Phylogenetic reconstruction of dinoflagellates from the genus *Symbiodinium* (Dinophyta) using ITS2 secondary structures

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Abstract

RNA secondary structures have been useful to identify cell machineries and processes, but recently the characters that these structures turn into have been used in phylogenetic reconstruction. This work showed the use of ITS2 (rDNA) secondary structures for inferring relationships between species, employing the method known as molecular morphometrics. This method uses morphological characteristics belonging to secondary structures (such as the number of nucleotides in helixes, bulges, internal and external loops) as phylogenetic characters. In the present study, dinoflagellates from the genus *Symbiodinium,* commonly known as zooxanthellae*,* were selected as model group, which have phylogenetic reconstructions with other molecular regions and markers, and relationships within the group are well known. Twenty new secondary structures were predicted for a total of 45 secondary structures used to construct a morphometrics matrix. These secondary structures had the distinctive characteristics for eukaryotes (an internal ring and four helixes). A total of three data sets were obtained to carry out phylogenetic analyses: 1) the morphometrics matrix, 2) the primary sequence alignment corrected by secondary structure, and 3) a combined matrix, that is, a combination of primary sequence and secondary structure's morphology. We found that the phylogenies obtained with molecular morphometrics information were partially congruent with phylogenies recovered with primary data only, the combined data, and previous reported phylogenies with other molecular regions. We corroborated the utility of secondary structures in improving the primary sequence alignments and combining data, which enhances most of ITS2 information, making secondary structure an excellent tool in phylogenetic systematics. Nonetheless, more studies are required in order to elaborate a model of evolution for ITS2 secondary structures.

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Introduction

Ribosomal genes are one of the most popular sequence regions used in phylogenetic systematics to infer relations amid groups of different taxonomic levels [e.g. 1, 2]. The eukaryotic nucleus is composed of three ribosomal genes known as 28S or large subunit, 5.8S and 18S or small subunit, two internal transcribed spacers, the ITS1 (between the 18S and 5.8S) and the ITS2 (between the 5.8S and 28S), an external transcribed spacer (ETS), and the non-transcribed intergenic spacers (IGS) [3]. These genes belong to a multigene family, thus their copies are repeated in tandem and disperse in the genome [4]. The entire ribosomal region is subject to concerted evolution [5] implying that the copies are homogenized in the genome, which enables its use in phylogenetic analysis as a single gene [6].

The sequence of the ITS2 is of particular interest because it has been recognized as a region with the capability to resolve relations at various taxonomic levels, from order to subspecies [3]. Additionally, it is easy to amplify via PCR and sequence given that primers are design on the conserve regions that flank it (5.8S and 28S) and has a size no bigger than 350 bp [6]. These characteristics make the ITS2 an attractive sequence for molecular systematic studies. However, the primary sequence of the ITS2 exhibits great variability [3, 7] making difficult the finding of homologous sites for alignments[8]. Nonetheless, the ITS2 has the advantage of having a conserved secondary structure in many eukaryotes[4, 9] due to the function that this structure has in the maturation of ribosomal RNA [4, 10, 11]. Therefore, it has been used by various authors in improving alignments and posterior use in phylogenetic analysis [e.g. 1, 2, 7].

The form of the ITS2 secondary structure was determined through crystallography studies [12] and through computational software such as Mfold [13] and ARB [14], which predict the structures folding the molecule using the method of minimum Gibbs free energy [15, 16]. The ITS2 structure is characterized by having a central ring and four helixes that have some features as helix III is the longest, helix II is the most stable and has a pyrimidine-pyrimidine internal loop, and helix IV is the shortest [3, 9, 17, 18].

These conserve characters are present in a wide range of organisms distantly related owed to the existence of compensatory base changes (CBC's) that maintain base pairings in the helixes of the structures[3, 19].

It has been suggested the use of secondary structures to obtain direct information in order to reconstruct phylogenies, owed to the presence of these conserved characters, in a method known as molecular morphometrics [20, 21]. The mentioned method is based in assuming that the information retained from the secondary structure may be as phylogenetic informative as the information obtained from primary sequence alignments [21]. The morphological matrix is constructed counting the number of nucleotides in the secondary structures' features, which are helixes, bulges, internal and external loops. The matrix is further used to carry out phylogenetic analyses [20, 21]. This method has proven to be reliable given that it has been used to study relations amid species of different group of organisms. Some examples using secondary structures in inferring phylogeny include the ITS2 secondary structures of genera and species of octocorals [8, 22, 23], 28S and 18S secondary structures of arthropods orders [21, 24], and the phylogeny of the universal tree of life [20].

We used zooxanthellae from the genus *Symbiodinium* as model group in order to examine the use of molecular morphometrics in their phylogeny given that several phylogenetic studies with different regions, such as ribosomal [25], mitochondrial [26], and chloroplast [25, 27] have been used. Additionally, some ITS2 secondary structures of most of *Symbiodinium* clades have been previously reported [18]. Zooxanthellae are endosymbionts of coral polyps and other invertebrates [e.g. 25, 28]. They are also an essential component of coral reefs since they have a significant contribution in the host productivity, survival, and success [e.g. 29]. Zooxanthellae are grouped in eight different lineages or clades (A-H) [25], each one with great genetic types that show distinct geographic and ecological patterns and host specificity [30]. For these reasons, the aim of this study was to analyze the phylogenetic congruence of molecular morphometrics data from ITS2 secondary structures of different zooxanthellae clades. Additionally, we compared this data with phylogenies obtained with molecular data and a combined matrix

from both types of data. This combined matrix allowed a phylogenetic analysis of all the information contained from the data collected.

