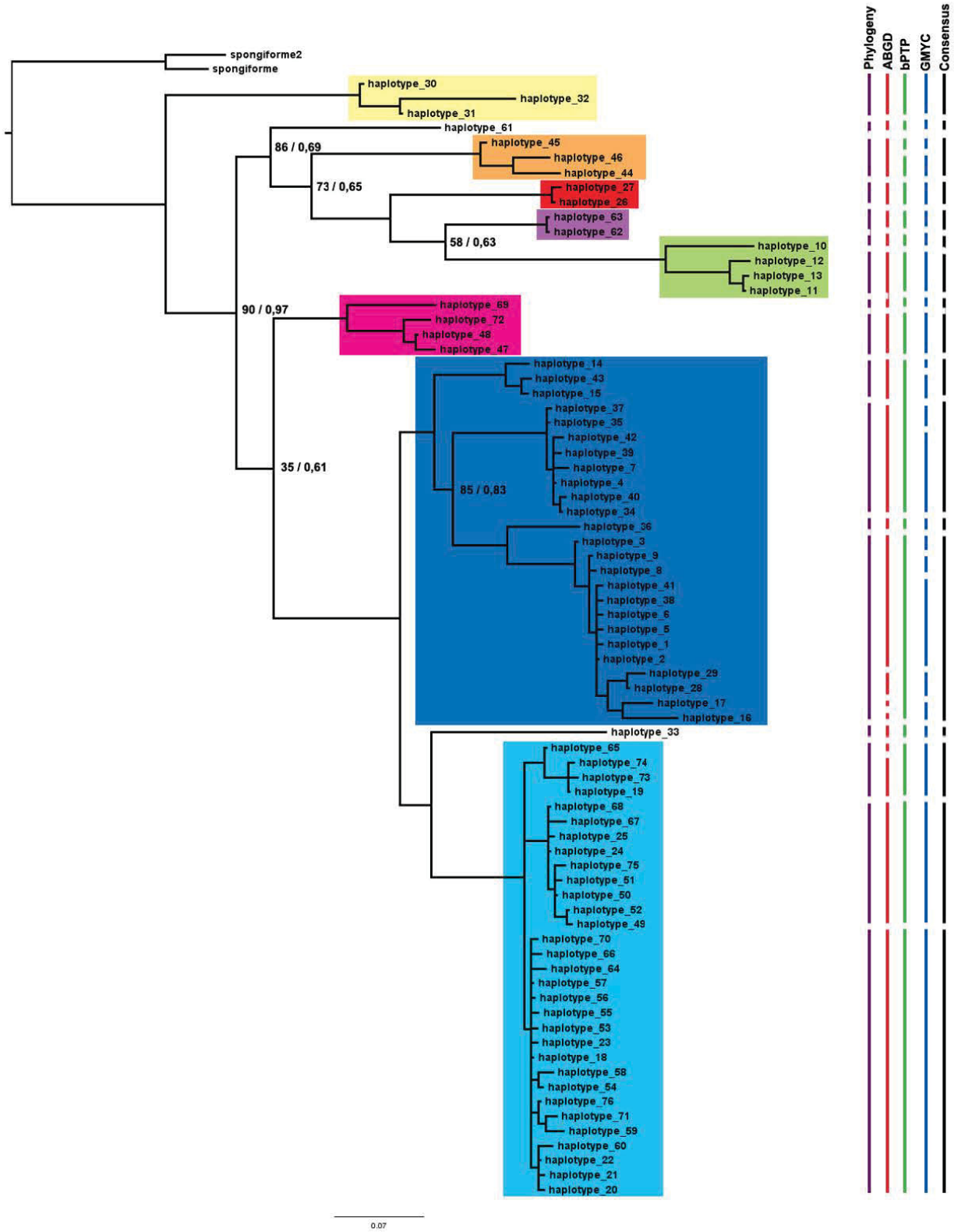

Figure 7: Results from species delimitation analysis showing the suggested species for each method (Phylogeny, ABGD, bPTP, GMYC) and the majority consensus between methods.

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Morphology study

Seven samples that rendered sequences also had developed larvae for study and all of them belonged to a same taxonomy unit in clade A1, while 24 other colonies with developed larva were collected along the Brazilian coast and given the fact that we only found haplotypes of clade A1 in this region, we assume that all those colonies also belonged in Clade A1 (Table 1 in appendix). We confirmed the great variation of characters both between and within colonies of this clade. Colonies showed a variety of color and texture (Fig. 8), some had a flesh consistency, and some were thinner, with a large diversity of shapes and occupying a manifold diversity of substrates. Some colonies collected during the study did not have zooids covered by the black squamous epithelium described for this species, and some colonies had both zooids with and without the squamous epithelium, observed in both alive and preserved exemplars. Zooids can be seen from the outside of the tunic uniformly spread or in clumps which are composed by groups of 10 to 12 zooids connect to the basal layer of colony by connectives that emerge from the base and then branch to each zooid.

