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Multi-gene analysis of *Symbiodinium* dinoflagellates: a perspective on rarity, symbiosis, and evolution

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ABSTRACT

Symbiodinium, a large group of dinoflagellates, live in symbiosis with marine protists, invertebrate metazoans, and free-living in the environment. *Symbiodinium* are functionally variable and play critical energetic roles in symbiosis. Our knowledge of *Symbiodinium* has been historically constrained by the limited number of molecular markers available to study evolution in the genus. Here we compare six functional genes, representing three cellular compartments, in the nine known *Symbiodinium* lineages. Despite striking similarities among the single gene phylogenies from distinct organelles, none were evolutionarily identical. A fully concatenated reconstruction, however, yielded a well-resolved topology identical to the current benchmark *nr28S* gene. Evolutionary rates differed among cellular compartments and clades, a pattern largely driven by higher rates of evolution in the chloroplast genes of *Symbiodinium* clades D2 and I. The rapid rates of evolution observed amongst these relatively uncommon *Symbiodinium* lineages in the functionally critical chloroplast may translate into potential innovation for the symbiosis. The multi-gene analysis highlights the potential power of assessing genome-wide evolutionary patterns using recent advances in sequencing technology and emphasizes the importance of integrating ecological data with more comprehensive sampling of free-living and symbiotic *Symbiodinium* in assessing the evolutionary adaptation of this enigmatic dinoflagellate.

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INTRODUCTION

Dinoflagellates in the genus *Symbiodinium* are essential components of coral reef ecosystems in their role as photosynthetic endosymbionts of a myriad of marine organisms belonging to at least five distinct phyla: Foraminifera, Porifera, Cnidaria, Mollusca, and Platyhelminthes (*Trench, 1993*). Although highly predominant within benthic hosts, symbiotic associations have also been reported in the pelagic medusa *Cotylorhiza tuberculata* (*Astorga, Ruiz & Prieto, 2012*). Perhaps best known for their relationship with scleractinian corals, *Symbiodinium* spp. underpin the productivity and calcification that creates coral skeletons and the structures known as coral reefs that serve as habitat for the immense biodiversity these coastal ecosystems support.

Research conducted during the last two decades has allowed extensive genotyping of endosymbiotic *Symbiodinium* in both the Western Atlantic and Indo-Pacific Oceans and across benthic host taxa at a variety of spatial and temporal scales (reviewed in [Coffroth & Santos, 2005](#); [Franklin et al., 2012](#); [Stat, Carter & Hoegh-Guldberg, 2006](#)). Several recent studies have also begun to describe *Symbiodinium* diversity in free-living environments, including the water column ([Manning & Gates, 2008](#); [Pochon et al., 2010](#); [Takabayashi et al., 2012](#)), sediments ([Pochon et al., 2010](#); [Porto et al., 2008](#); [Takabayashi et al., 2012](#)), coral sand ([Hirose et al., 2008](#)), coral rubble ([Coffroth et al., 2006](#)), on the surface of macroalgal beds ([Porto et al., 2008](#); [Venera-Ponton et al., 2010](#)), and in fish feces ([Castro-Sanguino & Sánchez, 2012](#); [Porto et al., 2008](#)).

Historically, the pioneering work of [Rowan & Powers \(1992\)](#) divided the genus *Symbiodinium* into three phylogenetic groups referred to as clades A-C using nuclear small subunit ribosomal (*nr18S*) sequences. Despite the conserved nature of this marker, sequence variation between clades is comparable to other dinoflagellate taxa placed in different orders ([Rowan & Powers, 1992](#)). Later, the use of more variable nuclear large subunit ribosomal (*nr28S*) sequences was applied across broader host taxa and geographic scales ([Santos et al., 2002](#); [Pawlowski et al., 2001](#); reviewed in [Baker, 2003](#)), and ultimately led to the molecular classification of *Symbiodinium* into nine lineages ([Pochon & Gates, 2010](#)), clades A through I ([Table 1](#)). Clades D, F, and G have been further divided into sub-clades D1-D2, F2-F5, and G1-G2, respectively, using *nr28S* and the chloroplast large subunit ribosomal DNA (*cp23S*) domain V ([Hill et al., 2011](#); [Pochon, LaJeunesse & Pawlowski, 2004](#); [Pochon et al., 2006](#)). Comparative phylogenetic reconstructions have yielded similar evolutionary relationships among *Symbiodinium* clades using *nr28S* and *cp23S* genes ([Santos et al., 2002](#); [Pochon & Gates, 2010](#)), as well as when using the coding region of the plastid-encoded photosystem II protein D1 (*psbA*; [Takishita et al., 2003](#)), the mitochondrial cytochrome oxidase I (*coI*; [Takabayashi, Santos & Cook, 2004](#)), and mitochondrial cytochrome b (*cob*; [Zhang, Bhattacharya & Lin, 2005](#); [Sampayo, Dove & LaJeunesse, 2009](#)). However, compared to other markers the *nr28S* gene typically yields better-resolved phylogenies and is therefore considered as a 'benchmark gene' for clade-level analysis of *Symbiodinium* ([Pochon et al., 2012](#)).

The nine existing clades and eight sub-clades of *Symbiodinium* have been largely delineated based on host-symbiont associations ([Table 1](#)). For example, clades A, B, C, and D1 most commonly associate with Molluscan and Cnidarian hosts, clades B, D2, and G2 with Poriferan hosts, and clades F, G1, H, and I with Foraminifera. To date, the majority of *Symbiodinium* clades have also been found in the free-living environment ([Table 1](#)), particularly clades A and B which appear to contain a high number of unique strains that may be exclusively adapted to a free-living mode of life ([Coffroth et al., 2006](#); [Hirose et al., 2008](#); [Takabayashi et al., 2012](#); [Yamashita & Koike, 2013](#)). However, representatives from all clades are likely to be soon characterized from the free-living environment as novel sequencing technologies now provide researchers with unprecedented screening sensitivity and ability to quickly design novel *Symbiodinium*-specific markers with increased resolution. To date, a number of high-resolution markers have been employed for fine-scale studies

Table 1 Summary of existing *Symbiodinium* lineages. The nine clades (A–I) and eight sub-clades (D1–D2, F2–F5, and G1–G2) that constitute the genus *Symbiodinium*, with selected literature highlighting the habitat prevalence/preference of each lineage.