Methods

ITS2 Sequences

For the phylogenetic analyses, twenty-four ITS2 sequences and their secondary structures, published by [18], were used belonging to the genus *Symbiodinium*. Additionally, sequences of zooxanthellae associated to different marine hosts found on Genbank databases, 13 sequences of zooxanthellae from the sponges genus *Cliona* (EF134611-EF134623, Granados et al., submitted), one sequence of zooxanthellae from zoanthids (Granados et al., unpubl.), five sequences of zooxanthellae form foraminiferans [31], the free-living strain clade A[32], and the outgroup *Gymnodinium* sp. (AF201747) for a total of 45 sequences used.

ITS2 Secondary Structures

Sequences obtained from Granados et al. (submitted), Granados et al. (unpubl.) and [31] were folded in the software Mfold v3.2 [13] and further edited in the graphics program CorelDraw v2.3. We generated a parenthesis format of the secondary structures for each sequence with the program CBC analyzer [33]. These sequences were aligned in the program 4SALE, which aligns using a modified algorithm of CLUSTALW and synchronous the alignment of sequences with their secondary structures [34]. A matrix of CBC's between pairs of sequences was also generated with 4SALE.

Molecular Morphometrics

Morphological characters from the secondary structures were determined following the nomenclature proposed by [17] and used by [18]. Structures' characteristics (helixes, internal loops, external loops and bulges) were established in the 5'-3' direction of the sequence and measured according to the length (number of nucleotides) of each feature.

We used these measures to create a character/species matrix in order to use for multiple phylogenetic analyses (Supplementary Material A).

Phylogenetic Analyses

Three phylogenetic analyses were carried out with the aligned primary sequences. A maximum parsimony analysis was run in PAUP* 4.0b10 [35]using heuristic search and branch support with 1,000 bootstrap replicates. A maximum likelihood analysis was done in the portal CIPRES v1.13 (www.phylo.org) with the tool RAxML [32]
using the parameters of the nucleotide model of substitution general time reversible (GTR+G) and branch support with 1,000 bootstrap replicates. Finally, we did Bayesian inference using MrBayes [36] employing the parameters from the nucleotide model of substitution GTR+G obtained from AIC (Akaike information content) inferred in the program MrModelTest [37]. The analysis was carried out with one million generations, four Markov Monte Carlo chains, burn-in 500, and a sample frequency of 100 generations.

For the molecular morphometrics, we did maximum parsimony using Wagner parsimony (i.e. ordered characters), heuristic search, and branch support with 1,000 bootstrap replicates. Additionally, we carried out Bayesian inference using the model Mk for molecular characters proposed by [38], which assumes that each linage is always in one possible state, and none of the character states will be considered plesiomorphic or apomorphic *a priori*. The morphological matrix was partitioned in five sets corresponding to each helix of the secondary structure. For this analysis, we had to recode the character "length of helix III" given that MrBayes only accepts ten character states (0-9) [36]. The parameters used for this data were as previously described.

Finally, we did Bayesian inference for the combined matrix, that is, data from the primary sequences and from the molecular morphometrics. This matrix was partitioned in six sets: one set corresponded to the primary sequence and five sets corresponded to the morphological matrix. The parameters used for this data were one million generations from four MCMC chains, burn-in 500 and a sample frequency each 100 generations.

Results

ITS2 Secondary Structures

We obtained for first time eight sequences belonging to *Symbiodinium* clade A, two sequences from clade B and three sequences from clade G engaging in symbioses with sponges from the genus *Cliona* (Granados *et al.*, submitted), one sequence of clade B associated to the zoanthid from the species *Parazoanthus parasiticus* (Granados *et al.*, unpubl.), five sequences of clade G associated to foraminiferans from the family Soritidae [31], and one sequence of a free-living dinoflagellate. Therefore, we have a total of 20 new secondary structures for the genus *Symbiodinium* (Fig. 1). The folded structures had Gibbs free energy values (ΔG) between -27.76 and -67 kcal at 37°C. The secondary structures belonging to clades A and G exhibited a structure with four helixes while clade B structures had five helixes (Fig. 1A, 1B). Furthermore, clade G secondary structures displayed a bifurcated helix IIIb (Fig. 1C), except the structure from type G4 (Fig. 1D). The helixes of the predicted secondary structures have the following sizes of base pairs: helix I between 8-14 bp, helix II between 12-16 bp, helix III between 15-20 bp, and helix IV between 4-7 bp. It was interesting to find that 93% had a loop of pyrimidines in helix II. Of all the predicted secondary structures, helix IIIb was the most variable one in characters and length (Fig. 1).

From the ITS2 sequences used in this study, we did not find CBC's among sequences belonging to the same zooxanthellae clade, except the sequence MedA, which showed between two and three CBC's with sequences from clade A and hemiCBC's with sequences from types G1a and G3 (Supplementary Material C). We also found between one and eight CBC's among structures from different *Symbiodinium* clades (Supplementary Material C). The secondary structures that exhibited the highest number of CBC's with respect to the rest were types E1, showing between two and eight, and D4, showing between one and six (Supplementary Material C). Most of the CBC's were found in the bases of helixes I and II and in the central and apical part of helix IIIb.

Phylogenetic Analyses

The maximum parsimony analysis carried out for primary sequence matrix had 411 characters and showed 406 variable characters, of which 265 were parsimony informative. The most parsimony tree had a length of 1121 steps with a consistency index of 0.657 and a retention index of 0.883. Table 1 indicates the node supports that were consistent in all the topologies obtained, hereafter shared nodes, which have support values of 63 to100.