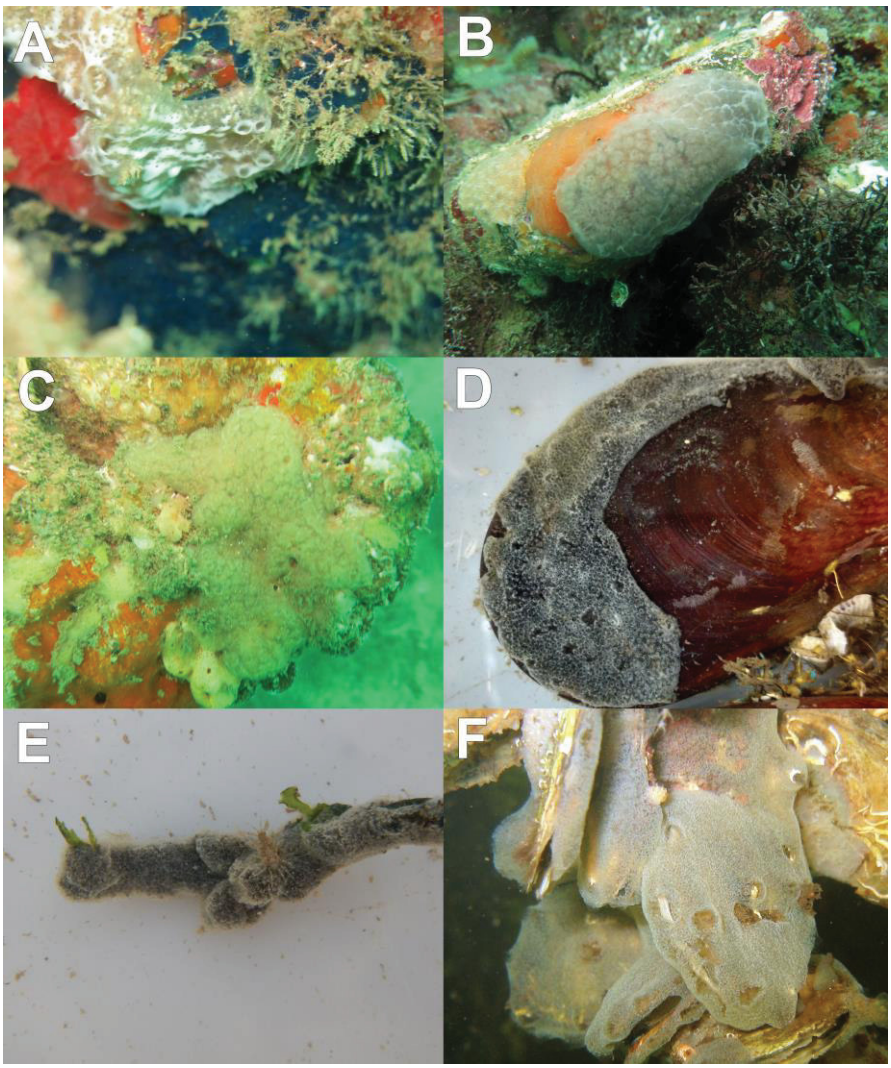

Figure 8: Colony appearance diversity from west Atlantic. A-C: Rio de Janeiro - Brazil, D: Paraná – Brazil, E: Rio Grande do Norte – Brazil and F: Bocas del Toro - Panama.

Most of the samples had contracted zooids and when with developed gonads, they were predominantly protandric. Well relaxed zooids were 1.3 to 1.5 mm long with oral siphon with six well defined long lobes and wide atrial opening. The thorax is large with four rows of stigmata, with 8-9, 7, 6-7, 5-7 stigmata in each row. There are at least 16 oral tentacles with three sizes. The retractor muscle projects close to the oesophagic-rectal peduncle ending right after the abdomen. Four stolonic vessels, two long and two short, emerge from the abdomen and end in ample terminal ampullae in the tunic. There are two follicle testis with a straight vas deferens.

Larvae were found developing at the basal portion of colony and were usually surrounded by a thin layer of tunic. They were carefully observed when they had their

both embryos fully developed with visible stigmata. Larvae are mostly globular, with a long tail that goes from the half to a quarter of larval perimeter. There are three adhesive papillae positioned in line and two pairs of ectodermal ampullae. The oozooid is dorsal while the blastoozoid can be seen right ventrally to it.

Larval measurements can be found in Table 2 of the appendix. Trunk size varies from 381 to 1143 µm, while tail length 639 to 1483 µm, resulting in total size of larvae ranging from 798 to 2238 µm (Fig. 9A). The measures that had the largest variation were the ones corresponding to distances (K, L and N), what reflects the variation in shape of the animals (Fig. 9B). The distance from statocyte to the dorsal adhesive papillae (K) varies from 117 to 380 µm, between the statocyte and the end of trunk (L), 201 to 468 µm, and between statocyte and larvae ventral surface (N) 232 to 697 µm. The smallest variation was observed in calyx diameter of dorsal papillae (D - 43 to 90 µm), calyx length of dorsal papillae (E - 28 to 76 µm), peduncle width of dorsal papillae (F - 15 to 37 µm), peduncle width of dorsal ampullae (J - 17 to 65 µm), statocyte diameter (M - 11 to 23 µm), tail width at wider section (O 48 to 101 µm), and embryo stigmata length (Q - 10 to 26 µm).