Clade/ Sub-clade	<i>in hospite</i> /free- living	Habitat Preferences/Prevalence	References
A	<i>in hospite</i>	Cnidaria	(LaJeunesse, 2001; Reimer et al., 2006; Stat, Morris & Gates, 2008)
	<i>in hospite</i>	Mollusca	(Baillie, Belda-Baillie & Maruyama, 2000; Ishikura et al., 2004; LaJeunesse et al., 2010)
	<i>in hospite</i>	Plathelminthes	(Baillie, Belda-Baillie & Maruyama, 2000)
	free-living	Water column	(Manning & Gates, 2008; Pochon & Gates, 2010; Takabayashi et al., 2012)
	free-living	Sediment	(Pochon & Gates, 2010; Porto et al., 2008; Takabayashi et al., 2012)
	free-living	Reef sand/rubbles	(Coffroth et al., 2006; Hirose et al., 2008)
	free-living	Macroalgal beds	(Porto et al., 2008)
	free-living	Fish feces	(Castro-Sanguino & Sánchez, 2012; Porto et al., 2008)
B	<i>in hospite</i>	Cnidaria	(Coffroth, Santos & Goulet, 2001; LaJeunesse, 2001; Santos, Taylor & Coffroth, 2001)
	<i>in hospite</i>	Mollusca	(LaJeunesse, 2002)
	<i>in hospite</i>	Porifera	(Hunter, LaJeunesse & Santos, 2007)
	free-living	Water column	(Manning & Gates, 2008; Pochon & Gates, 2010; Takabayashi et al., 2012)
	free-living	Sediment	(Pochon & Gates, 2010; Porto et al., 2008; Takabayashi et al., 2012)
	free-living	Reef rubbles	(Coffroth et al., 2006)
	free-living	Macroalgal beds	(Porto et al., 2008)
	free-living	Fish feces	(Castro-Sanguino & Sánchez, 2012; Porto et al., 2008)
C	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon, LaJeunesse & Pawlowski, 2004)
	<i>in hospite</i>	Cnidaria	(Coffroth & Santos, 2005; LaJeunesse, 2005; Sampayo et al., 2007; Wagner et al., 2011)
	<i>in hospite</i>	Mollusca	(Baillie, Belda-Baillie & Maruyama, 2000; Ishikura et al., 2004; LaJeunesse et al., 2010)
	<i>in hospite</i>	Plathelminthes	(Baillie, Belda-Baillie & Maruyama, 2000)
	free-living	Water column	(Manning & Gates, 2008; Pochon & Gates, 2010; Takabayashi et al., 2012)
	free-living	Sediment	(Pochon & Gates, 2010; Porto et al., 2008; Takabayashi et al., 2012)
	free-living	Macroalgal beds	(Porto et al., 2008; Venera-Ponton et al., 2010)
	D1	<i>in hospite</i>	Cnidaria
<i>in hospite</i>		Mollusca	(Ishikura et al., 2004; LaJeunesse et al., 2010)
free-living		Water column	(Manning & Gates, 2008; Takabayashi et al., 2012)
D2	<i>in hospite</i>	Foraminifera	(Pochon et al., 2007; Garcia-Cuetos, Pochon & Pawlowski, 2005)
	<i>in hospite</i>	Porifera	(Carlos et al., 1999)
E	<i>in hospite</i>	Cnidaria	(LaJeunesse & Trench, 2000; LaJeunesse, 2001)
	free-living	Water column	(Carlos et al., 1999; Gou et al., 2003; Santos, 2004)
F2	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon & Gates, 2010)
	<i>in hospite</i>	Cnidaria	(Rodriguez-Lanetty, Cha & Song, 2002)
F3	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon & Gates, 2010)
F4	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon & Gates, 2010)
F5	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon & Gates, 2010)
G1	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon & Gates, 2010)
G2	<i>in hospite</i>	Cnidaria	(Bo et al., 2011; van Oppen et al., 2005)
	<i>in hospite</i>	Porifera	(Schoenberg & Loh, 2005; Schoenberg et al., 2008; Hill et al., 2011)
	free-living	Water column	(Takabayashi et al., 2012)
	free-living	Sediment	(Takabayashi et al., 2012)
	free-living	Fish feces	(Castro-Sanguino & Sánchez, 2012)
H	<i>in hospite</i>	Foraminifera	(Pochon et al., 2001; Pochon et al., 2006; Pochon et al., 2007; Pochon & Gates, 2010)
	free-living	Water column	(Manning & Gates, 2008)
I	<i>in hospite</i>	Foraminifera	(Pochon & Gates, 2010)

investigating the biogeography, host specificity, physiology, and ecological partitioning of specific strains within *Symbiodinium* clades, including microsatellite loci ([Thornhill et al., 2009](#)), the Internal Transcribed Spacer regions 1 and 2 of the nuclear ribosomal DNA ([van Oppen et al., 2005](#); [Iglesias-Prieto et al., 2004](#)), and recently the non-coding region of *psbA* ([LaJeunesse & Thornhill, 2011](#); [Thornhill et al., 2014](#)). However, none of these markers have yet been successfully employed for characterizing free-living populations of *Symbiodinium* due to clear challenges of specifically targeting *Symbiodinium* against the backdrop of the complex micro- and meio-eukaryotic diversity found in environmental samples. In an attempt to characterize novel markers for symbiotic and free-living *Symbiodinium*, [Pochon et al. \(2012\)](#) used available Expressed Sequence Tags (EST) libraries for *Symbiodinium* ([Leggat et al., 2007](#); [Voolstra et al., 2008](#)), to identify 84 candidate genes, and perform in-depth phylogenetic analyses of four relatively fast evolving genes (*coI*, *calmodulin*, *rad24*, and *actin*). Other more conserved genes, including the elongation factor 2 (*elf2*) and the *cob* genes, were also recovered from EST libraries and sequenced for future clade-level analysis.

Our current understanding on the divergence pattern and evolution of *Symbiodinium* clades is relatively limited. A standard molecular clock using *nr28S* sequence data, suggested that the ancestor of the *Symbiodinium* species complex evolved during the K-T boundary (65 MYA) in warm tropical waters ([Tchernov et al., 2004](#)), which corresponds to a major transition time from the extinct Mesozoic rudist-based reefs, to the modern scleractinian-dominated reefs. [Pochon & Pawlowski \(2006\)](#) later employed a relaxed molecular clock approach with *nr28S* data and suggested that *Symbiodinium* clades started to diversify from ancestral clade A some 50 MYA, in the beginning of Eocene. Their analysis revealed that the major diversifications of clades occurred during global cooling periods: the origination of *Symbiodinium* clades A, B, D, E, and G during the Eocene cooling, followed by a massive radiation that took place in all lineages since mid-Miocene (15 MYA).

To improve our understanding of *Symbiodinium* clade evolution, in this study, we present a 'clade-level' multi-gene analysis incorporating samples from all known *Symbiodinium* clades and sub-clades ([Table 2](#)). We selected two genes from three distinct organelles (nucleus: *nr28S* & *elf2*; chloroplast: *cp23S* & *psbA*; and mitochondria: *coI* & *cob*) to test the following hypotheses: (1) single gene phylogenies will yield statistically distinct clades relationships; (2) A six-gene concatenated tree will be statistically different from benchmark *nr28S*; and (3) Pair-wise relative substitution rate analyses will reveal compartment-specific differences in evolutionary rates among *Symbiodinium* clades and organelles. Our results are integrated within the current state of knowledge of free-living and endosymbiotic *Symbiodinium* lineages ([Table 1](#)) and may serve as a basis for future studies investigating evolutionary implications of rarity and symbiotic/free-living lifestyles among *Symbiodinium* dinoflagellates.

MATERIALS AND METHODS

DNA samples

Thirty-four DNA samples encompassing all known *Symbiodinium* clades (A-I) and sub-clades (F2-F5; D1-D2; G1-G2) were selected for phylogenetic analyses ([Table 2](#)).

Table 2. Description of *Symbiodinium* samples, host origin, and GenBank accession numbers of all DNAs used in this study.