The maximum parsimony analysis done for the morphometrics matrix had 77 characters with 53 parsimony informative characters. The most parsimony trees had a length of 658 steps, a consistency index of 0.46, and a retention index of 0.8. In the phylogeny obtained from this analysis, clade C appears as paraphyletic, including clade H and F, while the rest of the clades are monophyletic. The bootstrap support for shared nodes had values of 64 to 87 (Table 1).

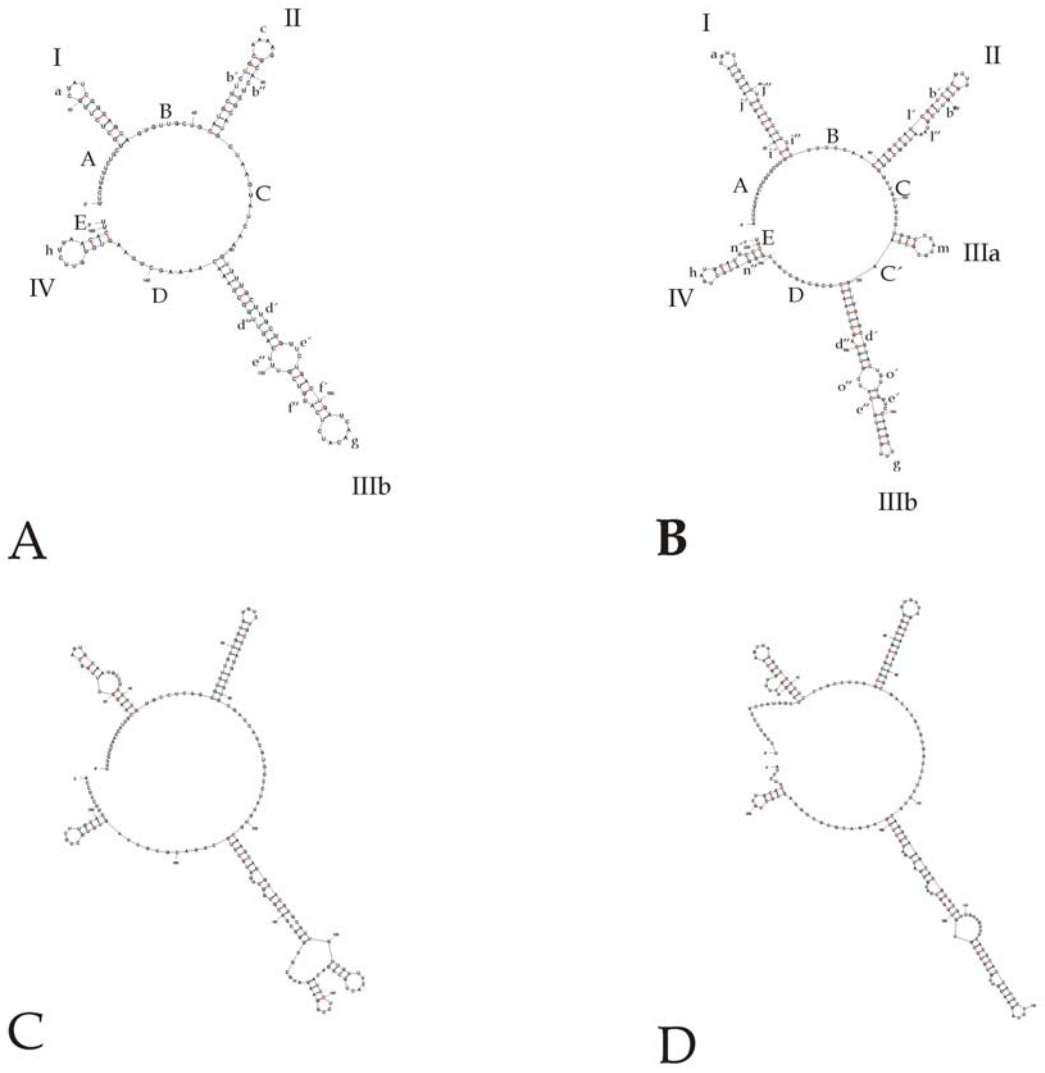

Figure 1. Predicted ITS2 secondary structures for different clades of the genus *Symbiodinium*. **A.-B.** Secondary structures showing the molecular morphometrics characters of the ITS2 secondary structures, following [17]. **A.** Typical secondary structure composed of four helixes, which corresponds to clade A, associated with the sponge *Cliona aprica*. **B.** Typical secondary structure composed of five helixes, which corresponds to clade B associated to *C. aprica*. **C.** Secondary structure of clade G associated to *C. varians*. Note the bifurcation of helix IIIb. **D.** Secondary structure of type G4, associated to foraminiferans of the family Soritidae.

The maximum likelihood analysis for the molecular data had the following nucleotide frequencies: A: 0.178, C: 0.245, G: 0.248 and T: 0.325. In this analysis, *Symbiodinium* A

is resolved as polyphyletic, clade G is resolved as paraphyletic, including D and the other clades as monophyletic. Bootstrap supports of shared nodes were between 89 and 99% (Table 1).