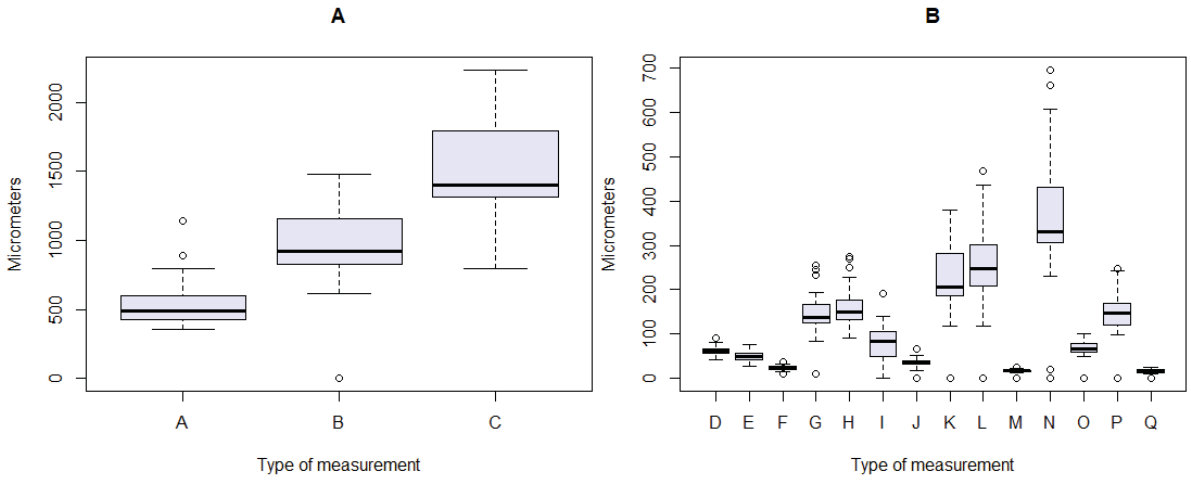

Figure 9: Variability of measurements taken from larvae. A-Q are the measurements in Table 1.

The Pearson's correlation test revealed highly correlated variables, showing that almost all the structures have a positive relationship with each other and grow in a

similar rate during the development (Fig. 11). Higher correlation was found between the size of trunk (B) and larval total length (C), The peduncle width of the dorsal ectodermal ampulla (J) had an almost constant size and had low correlation with other structures sizes and distances, likewise thorax length (P) and embryo stigmata

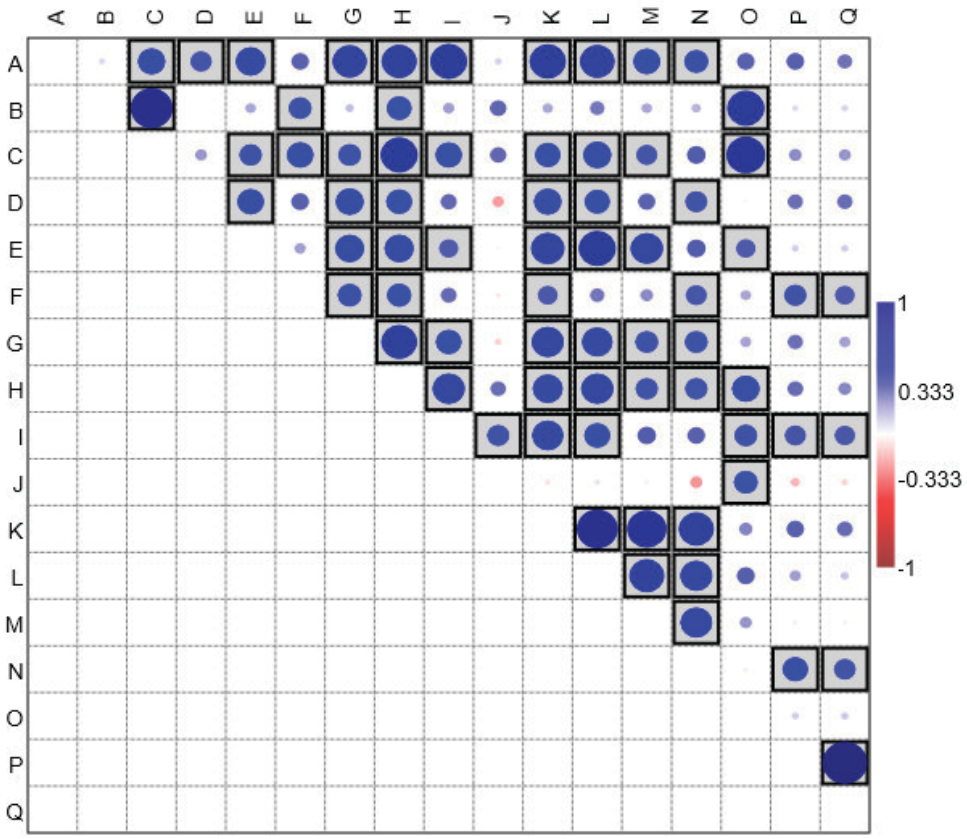

Figure 10: Pearson's correlation test between variables. Circle size is proportional to its color and squared boxes represent significant *p* values (<0,05).

Principal Coordinates Analysis (PCoA) first two vectors recovered together the two groups defined *a priori* when plotted the dissimilarity matrix along the multidimensional space. All Clade A1 samples that have DNA sequence matching samples were not different from the other samples, corroborating that they belong to the same group (Figure 11).