Sample#	Clade ^a	ITS2 ^b	Host origin	Isolate ID ^c	nr28S	elf2	cp23S	psbA	col	cob
1	C	C1	<i>Amphisorus hemprichii</i>	2359X [S]	JN558040	JN557869	JN557969	JN557844	JN557891	JN557943
2		C90	<i>Sorites</i> sp.	1355X [S]	JN558045	JN557871	JN557975	JN557846	JN557893	JN557945
3		C91	<i>Sorites</i> sp.	2467X [S]	JN558048	JN557872	JN557978	JN557847	JN557894	JN557946
4		C15	<i>Amphisorus hemprichii</i>	2361X [S]	JN558042	JN557870	JN557972	JN557845	JN557892	JN557944
5	H	H1	<i>Sorites</i> sp.	2382X [S]	JN558051	JN557873	JN557981	JN557848	JN557895	JN557947
6		H1a	<i>Sorites</i> sp.	2350X [S]	JN558053	JN557874	JN557984	JN557849	JN557896	JN557948
7	F2	F2	<i>Sorites</i> sp.	206I [S]	JQ247043	JQ277946	JQ247052	JQ277935	JQ277957	JQ277979
8		F2a	<i>Sorites</i> sp.	215I [S]	JQ247044	JQ277947	JQ247053	JQ277936	JQ277958	JQ277980
9	F3	F3.2	<i>Amphisorus hemprichii</i>	2551X [S]	JQ247046	JQ277949	JQ247055	JQ277938	JQ277960	JQ277982
10		F3.1a	<i>Amphisorus hemprichii</i>	455X [S]	JQ247045	JQ277948	JQ247054	JQ277937	JQ277959	JQ277981
11	F4	F4.1	<i>Sorites</i> sp.	5121X [S]	JQ247047	JQ277950	JQ247056	JQ277939	JQ277961	JQ277983
12		F4.8	<i>Sorites</i> sp.	2692X [S]	JQ247048	JQ277951	JQ247057	JQ277940	JQ277962	JQ277984
13	F5	F5.1	<i>Meandrina meandrites</i>	RT-133 [C]	JN558063	JN557876	JN557996	JN557851	JN557898	JN557950
14		F5.1d	<i>Simularia</i> sp.	Sin [C]	JN558069	JN557877	JN558000	JN557852	JN557899	JN557951
15		F1	<i>Montipora verrucosa</i>	Mv [C]	JN558066	JN557875	JN557997	JN557850	JN557897	JN557949
16		F5.2g	<i>Montastraea faveolata</i>	Mf [C]	JN558072	JN557878	JN558004	JN557853	JN557900	JN557952
17	B	B1	<i>Plexaura kuma</i>	704 [C]	JN558057	JN557879	JN557991	JN557854	JN557901	JN557953
18		B2	<i>Eumicea flexuosa</i>	Pflex [C]	JN558060	JN557880	JN557993	JN557855	JN557902	JN557954
19		B19a	<i>Plexaura kuma</i>	703 [C]	JN558055	JN557881	JN557987	JN557856	JN557903	JN557955
20	I	I1	<i>Sorites</i> sp.	OHU7 [S]	FN561559	JQ277955	FN561563	JQ277944	JQ277966	JQ277988
21		I2	<i>Sorites</i> sp.	OHU3 [S]	FN561560	JQ277956	FN561564	JQ277945	JQ277967	JQ277989
22	D1	D1	<i>Acropora</i> sp.	A001 [C]	JN558075	JN557882	JN558007	JN557857	JN557904	JN557956
23		D1a	unknown anenome	Ap02 [C]	JN558078	JN557883	JN558010	JN557858	JN557905	JN557957
24	D2	D1.1	<i>Marginopora vertebralis</i>	2485X [S]	JQ247049	JQ277952	JQ247058	JQ277941	JQ277963	JQ277985
25		D1.2	<i>Haliciona koremella</i>	HK [C]	JN558081	JN557884	JN558013	JN557859	JN557906	JN557958
26	G1	G2	<i>Marginopora vertebralis</i>	2479X [S]	JN558089	JN557885	JN558019	JN557860	JN557907	JN557959
27		G2b	<i>Marginopora vertebralis</i>	3590X [S]	JN558088	N/A	JN558017	JN557861	JN557908	JN557960
28	G2	G2.1*	<i>Cliona orientalis</i>	OR2 [S]	JQ247050	JQ277953	JQ247059	JQ277942	JQ277964	JQ277986
29		G2.2*	<i>Cliona orientalis</i>	RN3 [S]	JQ247051	JQ277954	JQ247060	JQ277943	JQ277965	JQ277987
30	E	E1	<i>Anthopleura elegantissima</i>	RT-383 [C]	JN558084	N/A	JN558015	JN557862	JN557909	JN557961

(continued on next page)

Table 2 (continued)

Sample#	Clade ^a	ITS2 ^b	Host origin	Isolate ID ^c	nr28S	elf2	cp23S	psbA	col	cob
31	A	A2_1	<i>Bartholomea annulata</i>	RT-23 [C]	JN558097	JN557887	JN558029	JN557864	JN557911	JN557963
32		A2_2	<i>Gorgonia ventalina</i>	RT-89 [C]	JN558100	JN557888	JN558032	JN557865	JN557912	JN557964
33		A3	<i>Pseudoplexaura porosa</i>	725 [C]	JN558091	JN557889	JN558021	JN557866	JN557913	JN557965
34		A13	<i>Plexaura kuna</i>	708 [C]	JN558094	JN557886	JN558027	JN557863	JN557910	JN557962
Outgroup1	<i>G. simplex</i>	N/A	N/A	CCMP419 [C]	JN558103	JN557890	JN558033	JN557867	JN557914	JN557966
Outgroup2	<i>P. betii</i>	N/A	N/A	PB-1 [C]	JN558106	N/A	N/A	N/A	JN557915	JN557967
Outgroup3	<i>P. glacialis</i>	N/A	N/A	CCMPI383 [C]	JN558108	N/A	JN558036	JN557868	JN557916	JN557968

Notes.

^a Letters A to H refer to the *Symbiodinium* clades, and lineages D1-D2, F2-F5, and G1-G2 are the *Symbiodinium* sub-clades.

^b Alpha-numeric names correspond to *Symbiodinium* ITS2 rDNA molecular taxonomy sensu *Pochon et al. (2007)*. Letters correspond to the *Symbiodinium* clades, and numbers correspond to a specific ITS2 sequence. All samples are genetically distinct, except for *Symbiodinium* A2, which was found in two distinct cultures and referred here to as A2_1 and A2_2. Types D1.1 and D1.2 corresponds to the symbionts of the foraminifer *M. vertebralis* and the sponge *Haliclona koremella*, respectively (see *Pochon et al., 2007* for details), and were previously described as belonging to *Symbiodinium* sub-clade D1 (*Garcia-Cuetos; Pochon & Pawlowski, 2005; Pochon & Pawlowski, 2006*), but reclassified here as sub-clade D2. Sub-clade D1 contains *Symbiodinium* strains that are commonly associated with Scleractinian corals, such as symbiont ITS2 types D1 and D1a (*Stat & Gates, 2011*). Types G2 and G2b belong to sub-clade G1 as shown in *Pochon et al. (2012)*.