In the Bayesian inference consensus tree for the primary sequences, we obtained that all clades were monophyletic (Fig. 2A). The phylogeny for molecular morphometrics data showed clade B and G as monophyletic, Clade C as paraphyletic, including H and F, while the rest of clades were polyphyletic (Fig. 2B). The phylogeny for combined data showed clade A as polyphyletic and the rest of clades were monophyletic (Fig. 2C). Comparing the three topologies, we observed that the topologies obtained from molecular data and combined data exhibited the phylogenetic pattern found by [18]. This pattern shows clades B, C, F, and H grouped together as a monophyletic group due to the synapomorphy of the presence of a fifth helix in the secondary structures (Fig. 1A-B and Fig 2A, 2C). However, the phylogenetic hypothesis generated by molecular morphometrics showed that the novelty of the fifth helix was a homoplasic character (Fig. 2B). We carried out map of morphological characters based on the topology obtained by Bayesian inference in the program MacClade . We obtained 11 synapomorphies of clade B members and clades C and G only showed three synapomorphies.

In the three topologies of Bayesian inference, the sequences from clades A and G, which are symbionts of sponges of the genus *Cliona*, were monophyletic within each corresponding clade. This result was different from *Cliona* hosting clade B that was grouped different in each tree (Fig.2).

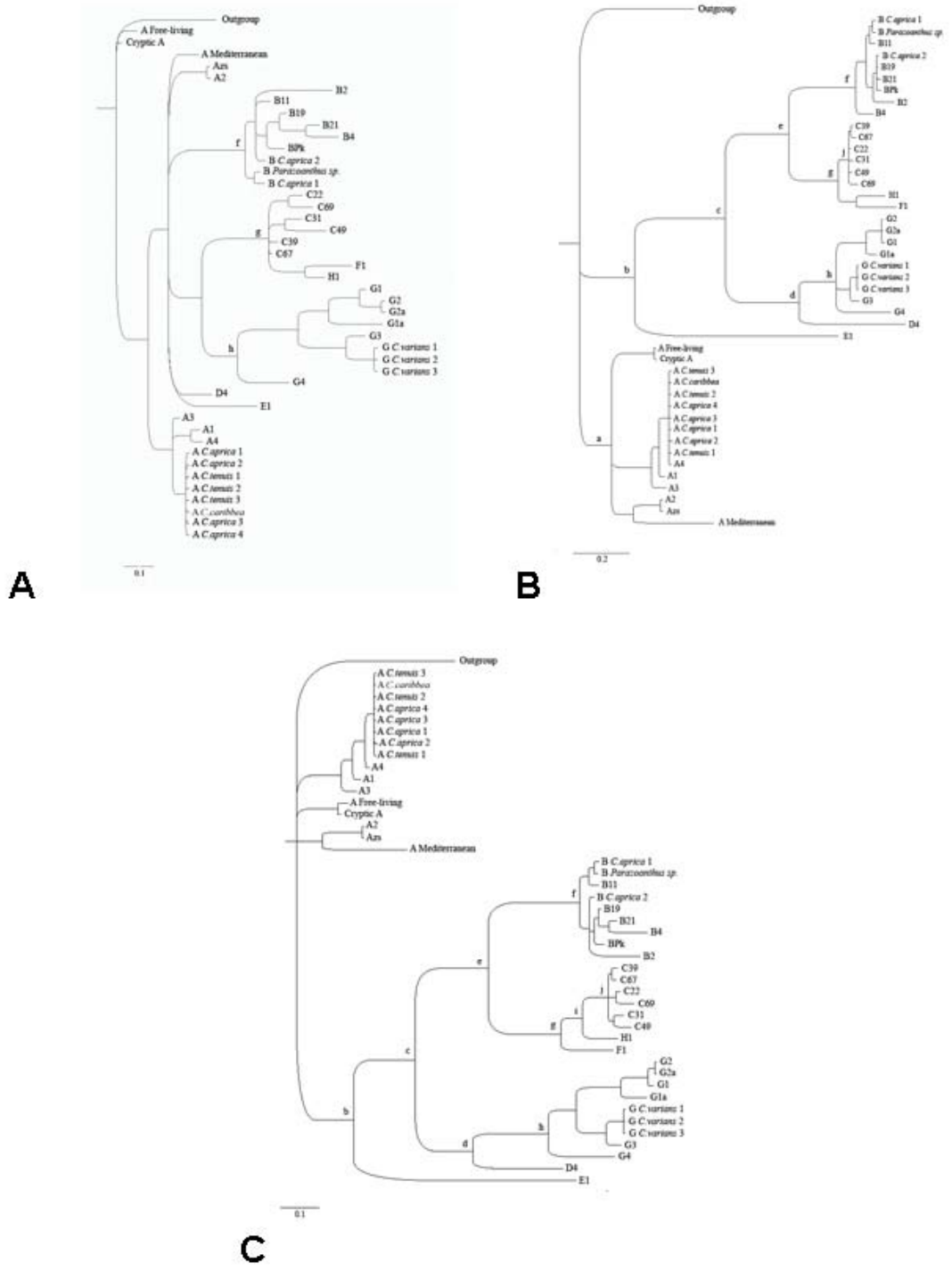

Figure 2. Phylogenetic hypothesis obtained using Bayesian inference. **A.** Topology corresponding to primary sequences under the substitution model of evolution HKY+G. **B.** Topology corresponding to molecular morphometrics under the model of evolution Mk. Characteristics of helix IIIb are mapped at the left. **C.** Topology corresponding to

combined data. Branch supports are labeled a-l and their values are presented in Table 1. Sequence information is found in Supplementary Materia A

The phylogeny for the primary sequence showed *a posteriori* probabilities from 0,68 to 1, while the phylogeny for combined data showed *a posteriori* probabilities from 0,53 to 1, and the phylogeny for morphological characters showed *a posteriori* probabilities from 0,84 to 1. However, the phylogeny from morphometric characters showed the lowest number of shared nodes compared whit the others two phylogenies (Fig. 2 and Table 1).