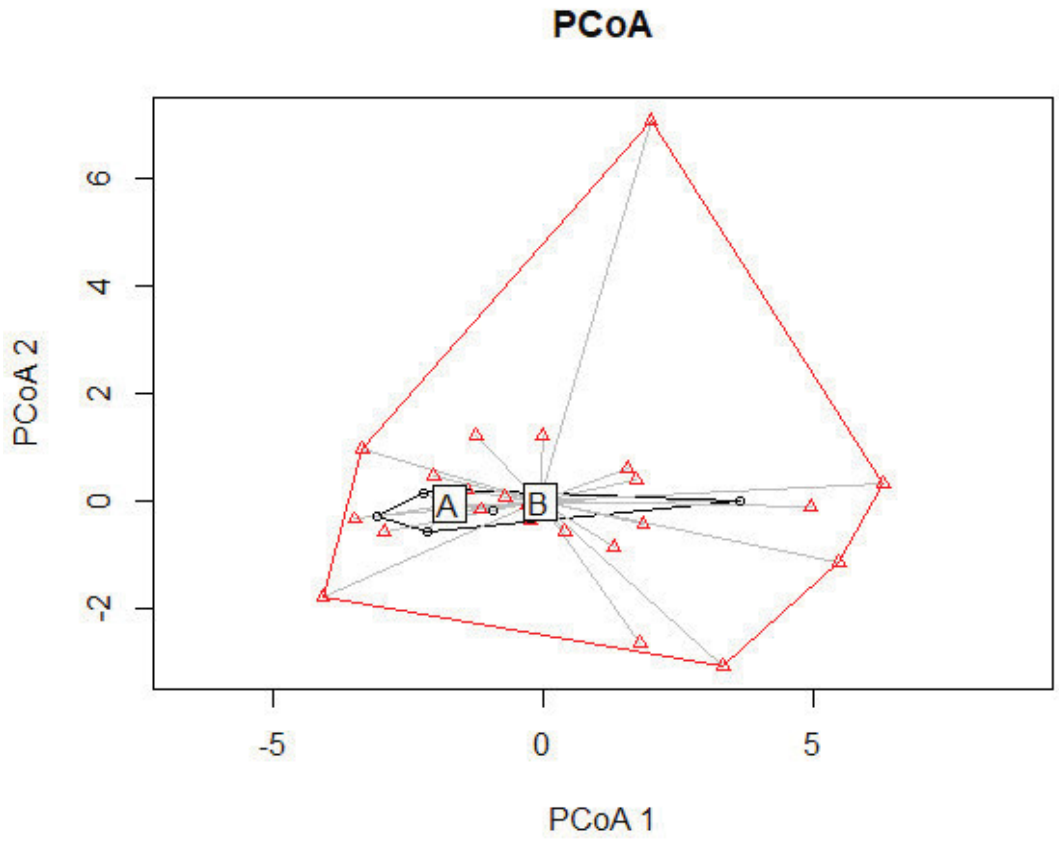

Figure 11: Principal Coordinates Analysis (PCoA) vectors showing groups distribution at multidimensional space. A represents the centroid of seven samples with mtDNA sequences (black) that belong in clade A1 and B represents the centroid of 25Brazilian samples analyzed (red), supposedly also belonging in clade A1.

Distribution of *D. listerianum* complex comprises almost all the globe, except by polar areas and it is present in all the Oceans, but it has been less reported in the Indian Ocean (Fig. 12).

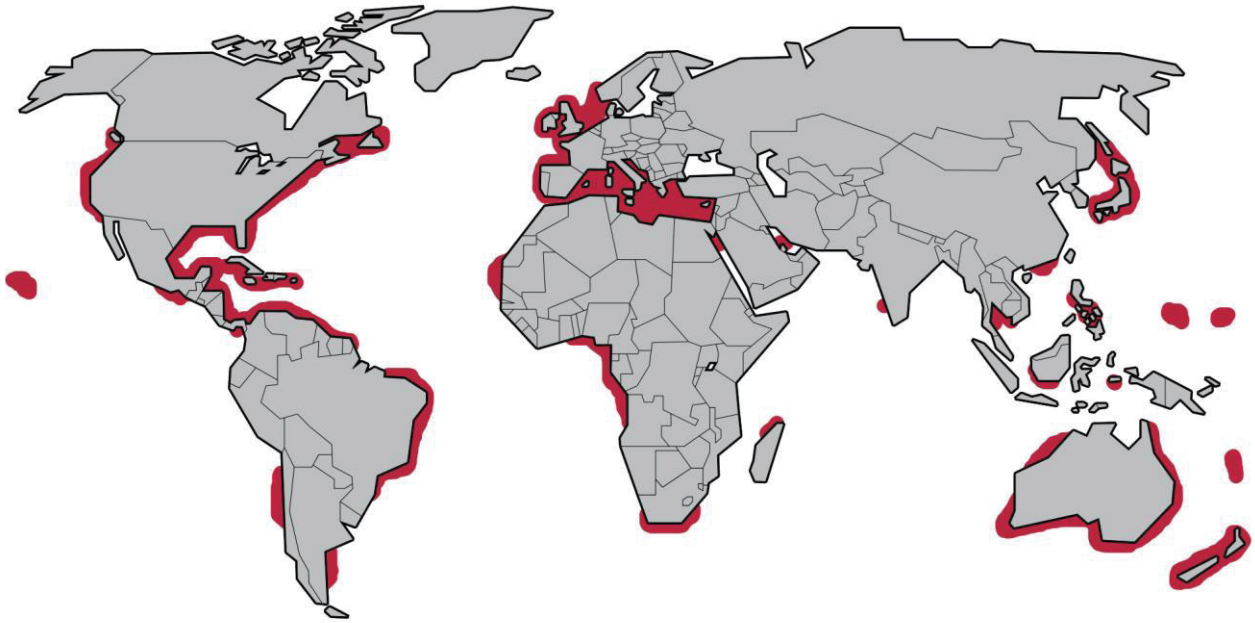

Figure 12: Distribution known for *D. listerianum* complex.