^c Samples ID are followed by [C] if DNA was extracted from a culture, or [S] if extracted from a symbiotic host. All GenBank accession numbers starting with the letters 'JQ' were obtained in the present study.

* Indicates new ITS2 sequences; novel types G2.1 and G2.2 belong to sub-clade G2 following *Hill et al. (2011)*.

These samples included fifteen axenic *Symbiodinium* cultures belonging to five clades/sub-clades (A, B, D, E, and F5), seventeen samples originally isolated from symbiotic soritid foraminifera (Pochon *et al.*, 2007; Pochon & Gates, 2010) belonging to six *Symbiodinium* clades/sub-clades (C, D2, F2-F4, G1, H, and I), and two samples extracted from the symbiotic bioeroding sponge genus *Cliona* and belonging to *Symbiodinium* sub-clade G2 (see Bo *et al.*, 2011; Hill *et al.*, 2011). Additionally, three cultured dinoflagellates, *Gymnodinium simplex* [CCMP 419], *Pelagodinium beii* (Siano *et al.*, 2010), and *Polarella glacialis* [CCMP 1383] were used as outgroups in our analyses following Pochon *et al.* (2012).

Genes selection, DNA extraction and sequencing

Six genes from three organelles were chosen for phylogenetic analyses. These include two nuclear genes (1) *nr28S* (D1-D3 region) [920 base-pairs] and (2) *elf2* [473 bp]; two chloroplast genes (3) *cp23S* (Domain V) [647 bp] and (4) the coding region of *psbA* [700 bp]; and two mitochondrial genes (5) *coI* [1057 bp] and (6) *cob* [906 bp]. Sequences for analysis were gathered from 26 samples obtained from a previous study (Pochon *et al.*, 2012), nine DNA samples were extracted and partially analyzed in other studies (Pochon *et al.*, 2007; Pochon & Gates, 2010) and further sequenced here to cover all genes using the primers and PCR cycling conditions described in Pochon *et al.* (2012), and two DNA samples were extracted from sponge tissues of the genus *Cliona* (courtesy of C. Schoenberg) and sequenced for all genes following Pochon *et al.* (2012) (see Table 2). The *psbA* gene was not reported in Pochon *et al.* (2012) and was PCR amplified in this study using the forward primer *psbA*_1.0 (5'-CWGTAGATATTGATGGWATAAGAGA-3') located at the 5' end of the coding region and the reverse primer *psbA*_3.0 (5'-TTGAAAGCCATTGTCTACTCC-3') located approximately 700 bp downstream from the 5' end and using standard thermocycling conditions with an annealing temperature of 52 °C. All sequences were obtained by direct sequencing, except for *nr28S* and *cp23S* sequences, which were cloned prior to sequencing in Pochon *et al.* (2012), and a single sequence per sample included in the present study. In all cases, the variability between cloned sequences of any given sample was minimal (e.g., see Figure S1 of Pochon *et al.*, 2012), ranging between 0 and 4 bp difference (data not shown). However, sequences showing the shortest branch length in each sample were selected (data not shown). In cases where several sequences showed the same short branch length, one sequence was randomly chosen among them and included in the analysis.

Phylogenetic analyses

DNA sequences were inspected and assembled using Sequencher v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) and manually aligned with BioEdit v5.0.9 sequence alignment software (Hall, 1999). Thirteen distinct DNA alignments were generated: six alignments corresponding to individual gene alignments, one fully concatenated alignment of all six genes (ALL Concat), and six partially concatenated alignments including all possibilities of five genes each (i.e., each alignment excluded one of the six gene candidates). Concatenated alignments were created using the 'join sequence files' option in TREEFINDER v12.2.0 (Jobb, von Haeseler & Strimmer, 2004). *elf2* was included in

these analyses despite two missing samples (see samples #27 and #30; [Table 2](#)), which were coded as missing data in all concatenated alignments. GenBank accession numbers for all investigated sequences are shown in [Table 2](#).

Each DNA alignment was analyzed independently under both Maximum-likelihood (ML) and Bayesian environments. Best-fit models of evolution were estimated for each alignment (see [Table S1](#)) using Modeltest v3.7 ([Posada & Crandall, 1998](#)). ML analyses were carried out using PhyML v3.0 ([Guindon et al., 2009](#)), and the reliability of internal branches was assessed using 100 bootstraps with subtree pruning-regrafting branch swapping. Bayesian tree reconstructions with posterior probabilities were inferred using MrBayes v3.2 ([Ronquist et al., 2012](#)), using the same model of DNA evolution as for the ML analyses. Four simultaneous Markov chains were run for 1,000,000 generations with trees sampled every 10 generations, with 50,000 initial trees discarded as “burn-in”, based on visual inspections. Concatenated alignments were run under ML and Bayesian environments as described above, with the alignments partitioned so that the specific model of evolution corresponded to each gene fragment.

Topological tests, rate calculations, and statistical analyses

To compare the topology of the various trees, approximately unbiased (AU) topological congruency tests ([Shimodaira, 2002](#)) were performed using site likelihood calculation in RaxML v7.2.5 ([Stamatakis, 2006](#)), followed by AU tests using CONSEL ([Shimodaira & Hasegawa, 2001](#)) with default scaling and replicate values. *elf2* was excluded from the single gene analyses due to missing data (samples #27 and #30; [Table 2](#)), but was included in the concatenated analyses (see above).

In order to determine evolutionary rates among *Symbiodinium* lineages for each of the six investigated genes, relative-rate tests (RRT) were performed using the program RRTREE v1.1 ([Robinson-Rechavi & Huchon, 2000](#)). Clades and sub-clades were compared in a pair-wise fashion with *G. simplex* as the outgroup. Relative rates of evolution (K-scores from RRTREE analysis above) were compared among clades and among cellular organelles using a two way ANOVA, followed by post hoc analysis with Tukey’s Honestly Significant Difference (THSD) test.

RESULTS

DNA alignments for the six investigated genes ranged between 473 (*elf2*) and 1,057 bp (*col*). Individual phylogenies were generated ([Fig. 1](#)), and each was compared to the topology obtained with the *nr28S* gene, which is the current molecular taxonomic benchmark for the clade-level classification of *Symbiodinium* ([Hill et al., 2011](#); [Pochon & Gates, 2010](#); [Pochon et al., 2012](#)). Overall, the cladal relationships were remarkably similar among the genes investigated, particularly the basal positions of clades A, D, E and G, and the derived positions of clades B, C, F, H, and I. *Symbiodinium* clades were relatively well resolved in the nuclear and chloroplastic genes, but not the mitochondrial genes, which placed clades C, F, and H in completely unresolved monophyletic groups (see [Figs. 1E](#) and [1F](#)). However, with the exception of *nr28S*, the relationships amongst clades were weakly supported for all markers, especially in the higher parts of the trees, and this was