Table 1. Branch supports and posterior probabilities of each topology inferred by the different phylogenetic analyses carried out to each data set. Nodes are named a-l. MP: maximum parsimony; ML: maximum likelihood; BI: Bayesian inference. Symbol "-" indicates absence of the node in that topology.

	Datos Moleculares			**Datos Morfométricos**		**Datos Combinados**
Nodos	**MP**	**ML**	**IB**	**MP**	**IB**	**IB**
a	93	-	0.68	69	-	-
b	94	-	0.71	-	-	0.73
c	83	98	0.99	-	-	0.92
d	99	95	0.99	-	-	0.99
e	69	95	1	-	-	1
f	100	99	1	64	1	1
g	100	92	1	84	1	1
h	99	-	0.74	87	0.84	1
i	-	-	-	-	-	0.53
j	91	89	0.66	-	-	0.95

Discussion

ITS2 Secondary Structures

The secondary structures of the internal transcribed spacer 2 have been considered an important region in order to better understand the molecular dynamics inside eukaryote cells. These structures have functions in the formation of essential organelles that preserve cellular life and, in general, of the organism [4, 10, 11]. In this study, we corroborated that the ITS2 secondary structure has conserve architecture in most eukaryotes [4, 6] obtaining that the structures of zooxanthellae from the genus

Symbiodinium fit with the model of a central ring and four helixes [3, 7, 17]. Furthermore, these structures fit with previous published characteristics for dinoflagellates [17, 18], where clades A and G structures showed four helixes and clade B structures had five helixes (Fig.1). The presence of an additional helix could be due to a split of helix III into two helixes [17]. The fifth helix found in clade B was also found in zooxanthellae clades C, F, and H [18].

Clade G secondary structures maintained the bifurcation of helix IIIb, which was previously reported for *Symbiodinium* G [18] and for the free-living dinoflagellate *Calciodinellum operosum* [17]. The bifurcation of helix IIIb has been explained as a result of substitutions that have occurred in the sequence, but have been conserved as CBCs and hemiCBCs in the bifurcations of the helixes [18]. The absence of the bifurcation of helix IIIb in type G4 is due to an insertion (5´-CACCAU-3´) and substitutions in the sequence that have preserved the base pairings of the helix. These mutational events may produce changes in the length and form of the structure [21] and be no-compensatory mutations, such as in the case of pseudogenes [e.g. 39]. Despite these changes, the length of the helixes was among reported values for secondary structures of the ITS2 in *Symbiodinium* [18].

The combination of conserved and variable sites in the secondary structures is owed to the function that each site has during RNA maturation. Helix II is highly because it is a binding site of proteins and plays a role in the transcription process [10]. The internal loop of pyrimidines in helix II is a conserve trait, which has been observed in the ITS2 secondary structures of various eukaryotes [4, 6, 40, 41], including the genus *Symbiodinium* [18] and other dinoflagellates [17]. The variability seen in helix IIIb may be due to more relaxed functional requirements [42], thus it shows phylogenetic signal.

The presence of characteristics in the secondary structures such as CBCs maintains nucleotide pairings that allow the formation of homologous helixes in different species [3, 19]. We found that the number of CBCs in the ITS2 sequences of *Symbiodinium* of the same clade (between 0-2bp) and among clades (between 1-8bp) are within the ranges

found by [19]. He showed that sequences of the same species did not have CBCs, while sequences of different species had at least 2 CBCs. Additionally, the number of CBCs increases proportionally with genetic distance.

Phylogenetic analyses and molecular morphometrics

Primary sequence alignments have been corrected using secondary structure, which have conserve architecture in most eukaryotes [3, 4, 6, 40]. This improves alignments of the ITS2 region that has great nucleotide variability given that it places homologous characteristics of the secondary structures in the alignment [2]. These alignments have had a major utility for phylogeny reconstructions in different groups including insects [1, 41], plants [2], algae [43], and dinoflagellates [44]. In this study, the *Symbiodinium* phylogeny obtained with molecular data, from the alignment with 4SALE, showed high congruency with previous phylogenies (ITS2 and partial LSU, [[20, 25]; mitochondrial *Cox1*, [26]; chloroplast 23S, [27]. Furthermore, this corroborates the advantages of ITS2 alignments in synchrony with secondary structures for its use in inferring phylogenies.

Molecular morphometrics is a new tool in phylogenetic systematics [20, 21]. It tries to answer relations among organisms using only the information from secondary structures of molecules regardless the high variability of the primary structure. The use of ITS2 secondary structures with molecular morphometrics had shown to be a good strategy for resolving phylogenies of groups of octocorals [8, 22, 23]. However, in other organism, such as basidiomycotas, its power of resolution has been limited [45]. In this study, we showed that phylogenies obtained solely from molecular morphometrics were not highly congruent with hypotheses based on molecular data, combined data (Fig.2), and previous published phylogenies of *Symbiodinium* [e.g. 25]. The phylogeny inferred with molecular morphometrics highly depends on the model of evolution used in the analysis of Bayesian inference (Mk model, [38]. However, this model should be used with caution given that its predictions (homogeneous change rate and free change of character states) may not be generalized to all morphological characters (for more details, [38]. Some authors have suggested the creation of a model of evolution for secondary structures able

to differentiate their variation events. The former idea will allow more confidence in the phylogenies inferred from this information [21, 45].