Discussion

This study found 33 new haplotypes of COI for *Diplosoma listerianum* in addition to the 43 haplotypes previously known around the world (Pérez-Portela *et al.* 2013) and revealed the existence of different lineages along the West Atlantic. The new phylogeny suggests that the *D. listerianum* complex is formed by seven clades, instead of four as proposed by Pérez-Portela *et al.* (2013), and the analysis of species delimitation indicated further subdivision of those clades into 17 taxonomy entities. The use of more than just one method for species delimitation gives us the necessary strength to support our results as closest as possible to reality, since the congruence between methods is reinforced and “splitter” methods are alleviated (Carstens *et al.* 2013). In the other hand, studies with a multiloci approach are necessary to confirm our results and have the potential to reveal even more intraspecific variation, as was already showed for a very known ascidian species (*B. schlosseri*) complex for which complete mitogenomes were sequenced (Griggio *et al.* 2014). The use of one single marker could generate an incomplete result (Maddison 1997) by ignoring the whole history of this lineage since it is telling us the story of one

gene, but previous studies using this mitochondrial approach were able to reveal a great hidden diversity in ascidians (Turon *et al.* 2003, Tarjuelo *et al.* 2004, Pérez-Portela & Turon 2008, Rius & Teske 2013).

In comparison to other species in Order Aplousobranchia, the 76 haplotypes of this COI fragment for *D. listerianum* is a very large number. In the same family, *Didemnum vexillum* Kott, 2002 (Stefaniak et al., 2009), has 10 known haplotypes. In Pseudodistomidae, *Pseudodistoma crucigaster* Gaill, 1972 revealed 11 haplotypes in the west Mediterranean Sea (Tarjuelo *et al.* 2004). In Phlebobranchia, *Ciona intestinalis* (Linnaeus, 1767) has 46 known haplotypes from samples from the United States and Canada (Zhan *et al.* 2012). In Stolidobranchia larger number of haplotypes are known: 58 for *Botryllus schlosseri* (Pallas, 1776) to the east coast of the United States and Europe (Nydam *et al.* 2017), 52 for *Microcosmus squamiger* (Michaelsen, 1927) in sequences from several regions of the world (Rius *et al.* 2008) and 171 for *Pyura chilensis* Molina 1782 from eight populations of the Chilean coast (Haye & Muñoz-Herrera 2013). Among the above species, some are known complexes like *Botryllus schlosseri* and others showed themselves much more connected like *Pyura chilensis* that is a single species. In this way, haplotype number is not a characteristic of a species complex but can lead us to find diversification within species. Besides a high number of haplotypes in our data, the haplotype network showed some structure with some clades (A1) with highly connected haplotypes and other very isolated clades (*e.g.* Clade B and C), supporting the existence of a complex.

In our analyzes clades A1 and A2 were 10.9% divergent, and the more distant clades were 20.2% divergent (Clades B and C), distance equal or larger than the interspecific divergence found in some other Aplousobranchia genera like *Clavelina* (15-20%) and *Pycnoclavella* (10-21%) (Pérez-Portela & Turon, 2008) and in Stolidobranchia genera, such as *Botryllus* (10-16,5%) (Bock *et al.* 2012). These comparisons give more support to the hypothesis that *D. listerianum* is indeed a cryptic species complex with high genetic diversity.

Samples from three Brazilian states Sergipe, Ceará and Maranhão correspond to new registers for this species complex, that now is recorded from 12 states in both natural and artificial substrates. While clade A2 is spread worldwide, the new determined clade A1 is distributed along the west Atlantic, from Florida to South Brazil with one occurrence at South Africa and another in the Pacific side of Panama. A few samples collected in the Atlantic coast of Canada failed to amplify and it was not possible to determine if Canadian population will contain clade A1, or A2, or both. The occurrence of Clade A1 right at the Pacific side of Panama Canal and the disjunct population found in South Africa by Pérez-Portela et al. (2013) can reinforce the human mediated transport (Lambert 2001). The canal is responsible for the transit of one thousand ships per month at least (Autoridad del Canal de Panama, 2018). Even passing through freshwater lakes that could be a barrier for marine organisms, some ships take less than a half day to pass by the Canal, and this could offer the chance for ascidians to cross this 'bridge' between oceans, hypothesis proposed before (Carman *et al.* 2011). In this study, 66% of Pacific samples from Panamá belonged in Clade A1, but although one of the haplotypes found in Pacific Panama was also found in the Atlantic side (19) the other was private (61). Transport coupled with previously recognized physiologic and reproductive characteristics (Bishop & Riland 1991; Bishop & Sommerfeldt 1996, Sommerfeldt et al. 2003, Rocha 1991, Sorte *et al.* 2010 and Lenz *et al.* 2011), may allow this complex of species to arrive and settle in different regions and provide more diversity to some localities.

While clades A1, A2 and C are geographically spread, Clades B, D, E, F and G are geographically restricted. Mexican samples from natural substrates in the Alacranes reef far away from the coast were allocated in two different clades, E and G, with 15.2% divergence between them, suggesting that these represent two native species to this region. Panamanian samples on the other hand, were allocated in six different clades, two of them exclusive to Panama: clade D has two haplotypes found in a marina by Pérez-Portela et al. (2013) and clade F has been found on settlement plates deployed in the Bocas Research Station dock, both in Bocas del Toro province. The lack of samples from natural substrates in this region preclude us to classify those exclusive clades as native or introduced, but the large traffic of recreational and transport boats and presence of Almirante port in the region suggest

that at least part of this diversity has been introduced in the region. Thus, the high biodiversity seen in the West Tropical Atlantic has its origin in both native and introduced haplotypes of the *Diplosoma listerianum* complex.