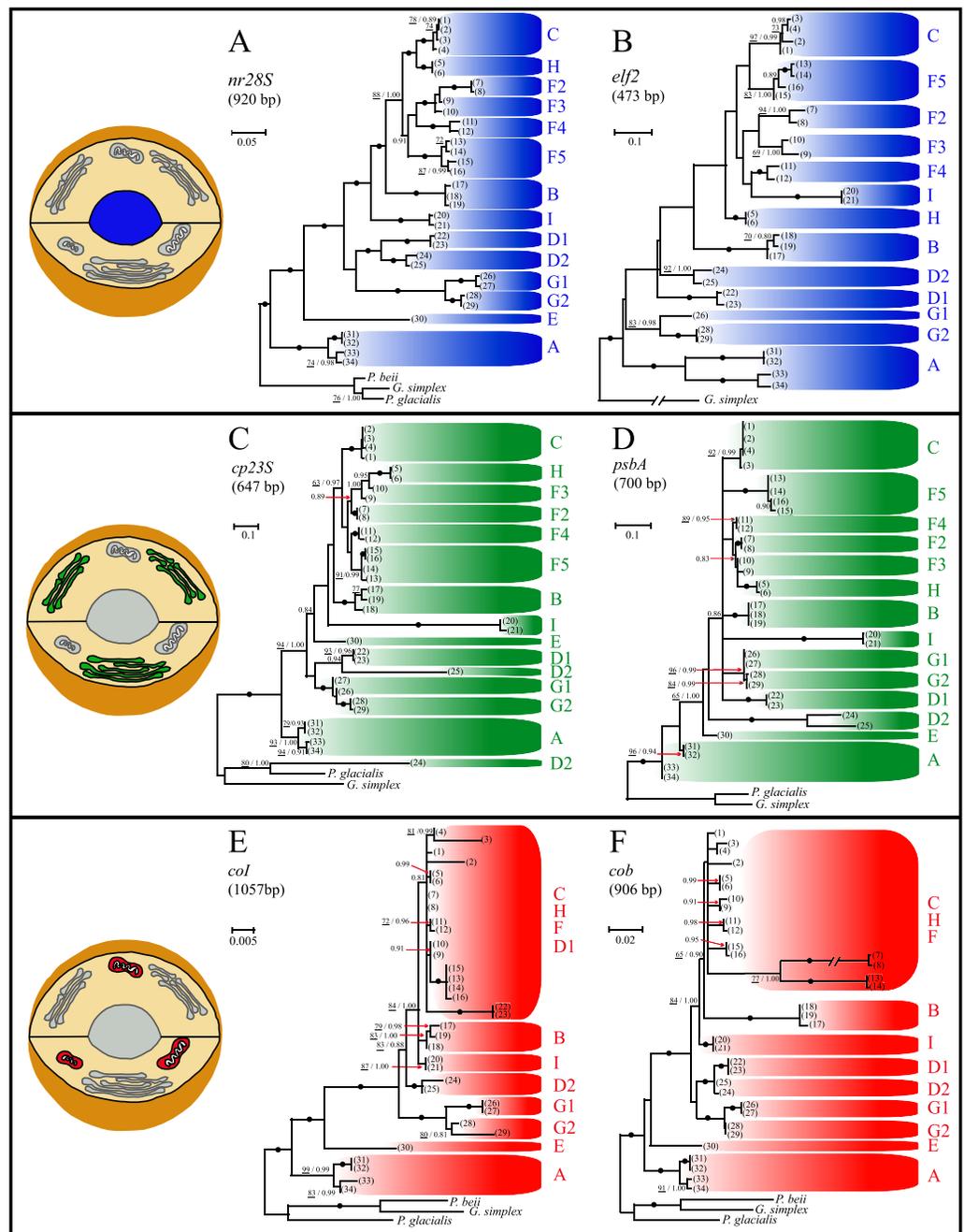


Figure 1 Single-gene phylogenies of *Symbiodinium* using two genes from three organelles. Best Maximum likelihood (ML) topologies for *Symbiodinium* clades and sub-clades A to I based on the nuclear genes (A) *nr28S* and (B) *elf2*, the chloroplastic genes (C) *cp23S* and (D) *psbA*, and the mitochondrial genes (E) *col* and (F) *cob*. Numbers in brackets refer to the *Symbiodinium* strains detailed in Table 2. Numbers at nodes represent the ML bootstrap pseudoreplicate (BP) values (underlined numbers; 100 BP performed) and Bayesian posterior probabilities (BiPP). Black dots represent nodes with <95% BP and BiPP of 1.0. Nodes without numbers correspond to BP and BiPP lower than 70% and 0.8, respectively. Nodes displaying BP lower than 50% were manually collapsed. The phylograms were rooted using the dinoflagellates *Gymnodinium simplex*, *Pelagodinium beii*, and/or *Polarella glacialis*. GenBank accession numbers are given in Table 2. Note: All clades are represented, except for clade E in the *elf2* phylogeny.

particularly evident for *psbA* where relationships between clades B, C, D, F, G, H, and I were completely unresolved (Fig. 1D). Furthermore, the relationships between sub-clades within clades D, F, and G showed contrasting results. Well-supported monophyly of all sub-clades was only observed in the *nr28S* gene (Fig. 1A). Notably however, clade G sub-clades (G1 and G2) formed a monophyletic group across all genes. In contrast, the monophyly of clade F and clade D sub-clades was only resolved with *nr28S* (Fig. 1A) and *nr28S* and *cob* (Figs. 1A and 1F), respectively. All *Symbiodinium* strains belonging to the same sub-clade grouped together across all genes, with two noteworthy exceptions. First, the four samples of sub-clade F5 (#14-16) separated into two groups in *cob* (Fig. 1F). Second, sample #24 (Table 2) of sub-clade D2 diverged significantly to the root of the tree in *cp23S* (Fig. 1C).

In order to increase the phylogenetic signal and assess which of the individual markers best reflects the most well resolved evolutionary history of *Symbiodinium*, a series of gene concatenation analyses were conducted. In total, seven distinct concatenated alignments were analyzed, including one fully concatenated alignment of all six genes (ALL Concat) consisting of a total length of 4,703 bp, and six partially concatenated alignments ranging in length from 3,646 bp (ALL except *coI*) and 4,230 bp (ALL except *elf2*), and including all possibilities of five genes each (see Methods). Phylogenetic analysis of the fully concatenated dataset (ALL Concat, Fig. 2) resulted in a highly resolved *Symbiodinium* tree with identical topology to *nr28S* gene, but with much stronger phylogenetic signal as evidenced by a significant increase in statistical support at all nodes (Fig. 2). Other concatenated alignments yielded weaker nodes support and unstable cladal relationships globally (data not shown).

Approximately unbiased (AU) topological congruency tests (Shimodaira, 2002) were used to verify whether any of the distinct phylogenies resulted in statistically identical topologies. First, pair-wise comparisons of single gene phylogenies (Fig. 1) resulted in significant *p*-values ($p < 0.05$) in all cases, indicating that the different genes have not followed identical evolutionary trajectories (see Table S2A). Second, concatenated topologies tested against single gene topologies, also resulted in significant *p*-values in all instances (data not shown). Third, pair-wise comparisons of single gene phylogenies to the concatenated topologies, revealed that the two longest genes, *coI* and *nr28S*, resulted in 5 and 6 significant topological comparisons, respectively (see Table S2B). Despite the relatively smaller size of *nr28S* (920 bp) compared to *coI* (1057 bp), *nr28S* was the only marker yielding a statistically identical topology to the fully concatenated topology (ALL Concat). The *nr28S* topology, however, was not identical to the best topology of the concatenated alignment excluding the *nr28S* gene fragment (see ALL except *nr28S* in Table S2B). Similarly, pair-wise comparisons of concatenated topologies revealed that significant *p*-values ($p < 0.05$) were only observed against the 'ALL except *nr28S*' topology (Table S2B).