The phylogeny with the best resolution and highest branch supports was inferred from combined data. This result shows the great utility of using combined data sets in reconstructing phylogenies given that it uses the primary sequence and molecular morphometrics from the ITS2 secondary structures. Nonetheless, these alternatives are poorly used (Herrera et al., unpubl.) and, in some cases, it had produced inconsistent results [23].

As conclusion, the use of ITS2 secondary structures helps in different ways in the inference of phylogenies: 1) synchronizing primary sequence alignments and 2) molecular morphometrics. Additionally, it has shown results congruent with previous phylogenies, which turns it in an excellent alternative for phylogenetic systematics. However, further studies are required in order to elaborate an evolutionary model of ITS2 secondary structures to obtain more trustable phylogenies. Some research had started in this process of developing the model of evolution for secondary structures of the ribosomal genes 28S and 18S [20, 46]

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Supplementary material

A. ***Information of ITS2 sequences from Symbiodinium used in this study.***

Nomenclature/type	Species	Associated Species	Accession Number
AFree-living			
Cryptica A	*Symbiodinium sp*		AF184948
Mediteranean A	*Symbiodinium sp*		DQ865210
A1	*Symbiodinium microadriaticum*		AF333505
A2Sp	*Symbiodinium pilosum*		AF333506
A3	*Symbiodinium sp*		AF333507
A4	*Symbiodinium pilosum*		AF333509
ACa1	*Symbiodinium sp*	*Cliona aprica*	EF134611
ACa2	*Symbiodinium sp*	*Cliona aprica*	EF134612
ACa3	*Symbiodinium sp*	*Cliona aprica*	EF134617
ACa4	*Symbiodinium sp*	*Cliona aprica*	EF134618
ACt1	*Symbiodinium sp*	*Cliona tenuis*	EF134613
ACt2	*Symbiodinium sp*	*Cliona tenuis*	EF134614
ACt3	*Symbiodinium sp*	*Cliona tenuis*	EF134615

ACc	*Symbiodinium sp*	*Cliona caribbea*	EF134616
Azs	*Symbiodinium sp*	*Zoantos sociatus*	
B2	*Symbiodinium sp*		EF333512
B4	*Symbiodinum muscatinei*		EF333510
B11	*Symbiodinium sp*		DQ865211
B19	*Symbiodinium sp*		DQ865212
B21	*Symbiodinium sp*		DQ865213
BPk	*Symbiodinium sp*		AF360575
BPa	*Symbiodinium sp*	*Parazoantus sp.*	
BCa1	*Symbiodinium sp*	*Cliona aprica*	EF134619
BCa2	*Symbiodinium sp*	*Cliona aprica*	EF134620
C22	*Symbiodinium sp*		AY239373
C31	*Symbiodinium sp*		AY258496
C39	*Symbiodinium sp*		AY258484
C49	*Symbiodinium sp*		AY589752
C67	*Symbiodinium sp*		AY686647
C69	*Symbiodinium sp*		AY589773
D4	*Symbiodinium sp*		DQ865214
E1	*Symbiodinium californium*		AF334659
F1	*Symbiodinium kawagutii*		AF333517
CG1	*Symbiodinium sp*		AJ291537
G1a	*Symbiodinium sp*	Foraminifero Soritidae	AM748597
G2	*Symbiodinium sp*	*Marginopora vertebralis*	AM748598
G2a	*Symbiodinium sp*	*Marginopora vertebralis*	AM748599
G3	*Symbiodinium sp*	Foraminifero Soritidae	AM748600
G4	*Symbiodinium sp*	Foraminifero Soritidae	AM748601
GCv1	*Symbiodinium sp*	*Cliona varians*	EF134621
GCv2	*Symbiodinium sp*	*Cliona varians*	EF134622
GCv3	*Symbiodinium sp*	*Cliona varians*	EF134623
H1	*Symbiodinium sp*		AJ291513

B. *Molecular morphometrics matrix based on ITS2 secondary structures' characters from zooxanthellae of the genus Symbiodinium.*