Colonies analyzed do not have contrasting patterns in their morphological characteristics that could permit differentiation of Clade A1 from other clades. On contrary, we found a large variation in colony color and texture, in the pigmentation of zooid's squamous epithelium and in measurements of larvae, within the range of variation which has been also observed in probable populations of clade A2 (Rowe 1966, Lahille 1890, Lafargue 1968). Some colonies had a large range in the size of larvae and there were also little differences on position of internal organs. Pérez-Portela *et al.* (2013) mentioned that they did not find any morphological characters useful to differentiate the clades in their study, but they did not mention if they had larvae available from all clades. We did not have larvae available from clades E, F and G neither and given that these clades are more distant from clades A1 and A2, there is a chance that some differences will be found. Other ascidian species complex, such as *Ciona* spp. also showed very subtle differences among cryptic species, and the use of a few measures in a morphometric analysis of larvae clarified how to recognize *C. robusta* from *C. intestinalis* (Pennati *et al.* 2015), after many years of study. Other species inside *Diplosoma* genus are well distinguished and well supported based on characters that was not studied in *D. listerianum* yet, like number of stigmata per row on each side of the zooid (Hirose & Oka, 2008), giving us incentive to continue with the species complex studies.

One of *D. listerianum* synonyms is *D. macdonaldi* (Herdman, 1886) was described using materials from Brazilian coast (Bahia state) and could possibly correspond to Clade A1 samples analyzed in this study, but its original description is very short, simple and does not describe larvae. Besides that, we did not have the opportunity to examine the type specimen, making this comparison infeasible.

Given its fast growth on artificial substrates and amenability to manipulation in lab conditions (Bishop & Ryland, 1991) we suggest that different populations of this complex should be cultivated in laboratory to facilitate proper relaxation and

addressing the morphological characters during growth and development of zooids, eggs and larvae to look for markers to differentiate species in the complex.

During decades, *D. listerianum* was used as a model species for physiological (Sorte *et al.* 2010, Lenz *et al.* 2011) and reproductive (Lane 1973, Bishop & Riland 1991, Bishop & Sommerfeldt 1996, Sommerfeldt *et al.* 2003) studies. It is highly possible that the results obtained by these authors cannot reflect the reality of the species complex and represent results valid for clade A2 only. Clade A2 alone was formed by four taxonomic entities determined by delimitation analysis, suggesting that this species is diversifying very fast and comparable to *B. schlosseri* (Griggio et al. 2014), and could be an excellent model to watch evolution in real time. The short life cycle, early acquisition of gonads and larvae, opportunistic colonization of open substrates, ability of epizoic growth and amenability to human transport can explain the creation of isolated populations of this complex and the fast evolution of these populations into different taxonomic entities.

Conclusion

We found a hidden molecular diversity for Clade A1 in Atlantic Ocean and determined that this clade is distributed in the West Atlantic, from Florida to South Brazil, with introduced populations in the Pacific side of Panama and South Africa. Although clade A2 is spread in all oceans, none of the samples sequenced in Brazil, Mexico or Florida belonged in this clade. In Africa, only samples from South Africa were studied by now, although the species is known from many Atlantic countries. This can reinforce the necessity of more research on these animals, including more sampling.

The genetic diversification was accompanied by phenotype diversification and we could not find morphological data to separate clade A1 from clade A2 (the more studied) in *D. listerianum* complex. The cultivation of populations from different clades could be an opportunity to follow sexual reproduction and developing larvae that could lead us to some diagnostic characteristics to separate clades. The use of new technologies for sequencing and the use of a phylogenomic approach will clarify further the taxonomic entities belonging in this complex and their distribution. Here,

we reinforce the necessity of a closer look at globally distributed species, specially ascidians, as they can represent more hidden biodiversity than formerly supposed.

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Appendix

Table 1: Specimens from Scientific Collection of Universidade Federal do Paraná used in the morphological study and corresponding sequences used in molecular studies when available.

DZUP code	Collection date	Preservative	Ocean	Country	State/ Province	City	Locality	Lat	Long	Collector	Haplotype
Did-083	11/04/1997	Formalin	Atlantic	Brazil	PR	Ilha de Currais		25º44'04" S	48º21'54" W	Faria, S.B.	
Dipl-035	03/03/2012	Formalin	Atlantic	Brazil	BA	Salvador	Marina Bonfim	12°55'13"S	38°30'38"W	Neves, I. M.	
Dipl-036	03/03/2012	Formalin	Atlantic	Brazil	BA	Salvador	Marina Píer Salvador	12°54'49"S	38°29'28"W	Neves, I. M.	
Dipl-043	01/09/2014	Ethanol	Atlantic	Brazil	RJ	Angra dos Reis	Marina Porto Galo	23°2'25.26"S	44°12'13.29"W	Skinner, L. F.	
Dipl-051	06/05/2011	Formalin	Atlantic	Brazil	RJ	Cabo Frio	Ilha Comprida 2	22º52'19"S	41º57'12"W	Skinner, L. F.	
Dipl-055	09/05/2014	Formalin	Atlantic	Brazil	RJ	Cabo Frio	Ilha de Papagaio	22º53'55"S	41º58'42"W	Skinner, L. F.	
Dipl-061	12/02/2011	Formalin	Atlantic	Brazil	ES	Guarapari	Iha Escalvada	20º42'00"S	40º24'30"W	Gamba, G.A.	
Dipl-091	14/02/2017	Formalin	Atlantic	Brazil	SC	Bombinhas	Praia Lagoinha	27°08'43"S	48°28'50"W	Rocha, R. M.	57
Dipl-095	04/09/2006	Formalin	Atlantic	Brazil	SC	Florianópolis	Ribeirão da Ilha	27°44'08"S	48°33'52"W	Rocha, R. M.	
Dipl-102	12/12/2005	Formalin	Atlantic	Brazil	SC	Penha		26°46'S	48°36'18"W	Rocha, R. M.	
Dipl-103	14/12/2016	Formalin	Atlantic	Brazil	PR	Pontal do Sul	Marina Ponta do Poço	25°32'55.88"S	48°23'17.74"W	Rocha, R. M.	