The variable branch lengths observed in the six phylograms (Fig. 1) are directly proportional to the amount of character change; hence the longest branches are indicative of increased evolutionary rates of any given *Symbiodinium* strain. In most cases, increased rates of *Symbiodinium* clades/sub-clades appeared to be gene-specific rather than a

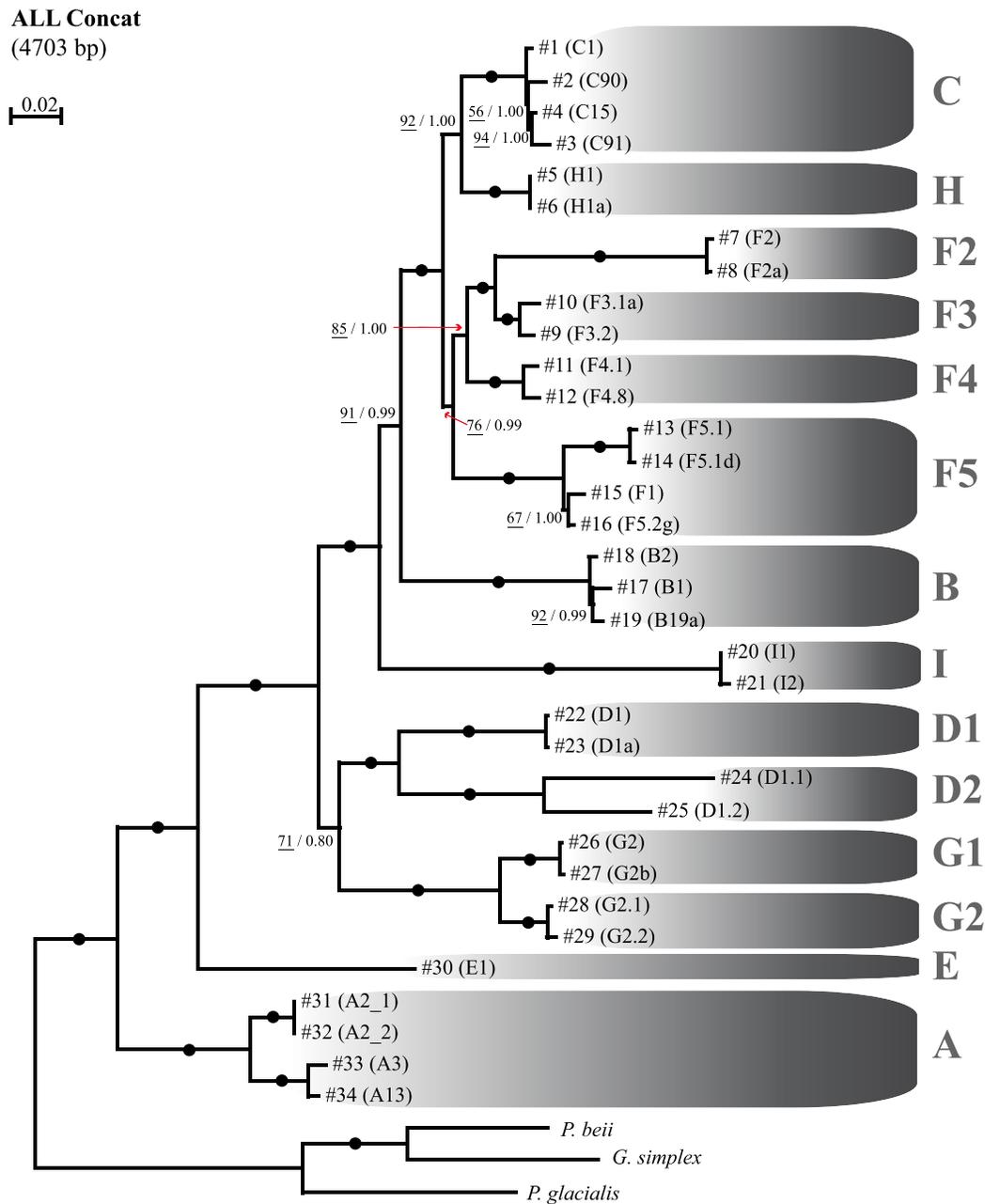


Figure 2 Best topology of *Symbiodinium* based on six concatenated genes. Maximum likelihood (ML) topology for *Symbiodinium* clades and sub-clades A to I based on fully concatenated DNA alignment (ALL Concat; 4,703 bp) of all six genes investigated in this study. The *Symbiodinium* strains within each clade/sub-clade are referred using the specific numbers and corresponding ITS2 names in brackets (Table 2, Fig. 1). Numbers at nodes represent the ML bootstrap pseudoreplicate (BP) values (underlined numbers; 100 BP performed) and Bayesian posterior probabilities (BiPP). Black dots represent nodes with 100% BP and BiPP of 1.0. The phylograms were rooted using the dinoflagellates *Gymnodinium simplex*, *Pelagodinium beii*, and *Polarella glacialis*.

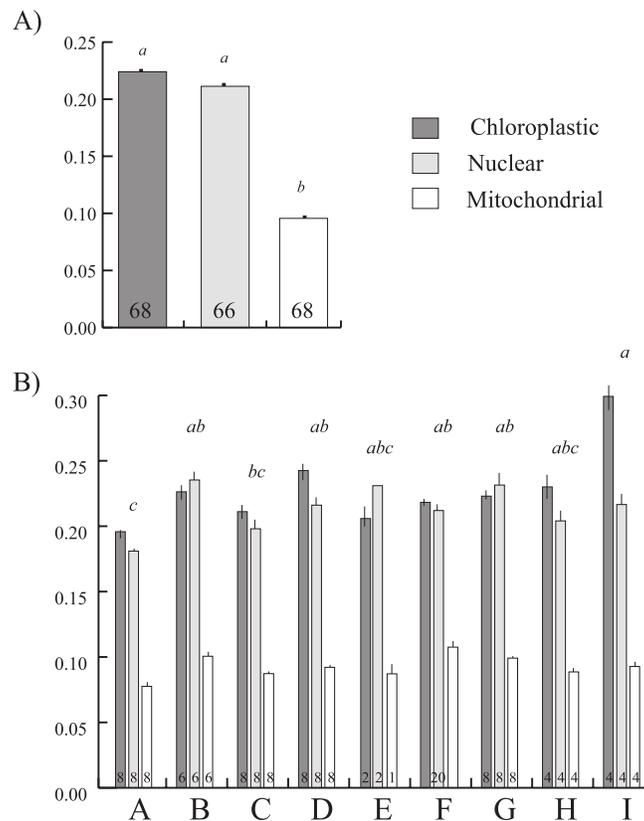


Figure 3 Comparison of relative rates of evolution among *Symbiodinium* organelles and clades. Plot of mean relative rates of evolution (mean \pm sem) across the (A) three organelles and (B) nine clades. Lower case, italicized letters above the bars represent post hoc THSD tests with significant differences between (A) the three organelles and (B) between clades (groups of three bars). Sample sizes are shown at the base of each bar, except clade E, where for each bar $n = 20$.