	A	I´	I"	a	i"	j´	j"	k´	k"	q´	B	II´	II"	b´	b"	c	l´	l"	p´	p"	ab´	ab"	ab´"	IIα´	IIα"	ac	IIβ	ad	C	IIIA	m	C´	IIIb	IIIb	d´	d"	e´	e"	t´	t"	f´	f"	o´	o"	r´	r"	s´	s"	g	v´	v"	v"´	IIIb	IIIb	IIIb	IIIb	w	x	y"	IIIb	IIIb	z"
AFr	0	0	4	4	0	0	0	0	0	0	6	2	3	1	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	2	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cry	1	0	3	4	0	0	0	0	0	0	7	2	3	1	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	2	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Med	2	1	1	4	0	0	0	0	0	0	7	4	1	1	1	4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	2	4	4	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
A3	3	1	1	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A1	4	1	1	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	4	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0
A4	4	2	2	4	0	0	0	0	0	0	7	0	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	4	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACa	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACa	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACt	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACt	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACt	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACc	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACa	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACa	4	2	2	4	0	0	0	0	0	0	7	2	1	2	2	5	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	3	1	1	3	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Azs	5	1	0	7	0	0	0	0	0	0	7	3	1	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	2	0	2	4	3	0	0	1	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
A2S	5	1	0	7	0	0	0	0	0	0	7	3	1	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	2	0	2	4	3	0	0	1	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	6	3	0	4	1	0	1	2	2	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	3	2	3	1	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
B11	6	4	1	8	1	0	1	0	0	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	4	4	6	1	4	1	0	1	3	0	1	1	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
B19	6	4	0	4	1	1	0	0	0	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	4	4	6	1	5	0	0	1	3	0	1	1	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
B21	6	4	0	4	1	1	0	0	0	0	7	1	2	1	2	8	0	0	1	1	0	0	0	0	0	0	0	0	4	4	6	1	4	0	0	1	3	0	1	1	0	0	2	1	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
B4S	6	4	0	4	1	1	0	0	0	0	7	1	2	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	4	4	6	1	6	3	0	1	3	0	1	1	0	0	2	1	2	1	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
BPk	6	4	0	4	1	1	0	0	0	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	4	4	6	1	7	1	0	1	5	6	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
BPa	6	5	1	4	1	0	1	0	0	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	4	4	6	1	7	2	0	1	3	0	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
BCa	6	5	1	4	1	0	1	0	0	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	4	4	6	1	7	1	0	1	3	0	0	0	0	0	3	2	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
BCa	6	4	0	4	1	0	1	0	0	0	7	5	2	1	1	4	0	3	0	0	0	0	0	0	0	0	0	0	4	4	6	1	4	0	0	1	3	0	0	0	0	0	3	2	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
C22	0	6	2	5	0	0	1	0	0	0	6	1	3	0	1	7	0	0	0	0	0	0	0	0	0	0	0	0	5	5	4	8	8	1	0	0	4	3	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0
C31	0	2	1	6	0	0	1	0	0	0	6	3	3	2	1	3	0	0	0	0	0	0	0	0	0	0	0	0	5	5	4	0	9	2	1	0	4	3	0	0	0	0	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0
C39	0	6	2	5	0	0	1	0	0	0	6	1	3	0	1	7	0	0	0	0	0	0	0	0	0	0	0	0	5	5	4	0	a	3	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0
C49	0	6	2	5	0	0	1	0	0	0	6	3	3	2	1	0	0	0	0	0	1	2	1	8	1	9	3	3	5	5	4	0	0	2	1	0	4	3	0	0	0	0	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0
C67	0	6	2	5	0	0	1	0	0	0	6	1	3	0	1	7	0	0	0	0	0	0	0	0	0	0	0	0	5	5	4	0	a	3	1	0	4	3	0	0	0	0	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0
C69	0	6	2	5	0	0	1	0	0	0	6	1	3	0	1	7	0	0	0	0	0	0	0	0	0	0	0	0	5	5	4	2	8	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0
D4	7	2	1	8	0	0	0	0	0	0	7	2	1	1	1	6	1	1	0	0	0	0	0	0	0	0	0	0	6	0	0	0	b	2	0	1	3	3	1	0	0	0	2	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
E1	5	7	1	5	0	0	0	0	0	0	8	8	1	0	0	8	0	7	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	1	0	0	2	2	1	0	1	0	2	1	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0
F1	4	7	4	7	0	0	1	0	0	0	7	2	1	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	7	5	3	0	9	4	1	1	4	3	0	0	0	0	0	0	0	0	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	0
CG	1	6	0	3	0	0	5	1	0	0	8	2	3	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	c	4	0	2	0	0	0	1	0	0	0	1	0	0	0	0	7	0	6	0	6	0	4	0	7	3	1	0	0	0
GSo	2	6	0	3	0	0	5	0	0	1	8	3	2	0	0	4	1	1	0	0	0	0	0	0	0	0	0	0	8	0	0	0	c	2	0	2	0	0	0	1	0	0	0	1	0	0	0	0	7	3	4	0	4	2	4	0	3	6	0	0	0	0
GM	1	6	0	3	0	0	5	1	0	1	8	2	3	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	c	1	0	2	0	0	0	1	0	0	0	1	0	0	0	0	7	3	3	0	6	0	5	2	7	3	1	0	0	0
GM	1	6	0	3	0	0	5	1	0	1	8	2	3	0	1	4	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	c	1	0	2	0	0	0	1	0	0	0	1	0	0	0	0	7	3	3	0	6	0	5	2	7	3	1	0	0	0
GSo	6	6	0	3	0	1	5	1	0	0	8	2	4	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	9	3	0	2	0	0	2	2	0	0	0	1	0	0	0	0	7	0	7	1	3	2	2	0	6	7	0	0	0	0
GSo	2	6	0	5	0	4	0	0	0	0	8	2	3	1	1	6	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	c	3	0	2	0	0	1	1	0	0	0	2	0	0	0	0	7	8	1	0	0	0	0	0	0	0	0	10	2	2
GC	5	6	0	3	0	1	5	1	0	0	8	0	5	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	9	4	0	2	0	0	2	2	0	0	0	1	0	0	0	0	7	0	8	0	2	2	2	0	6	7	0	0	0	0
GC v2	5	6	0	3	0	1	5	1	0	0	8	0	5	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	9	4	0	2	0	0	2	2	0	0	0	1	0	0	0	0	7	0	8	0	2	2	2	0	6	7	0	0	0	0 0 1

C. *CBCs (compensatory base changes) matrix between ITS2 sequences from Symbiodinium spp. calculated by the program 4SALE.*