Dipl-105	14/02/2017	Formalin	Atlantic	Brazil	SC	Bombinhas	Praia Lagoinha	27°08'43”S	48°28'50”W	Rocha, R. M.	
Dipl-106	14/12/2016	Formalin	Atlantic	Brazil	PR	Pontal do Sul	Marina Ponta do Poço	25°32'55.88"S	48°23'17.74"W	Rocha, R. M.	59
Dipl-112	14/12/2006	Formalin	Atlantic	Brazil	SC	Florianópolis	Ribeirão da Ilha	27°44'08”S	48°33'52”W	Rocha, R. M.	
Dipl-124	22/09/2006	Formalin	Atlantic	Brazil	SC	Penha		26°46'S	48°36'18"W	Rocha, R. M.	
Dipl-132	07/07/2016	Formalin	Atlantic	Brazil	MA	São José de Ribamar	Praia de Panaquatira	02°29'19.13''S	044°02'7.40'' W	Paiva, S. V.	18
Dipl-144	10/11/2016	Formalin	Atlantic	Brazil	BA	Salvador	Marina Mercado Modelo	12°58'21"S	38°30'55"W	Teixeira, J. A.	
Dipl-146	10/11/2016	Formalin	Atlantic	Brazil	BA	Salvador	Marina Mercado Modelo	12°58'21"S	38°30'55"W	Teixeira, J. A., Rocha, R. M.	
Dipl-148	14/02/2017	Formalin	Atlantic	Brazil	SC	Bombinhas	Praia da Lagoinha	27°08'43”S	48°28'50”W	Rocha, R.M, Teixeira, J.A.	57
Dipl-179	12/12/2005	Formalin	Atlantic	Brazil	SC	Penha	Cultivo Penha	26°46'S	48°36'18"W	Rocha, R.M	
Dipl-182	28/03/2017	Formalin	Atlantic	Brazil	ES	Guarapari	Escalvada face norte	20°41'55,3”S	40°24'20,8”W	Rocha, R.M, Paiva, S. V.	
Dipl-184	28/03/2017	Formalin	Atlantic	Brazil	ES	Guarapari	Escalvada face norte	20°41'55,3”S	40°24'20,8”W	Rocha, R.M, Paiva, S. V.	
Dipl-198	14/12/2007	Formalin	Atlantic	Brazil	BA	Salvador	Quebramar Sul	12o58’22”S	38o31’09”W	Rocha, R.M	
Dipl-204	12/12/2007	Formalin	Atlantic	Brazil	BA	Salvador	Quebramar Sul	12o58’22”S	38o31’09”W	Rocha, R.M	
Dipl-206	29/03/2011	Formalin	Atlantic	Brazil	RJ	Cabo Frio	Ilha de Papagaio	22º 53.736' S	41º 59.13' W	Rocha, R.M	

Dipl-223	13/12/2017	Formalin	Atlantic	Brazil	RN	Natal	Baixa do Cotovelo	5°55'28,6" S	35°7'20,7" W	Rocha, R.M.	**58**
Dipl-224	20/06/2017	Formalin	Atlantic	Panama	Bocas del Toro	Bocas del Toro	STRI Dock	9°21'3.89"N	82°15'26.00"W	Rocha, R.M.	
Dipl-234	29/06/2017	Formalin	Atlantic	Panama	Bocas del Toro	Bocas del Toro	Solarte	9°18'36.35"N	82°11'44.11"O	Rocha, R.M.	
Dipl-241	03/07/2017	Formalin	Atlantic	Panama	Bocas del Toro	Bocas del Toro	Marina Bocas	9°20'9.54"N	82°14'46.87"W	Rocha, R.M.	**64**
Dipl-247	16/12/2007	Formalin	Atlantic	Brazil	BA	Salvador	Porto da Barra	13º00'14"S	38º32'01"W	Rocha, R.M.	
Dipl-251	26/01/2012	Formalin	Atlantic	Brazil	ES	Guarapari	Naufrágio	20°41'23"S	40°23'24"W	Gamba, G. A.	

Table 2: Measurements taken from larva. Code numbers correspond to DZUP scientific collection numbers and the letters A – Q to measurements described in Table 02.