character state maintained across all markers. K-scores from relative rate tests were coupled with ANOVA to compare the relative rates of evolution among the clades and organelles (Fig. 3) examining all clades across the three markers. There was no significant interaction of clade and organelle ($F_{16,175} = 1.57$, $p = 0.081$), indicating that the pattern of changes in rates of evolution among clades were similar across organelles. Overall the general pattern of slower relative rates of evolution for some of the basal clades (A, E) and faster rates in more derived clades (C, F, H, and I) is held across organelles. However, organelles differed in their relative rates of evolution ($F_{2,175} = 248.9$, $p = 0.0001$), driven by rapid rates in the chloroplastic and nuclear compartments in comparison to the mitochondrial compartment (Fig. 3A), with the most rapid rates found in the chloroplastic markers due to the high evolutionary rates of clade I and sub-clade D2 (see Figs. 1C and 1D). Additionally, there was a significant difference between clades ($F_{8,175} = 3.87$, $p = 0.0003$) driven by the slow rates of clade A, and the rapid rates of clade I (Fig. 3B).

DISCUSSION

Multi-gene analysis supports nr28S as a benchmark lineage marker

Our knowledge of *Symbiodinium* evolution has historically been constrained by the limited number of phylogenetic markers that have been applied to this group. To date, less than 15 DNA loci have been used to examine *Symbiodinium* diversity in a phylogenetic context (LaJeunesse & Thornhill, 2011; Pochon et al., 2012; Rowan & Powers, 1992; Sampayo, Dove & LaJeunesse, 2009; Takabayashi, Santos & Cook, 2004; Takishita et al., 2003; van Oppen et al., 2001), and evolutionary relationships among all existing *Symbiodinium* lineages have never been inferred using more than two concatenated genes (Pochon & Gates, 2010). This study is the first to perform a multi-gene analysis using six markers representing three cellular organelles and integrating biological samples from all known clades and selected sub-clades that encompass the genus *Symbiodinium*. In spite of the overall similarity among the trees for each nuclear, chloroplastic and mitochondrial gene (Fig. 1), their topologies were statistically different (Table S2). This reflects within and among clade differences inherent to the individual markers. Most notably being the unstable positions of clades D, E, F5 and H, as well as weak support for among clade relationships observed in most markers investigated. Long-branch attraction artifacts (Felsenstein, 1985) most likely accounted for the placement of sub-clade D2 (sample #24) at the root of the tree in the chloroplast 23S topology, and for the monophyly of samples #7, 8, 13, and 14 in the *cob* topology. While the markers investigated here are conserved genes that have *a priori* limited utility for finer scale (i.e., within clade) analysis, each contains a unique set of characteristics, including variable cladal resolution and/or evolutionary rates (e.g., see samples #2 and #3 in *coI* or samples #7, 8, 13, 14 in *cob*), hence each marker has the potential to address different questions. These differences thus support our previous conclusion that no one gene fits all of the taxonomic questions being asked in the genus *Symbiodinium* (Pochon et al., 2012).

Our fully concatenated analysis, incorporating all investigated genes and totaling 4,703 bp, resulted in a highly resolved phylogeny that was statistically identical to the *nr28S* gene, a gene used as the benchmark for assigning *Symbiodinium* lineages (Fig. 2; Table S2). The fact that the concatenated nuclear, chloroplastic, and mitochondrial genes display overall similar evolutionary histories, suggests that the molecular taxonomy of the currently recognized *Symbiodinium* clades using *nr28S* is robust (Pochon et al., 2006; Pochon & Gates, 2010), and that the points of clade differentiation are ancient, allowing for a concerted evolution of these conserved genes across genomes. These new results support a sequential evolution of *Symbiodinium* clades A/E/G1-G2/D1-D2/I/B/F2-F5/H/C, from most ancestral to most derived, respectively. It appears that there is a level of constraint in the system, with recombination likely being a rare event (Santos & Coffroth, 2003; but see Chi, Parrow & Dunthorn, 2014), a feature that maintains separation among lineages.

Compartment specific evolution and link to environmental preference/prevalence

Dinoflagellates are characterized by several genetic distinguishing features, including large genome size, and complex architecture and gene regulation (*Barbrook et al., 2010; Hackett et al., 2004; Howe, Nisbet & Barbrook, 2008*). One prominent feature is the large number of genes that have relocated from the ancestral organellar genome to the nucleus, resulting in a significant reduction in plastid and mitochondrion genomes. For example, the few genes that remain in the plastid of peridinin-containing dinoflagellates are primarily the core subunits of the photosystem (including *cp23S*), and the cytochrome *b6f* and ATP synthase complex (about 16 genes including *psbA*) (*Hackett et al., 2004*). Similarly, the mitochondrial genome of dinoflagellates has been reduced to three protein-coding genes (*coI*, *coIII*, and *cob*), but also contains a large number of non-functional fragments separated by repetitive non-coding DNA (*Barbrook et al., 2010; Waller & Jackson, 2009*). Despite the fact that the six *Symbiodinium* genes investigated here are only a very small subset of the *Symbiodinium* genome, they are physically separated in three cellular compartments, each with distinct evolutionary constraints and potential. For example, our comparisons of evolutionary rates between markers revealed that the differences among cellular compartments was primarily driven by the dissimilarity in the rates of evolution in *cp23S* and *psbA* in *Symbiodinium* lineages D2 and I (*Figs. 1 and 2*).

A possible explanation is that the increased evolutionary rates reflect rarity and adaptation to marginal habitats. It has been posited that rare taxa are important in driving evolutionary trajectories and innovations (*Holt, 1997*). Rarity in terms of small population size and isolation can drive high rates of adaptation and speciation (e.g., peripheral speciation; *Mayr, 1963*), as mutations in rare species are more likely to accumulate in the periphery of the founding population's habitat where rare species may be subjected to persistent directional selection in the absence of gene flow, as they colonize new areas (*Garcia-Ramos & Kirkpatrick, 1997*). Such a scenario is supported by the fact that lineages D2 and I have only been documented on few occasions (*Carlos et al., 1999; Pochon et al., 2007; Pochon & Gates, 2010*), despite numerous *Symbiodinium* surveys conducted over the last 20 years in both the Western Atlantic and Indo-Pacific Oceans targeting a diversity of host taxa, as well as free-living communities, and crossing a variety of spatial and temporal scales (reviewed in *Coffroth & Santos, 2005; Stat, Carter & Hoegh-Guldberg, 2006*). In addition, *Symbiodinium* D2 and I have only been detected in the Hawaiian Archipelago and Micronesia (Guam and Palau), some of the most isolated island groups in the world and areas known for harboring high levels of endemism in marine biodiversity (*Hughes, Bellwood & Connolly, 2002; Pauley, 2003*). Both lineages have been suspected to either be free-living because of the manner in which the sample was isolated (*Carlos et al., 1999*), or recently ingested free-living strains due to their apparent rarity in nature (*Pochon & Gates, 2010*).

