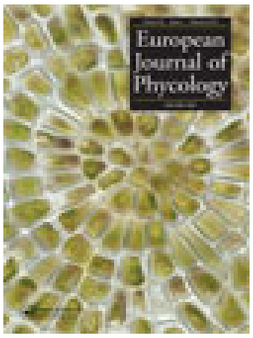

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Phylogenetic analysis of symbiont transmission mechanisms reveal evolutionary patterns in thermotolerance and host specificity that enhance bleaching resistance among vertically transmitted *Symbiodinium*

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Phylogenetic analysis of symbiont transmission mechanisms reveal evolutionary patterns in thermotolerance and host specificity that enhance bleaching resistance among vertically transmitted *Symbiodinium*

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ABSTRACT

Vertical transmission of *Symbiodinium* symbionts between generations of coral hosts has been hypothesized to result in superior matches between host and symbiont physiologies, and to form holobionts that are generally more resistant to thermal stress. Alternatively, horizontal transmission, with its greater potential for assembling physiologically diverse associations as well as being capable of substituting symbionts in response to stimuli, may result in holobionts that are generally more resistant to thermal stress. While the most common mode of transmission among Scleractinia–*Symbiodinium* symbioses is horizontal, mixed-modes transmission only occurs in vertically transmitting corals, allowing the maintenance of highly specialized associations across generations as well as transiently critical relationships. These advantages of mixed-modes transmission may serve to rescue otherwise susceptible corals, or alternatively, reinforce otherwise resistant corals, depending upon the other attributes of vertically transmitted *Symbiodinium* phylotypes. Here we ask if vertically transmitted symbionts tend to be more thermotolerant and specific. Because significant relationships between traits can be overestimated or obscured by patterns of shared evolutionary history, we inferred a novel molecular phylogeny for 97 *Symbiodinium* phylotypes representing clades A–F to evaluate the relationship between phylotype transmission-mode, thermotolerance and specificity to coral hosts. Thermotolerance and specificity have been independently derived multiple times during the evolutionary history of *Symbiodinium*, and cannot be predicted by clade membership. The probability of phylotype transmission being predominantly vertical increased by more than 200% across the observed ranges of increase of thermotolerance and specificity, even though phylotype thermotolerance is not correlated with host specificity. Higher thermotolerance and specificity of vertically transmitted *Symbiodinium* may contribute to robust bleaching resistance among vertically transmitting corals that could reinforce the potential benefits of mixed-modes transmission.

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KEYWORDS Climate change; coral bleaching; mixed-modes transmission; specificity; symbiosis; thermotolerance; phylotype trait evolution

Introduction

Coral reef ecosystems support as many as 1.3 million species (Fisher *et al.*, 2015) and more than 500 million people (Wilkinson, 2008). Productivity and calcification of the scleractinian corals that build this ecosystem depend upon mutualistic endosymbiosis with photosynthetic *Symbiodinium* to provide most of their fixed carbon (Muscatine, 1990). When disrupted by the bleaching response, the loss of *Symbiodinium* results in decreased competitive ability, reproduction, growth and repair, and increased bioerosion, disease, predation and mortality of the coral (Brown, 1997; Jokiel, 2004; Jones, 2008; McClanahan *et al.*, 2009); these effects of bleaching can cause systematic ecosystem degradation and collapse. Coral bleaching is induced by severe, rapid or prolonged thermal stress (Fitt *et al.*, 2001), all of which are becoming more prevalent and widespread as climate change intensifies (Wilkinson, 2008; Hughes *et al.*, 2017). The recent global bleaching event, only the third in history, had been continuously degrading coral reef ecosystems (alternating between the northern and southern hemispheres as the seasons change) since the summer of 2014.