	AFree	CrypA	ACa3	ACa4	Acc	ACt3	ACt2	Aca1	Aca2	ACt1	A4	A1	A3	A2Sp	AZs	MedA	**E1**	BPa	BCa1	B11	BCa2	B19	B21	BPk	B4Sm	B2	C39	C67	C22	C69	C31	C49	H1	F1	G2	G2a	CG1	G1a	GCv1	GCv2	GCv3	G3	G4	**D4**
AFree		0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	**6**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	3	1	**4**
CrypA	0	.	0	0	0	0	0	0	0	0	0	0	0	2	2	2	**6**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	3	1	**4**
ACa3	0	0	.	0	0	0	0	0	0	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
ACa4	0	0	0	.	0	0	0	0	0	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
Acc	0	0	0	0	.	0	0	0	0	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
ACt3	0	0	0	0	0	.	0	0	0	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
ACt2	0	0	0	0	0	0	.	0	0	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
Aca1	0	0	0	0	0	0	0	.	0	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
Aca2	0	0	0	0	0	0	0	0	.	0	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
ACt1	0	0	0	0	0	0	0	0	0	.	0	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
A4	0	0	0	0	0	0	0	0	0	0	.	0	0	0	0	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
A1	0	0	0	0	0	0	0	0	0	0	0	.	0	1	1	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
A3	0	0	0	0	0	0	0	0	0	0	0	0	.	1	1	3	**5**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	2	1	**4**
A2Sp	2	2	0	0	0	0	0	0	0	0	0	1	1	.	0	2	**6**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	5	5	5	4	3	3	3	5	3	**5**
AZs	2	2	0	0	0	0	0	0	0	0	0	1	1	0	.	2	**6**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	5	5	5	4	3	3	3	5	3	**5**
MedA	2	2	3	3	3	3	3	3	3	3	3	3	3	2	2	.	**8**	3	3	3	3	3	4	3	4	2	3	3	3	3	3	3	2	3	3	3	3	2	1	1	1	3	2	**4**
E1	6	6	5	5	5	5	5	5	5	5	5	5	5	6	6	8	**.**	7	7	7	7	7	8	7	7	7	6	6	6	6	5	6	3	6	4	4	4	4	3	3	3	3	2	**3**
BPa	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**7**	.	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
BCa1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**7**	0	.	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
B11	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**7**	0	0	.	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
BCa2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**7**	0	0	0	.	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
B19	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**7**	0	0	0	0	.	0	0	0	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
B21	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	**8**	0	0	0	0	0	.	0	0	0	2	2	2	2	2	2	2	2	3	3	3	2	1	1	1	2	2	**3**
BPk	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**7**	0	0	0	0	0	0	.	0	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
B4Sm	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	**7**	0	0	0	0	0	0	0	.	0	1	1	1	1	1	1	1	1	2	2	2	1	0	0	0	1	1	**2**
B2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	**7**	0	0	0	0	0	0	0	0	.	0	0	0	0	0	0	0	0	2	2	2	1	0	0	0	1	1	**0**
C39	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**6**	1	1	1	1	1	2	1	1	0	.	0	0	0	0	0	0	2	4	4	4	3	1	1	1	2	2	**5**
C67	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**6**	1	1	1	1	1	2	1	1	0	0	.	0	0	0	0	1	2	4	4	4	3	1	1	1	2	2	**5**
C22	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**6**	1	1	1	1	1	2	1	1	0	0	0	.	0	0	0	1	2	4	4	4	3	1	1	1	2	2	**5**
C69	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**6**	1	1	1	1	1	2	1	1	0	0	0	0	.	0	0	0	1	4	4	4	3	1	1	1	2	2	**3**
C31	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**5**	1	1	1	1	1	2	1	1	0	0	0	0	0	.	0	1	2	4	4	4	3	1	1	1	2	1	**5**
C49	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**6**	1	1	1	1	1	2	1	1	0	0	0	0	0	0	.	1	2	4	4	4	3	1	1	1	2	2	**5**
H1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	**3**	1	1	1	1	1	2	1	1	0	0	1	1	0	1	1	.	2	4	4	4	3	1	1	1	2	1	**5**
F1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	**6**	1	1	1	1	1	2	1	1	0	2	2	2	1	2	2	2	.	4	4	4	3	1	1	1	2	1	**4**
G2	3	3	3	3	3	3	3	3	3	3	3	3	3	5	5	3	**4**	2	2	2	2	2	3	2	2	2	4	4	4	4	4	4	4	4	.	0	0	1	0	0	0	1	0	**6**
G2a	3	3	3	3	3	3	3	3	3	3	3	3	3	5	5	3	**4**	2	2	2	2	2	3	2	2	2	4	4	4	4	4	4	4	4	0	.	0	1	0	0	0	1	0	**6**
CG1	3	3	3	3	3	3	3	3	3	3	3	3	3	5	5	3	**4**	2	2	2	2	2	3	2	2	2	4	4	4	4	4	4	4	4	0	0	.	1	0	0	0	1	0	**6**
G1a	2	2	2	2	2	2	2	2	2	2	2	2	2	4	4	2	**4**	1	1	1	1	1	2	1	1	1	3	3	3	3	3	3	3	3	1	1	1	.	0	0	0	1	0	**6**
GCv1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	1	**3**	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	.	0	0	0	0	**5**
GCv2	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	1	**3**	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	.	0	0	0	**5**
GCv3	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	1	**3**	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	.	0	0	**5**
G3	3	3	2	2	2	2	2	2	2	2	2	2	2	5	5	3	**3**	1	1	1	1	1	2	1	1	1	2	2	2	2	2	2	2	2	1	1	1	1	0	0	0	.	0	**5**
G4	1	1	1	1	1	1	1	1	1	1	1	1	1	3	3	2	**2**	1	1	1	1	1	2	1	1	1	2	2	2	2	1	2	1	1	0	0	0	0	0	0	0	0	.	**5**
D4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5	4	**3**	2	2	2	2	2	3	2	2	0	5	5	5	3	5	5	5	4	6	6	6	6	5	5	5	5	5	**.**