The high rates of evolution in chloroplastic genes in *Symbiodinium* sub-clade D2 and clade I might also reflect a relatively recent transition from free-living to symbiotic lifestyles. These habitats are extremely different in nature and composition, with free-living environments exhibiting high levels of environmental variability and unpredictability,

while symbiotic habitats are relatively more predictable being spatially constrained and influenced by the biology of the host. These environmental differences undoubtedly drive the very different morphologies of *Symbiodinium* found in these two habitats, with free-living *Symbiodinium* flagellated and motile, and symbiotic *Symbiodinium* encysted and immotile. In terms of evolutionary trajectories, such differences in environment must exert a profound influence. *Symbiodinium* strains evolving predominantly in symbiosis must have adapted particular biochemical and chloroplastic functions in an environment that bears little or no resemblance to a free-living setting. Previous studies on the transition between symbiotic and free-living habitat show that changes in evolutionary rate occur in bacteria that have transitioned from free-living to a symbiotic lifestyle and mutualism (Lutzoni & Pagel, 1997; Moran, 1996). In addition, in some ectomycorrhizal assemblages, changes in evolutionary rate correspond to reversing from symbiotic to free-living lifestyle (Hibbett, Gilbert & Donoghue, 2000). Further, rapid and extreme environmental changes may favor the survival of rare and transitioning species, as their existing phenotypic diversity may contain traits pre-adapted to a changing environment (Holt, 1997).

Our examination of evolutionary rates for multiple markers and organelles provides an opportunity to begin addressing the implications for gene and genome evolution due to symbiotic lifestyle and dissimilarities in organellar genome constraints. Here we see the significantly slower evolutionary rates of *Symbiodinium* clade A compared to other clades as well as overall slower relative rates of the mitochondrial compartment across all clades (Fig. 3). As recently highlighted by Decelle (2013), the predominance of a particular lifestyle in marine microalgae (i.e., symbiotic versus free-living) can have important implications in genome evolution. *Symbiodinium* clade A is a basal lineage known to date back to at least 50 MYA and which has possibly survived through the climatic vicissitudes of the K-T boundary (Tchernov et al., 2004; Pochon et al., 2006). This clade easily overgrows other *Symbiodinium* clades in culture (Carlos et al., 1999) and shows attributes of parasitism in scleractinian corals (Stat, Morris & Gates, 2008). Additionally, clade A contains a high number of unique strains that may never establish symbiotic relationships (e.g., Coffroth et al., 2006; Hirose et al., 2008; Yamashita & Koike, 2013), and evolves at a similar rate to its close pelagic dinoflagellate relatives, contrasting with all other *Symbiodinium* clades which on average evolve six times faster based on *nr18S* sequence analyses (Shaked & de Vargas, 2006). As discussed by Decelle (2013) these differential traits and pressures of clade A, such as prevalence in the free-living environment with an occasional symbiotic lifestage (i.e., planktonic symbionts) provide a situation where the genomes are primarily influenced by external environmental pressures rather than host controlled traits. The resulting pressures are more likely to establish sexual exchanges within larger free-living populations, minimizing genomic impacts with often comparatively slower rates of evolution. In contrast, lineages that spend most of their lifecycle *in hospite*, which is arguably the case for most *Symbiodinium* clades (Table 1), tend to develop a certain dependence on the host which can lead to comparatively higher rates of change due to genome reduction and higher genetic drift associated with the absence of purifying selection through sexual recombination (Lynch, Koskella & Schaack, 2006).

Our analysis of relative-rates of evolution also indicated that mitochondrial genes evolved approximately twice slower than nuclear and chloroplastic genes. This result appears to contrast markedly with the recent study of *Roy-Smith & Keeling (2012)*, which showed that silent site divergence of the mitochondrial genome in other protists with secondary red-algal derived plastids evolve 5–30 times faster than the divergence of their plastid genomes. These contrasting results may in part be due to the differences in DNA bases of a few selected genes in our study in comparison to the silent site divergence of complete mitochondrial and plastid genomes in *Roy-Smith & Keeling (2012)*. Nevertheless, as there is evidence that our results from a subset of genes matches those of land plants and green algae, with more rapid rates of divergence in the plastid organelle, additional work is needed to further explore the implications of transitions between the free-living and symbiotic state for *Symbiodinium*, with a goal of gaining a more comprehensive understanding of the dynamics and mechanisms behind the different evolutionary trajectories observed in this study. Additionally, the increasing use of next-generation sequencing for characterizing entire *Symbiodinium* genomes (e.g., *Barbrook, Voolstra & Howe, 2014*) is an exciting avenue that provides unprecedented opportunities for the investigation of novel markers and paves the way for much more comprehensive phylogenomics studies to come.

CONCLUSIONS

Our study examines the performance of six genetic markers from three organelles in samples representing all currently documented lineages of *Symbiodinium*. As such it represents a comprehensive phylogenetic reconstruction of *Symbiodinium*, and highlights differences in the taxonomic resolution of each marker and their relative value in addressing a variety of evolutionary questions. Our series of phylogenetic analyses were conducted to address three working hypotheses. Despite striking similarities among the single gene phylogenies from distinct cellular compartments, none were evolutionarily identical confirming our first hypothesis. This result reflected within and among clade differences inherent to the individual markers. Our second hypothesis, however, was rejected and showed that a supermatrix tree incorporating all investigated genes (4,703 bp alignment) resulted in a highly resolved phylogeny that was statistically identical to the *nr28S* gene. This result provides additional support for the use of *nr28S* as a ‘clade-level’ benchmark gene for *Symbiodinium*. Finally, compartment-specific differences in evolutionary rates among *Symbiodinium* clade and gene organelle were revealed confirming our third hypothesis. Highest evolutionary rates were observed within the chloroplastic compartment, a pattern that was largely driven by fast evolving *Symbiodinium* clades D2 and I, two lineages that are rare in nature and which may be transitioning between free-living and symbiotic states. As such, rarity appears to associate with evolutionary innovation in a key functional compartment in *Symbiodinium*. The identification of different evolutionary trajectories in chloroplast genes that link with habitat and prevalence suggests that this organellar compartment is evolutionarily plastic and responsive. This finding may have important implications for our understanding of evolutionary processes that underpin a

symbiotic lifestyle in this essential dinoflagellate group. Our analysis further revealed that investigated mitochondrial genes evolved approximately twice slower than nuclear and chloroplastic genes, an observation that contrasts with comparatively fast mitochondrial rates previously documented in non-symbiotic protists with secondary red-algal derived plastids. Together these results further highlight the need for deeper genome sequencing for a variety of *Symbiodinium* taxa with rapidly advancing next-generation sequencing approaches to understand the evolution of these enigmatic yet critical symbionts.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests. Xavier Pochon is an employee of The Cawthron Institute.

Author Contributions

- Xavier Pochon conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

- Hollie M. Putnam conceived and designed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Ruth D. Gates conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

All sequences have been deposited in GenBank. A detailed table is provided in the manuscript. All sequence accession numbers starting with “JQ” are novel:

JN558040 JN557869 JN557969 JN557844 JN557891 JN557943
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