Code	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
Did083	797.74	0	797.74	90.16	56.45	28.717	254.82	153.89	90.54	0	380.45	353.94	20.38	606.59	0	201.49	19.14
Dipl035	411.2	814.82	1226.02	43.1	37.78	19.98	129.1	115.08	9.866	34.74	117.54	158.1	13.95	264.2	58.5	119.962	12.35
Dipl036	536.06	1043.6	1579.66	64.85	55.982	21.049	115.61	119.15	90.18	31.15	228.32	250.41	16.72	306.7	76.6	101.32	18.2
Dipl043	477.98	889.83	1367.81	63.64	28.6	25.54	130.91	157.41	68.54	43.67	-	-	-	-	71.56	159.36	17.03
Dipl051	894.02	1344.06	2238.08	75.26	58.76	24.25	244.66	273.5	126.48	32.53	367.26	468.41	21.62	696.6	86.37	242.23	25.885
Dipl055	732.09	1365.74	2097.83	72.45	66.8	31.91	193.6	227.11	111.6	32.02	289.9	435.525	20.61	662.02	87.59	231.1	20.4
Dipl061	597.505	990.246	1587.75	80.037	53.689	27.043	139.879	189.83	93.722	32.95	273.82	296.908	17.59	455.093	66.563	140.454	15.764

Dipl091	381.06	923.27	1304.33	56.21	33.9	21.25	100.39	105.36	48.47	34.7	161.36	211.83	12.36	231.68	53.307	97.35	12.74
Dipl095	502.76	1059.66	1562.42	63.71	59.5	28.6	170.25	167.67	-	-	201.78	216.1	18.05	366.594	65.5	145.33	19.14
Dipl102	386.9	1060.51	1447.41	53.39	34.41	18.01	111.5	133.29	44.77	33.39	162.71	186.04	11.86	249.32	48.31	111.33	11.52
Dipl103	488.092	856.041	1.344.133	62.835	45.113	17.6	126.647	175.431	115.94	43.63	195.85	214.5	14.93	331.73	57.64	148.57	21.44
Dipl105	476.86	901.24	1378.1	57.7	48.88	21.28	10.61	126.06	42.76	44.8	202.68	245.82	16.8	323.5	57.941	146.63	18.08
Dipl106	417.76	805.76	1223.52	50.83	42.43	29.52	135.74	148.87	54.124	31.06	168.8	229.94	15.76	310.45	52.86	128.28	10.29
Dipl112	356.17	1018.42	1374.59	67.22	48.37	23.35	129.6	138.92	49.33	27.22	188.11	117.06	17.16	306.85	67.54	154.65	16.64
Dipl124	599.43	870.37	1469.8	70.78	58.23	20.95	164.34	167.92	112.13	39.79	287.74	333.26	18.9	376.92	82.33	174.6	21.45
Dipl132	484.197	840.45	1.324.647	52.165	41.805	19.35	129.381	115.92	50.9	33.61	205.23	271.66	18.1	322.18	63.4	-	-
Dipl144	451.45	638.7	1090.15	64.33	58.51	16.24	137.39	148.88	36.9	31.87	199.28	235.41	17.3	354.14	61.27	150.7	15.16
Dipl146	425.77	770.8	1196.57	63.8	47.15	9.63	92.75	118.62	-	-	184.46	247.472	15.11	325.35	58.9	-	-
Dipl148	516.81	852.41	1369.22	53.07	55.887	19.56	142.88	132.42	61.439	45.42	199.92	280.946	15.92	326.68	60.66	121.09	14.87
Dipl179	416.43	1047.02	1463.45	66.14	42.9	20.72	124.043	136.75	70.44	38.35	183.61	238.22	17.18	303.91	58.28	131.58	15.21
Dipl182	597.32	1303.94	1901.26	61.64	49.58	24.54	165.09	219.25	96.59	65.86	269.44	289.38	23.99	522.8	82.81	-	-
Dipl184	759.1	1457.68	2216.78	77.09	55.68	33.29	193.14	270.06	140.68	42.04	341.38	396.85	17.72	515.97	101.45	247.74	25.64
Dipl198	548.81	1259.28	1808.09	58.38	47.52	31.34	166.58	176.05	112.63	37.3	297.45	206.75	20.27	425.4	74.82	201.06	26.06
Dipl204	518.42	879.72	1398.14	57.85	53.7	21.44	138.71	142.72	96.14	37.86	231.45	305.54	12.45	369.64	69.445	145.62	15.34
Dipl206	770.88	1285.92	2056.8	70.15	76.01	22.67	232.05	249.65	124.67	51.32	307.25	401.52	19.605	19.6	99.56	-	-
Dipl222	399.46	617.95	1017.41	58	38.96	15.28	132.35	161.7	98.6	41.52	184.37	201.04	11.93	345.31	60.9	163.42	16.75
Dipl223	1143.11	701.89	1845	50.46	54.82	21.37	179.43	201.1	190.54	37.25	327.49	327.35	21.39	431.68	85.79	207.38	20.39
Dipl234	441.79	793.52	1235.31	57.47	39.52	18.21	84.02	91.06	83.93	37.22	242.09	197.14	17.08	308.9	74.55	165.99	17.84
Dipl241	432.24	921.75	1353.99	50.57	41.16	21.27	110.12	140.25	86.64	17.89	194.18	146.89	12.67	291.69	56.04	142.01	12.9
Dipl247	522.78	1482.82	2005.6	59.34	40.251	37.99	155.194	167.14	79.36	23.27	266.54	267	15.89	477.172	71.36	156.64	15.77
Dipl251	437.89	1346.74	1784.63	58	48.46	21.78	143.38	137.92	60.87	21.54	244.59	287.67	14.96	431.53	88.78	217.68	19.68