The responses of corals to thermal stress vary across colonies and taxa (Loya *et al.*, 2001; van Woesik *et al.*, 2011; Swain *et al.*, 2016, 2017*b*) and can be influenced by the physiological capabilities of their diverse *Symbiodinium* symbionts (Sampayo *et al.*, 2008; Stat *et al.*, 2008*b*; LaJeunesse *et al.*, 2009; Grottoli *et al.*, 2014; Hume *et al.*, 2015; Silverstein *et al.*, 2015; Swain *et al.*, 2017*a*). Nine clades (A–I) and more than 400 subcladal phylotypes of *Symbiodinium* have been identified using molecular techniques (e.g. Pochon & Gates, 2010; Franklin *et al.*, 2012; Pochon *et al.*, 2014), and this genetic diversity encompasses significant physiological diversity that has a broad range of effects on holobiont function (Knowlton & Rohwer, 2003; Little *et al.*, 2004; Stat *et al.*, 2008*b*; Cantin *et al.*, 2009; McGinty *et al.*, 2012; Baker *et al.*, 2013; Starzak *et al.*, 2014).

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Therefore, the capacity for corals to acclimate to climate change is, in part, controlled by symbiont identity, and the boundaries of potential acclimation may be established by the ability of corals to form associations with physiologically diverse symbionts.

Coral–*Symbiodinium* associations are not reciprocally exclusive (Baker, 2003; Fabina *et al*., 2013), and therefore the potential for symbiont shuffling (adjusting relative abundance of current symbionts) or shifting (obtaining different symbionts) is clear and has been broadly discussed and documented (Buddemeier & Fautin, 1993; Baker *et al*., 2004; Fautin & Buddemeier, 2004; Berkelmans & van Oppen, 2006; Thornhill *et al*., 2006*b*; Jones *et al*., 2008; LaJeunesse *et al*., 2009; Stat *et al*., 2009; Cunning *et al*., 2015; Silverstein *et al*., 2015; Fig.1). How that potential materializes is a function of symbiotic partner fidelity (i.e. association with a genotype across lifetimes or generations) and specificity (i.e. diversity of phylotypes that are potential symbionts). *Symbiodinium* are transmitted from coral to coral vertically, via packaging of algal cells in ova that are then usually brooded (~90% of brooders transmit vertically), or horizontally, after propagules are released (~75% of spawners transmit horizontally) through the acquisition of *Symbiodinium* cells by larvae or juveniles from the environment (Richmond & Hunter, 1990; Stat *et al*., 2006; Baird *et al*., 2009; Fig. 1). Vertical transmission ensures the continuation of an association between host and symbiont genotype across generations (high fidelity; Fig. 1), excludes generalist phylotypes (high symbiont specificity:

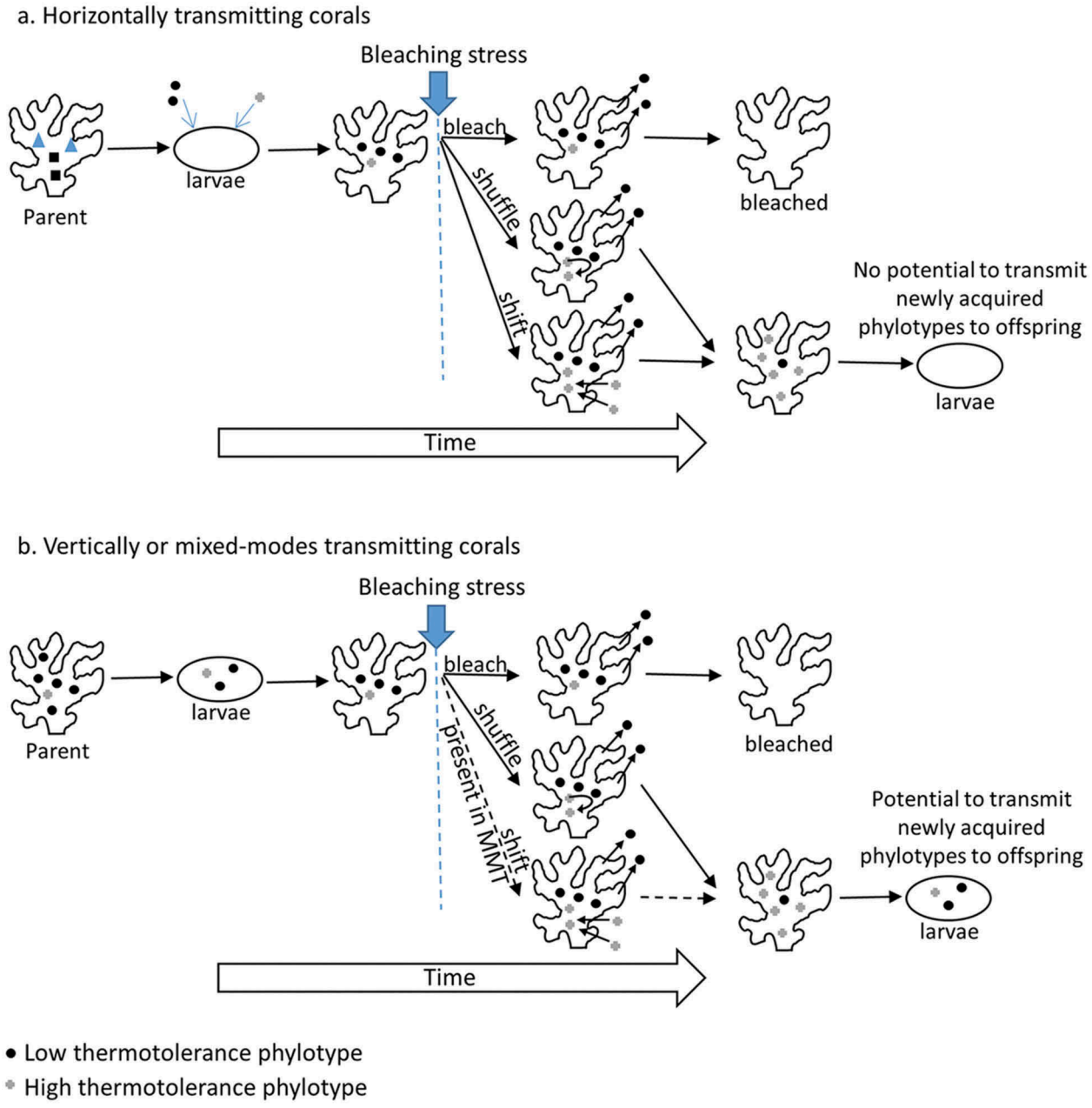


Fig. 1. Schematic of *Symbiodinium* phylotype acquisition, reapportionment and expulsion during bleaching for (a) horizontally and (b) vertically and mixed modes transmitting corals.

Douglas, 1998), may reduce the potential for shifting (and perhaps shuffling; Fay & Weber, 2012; Fig. 1) and should generally increase the specialization of the symbiosis by linking the fitness and evolutionary success of host and symbiont genotype (Sachs & Wilcox, 2006; Leigh, 2010). Conversely, horizontal transmission ensures variance in the association between host and symbiont genotype (i.e. low fidelity) through resampling of the environment with every generation (Fig. 1), allows associations with generalist phylotypes (i.e. low symbiont specificity: Douglas, 1998), may increase the potential for shifting and shuffling (Fig. 1) and should decouple the fitness and evolutionary success of host and symbiont genotypes (Sachs & Wilcox, 2006; Leigh, 2010) to permit greater variability in relationship outcomes (mutualism–parasitism).

While transmission modes are generally considered to be mutually exclusive, species-specific traits in corals, verifying this is an asymmetric challenge. Establishing the exclusive horizontal transmission of *Symbiodinium* is relatively uncomplicated: the asymbiotic condition can be verified by collecting ova as they are released, and using molecular and histological tools, the absence of *Symbiodinium* cells can be convincingly revealed (e.g. Harii *et al*., 2009; Fig. 1). Establishing the exclusive vertical transmission of *Symbiodinium* is a much greater challenge: the presence and identity of specific *Symbiodinium* genotypes can be verified from propagules as they are released (e.g. Byler *et al*., 2013; Fig. 1), but *Symbiodinium* could be horizontally acquired at any point between that initial release and eventual death of the colony (potentially over hundreds of years) and may occur transiently during disturbance (e.g. Thornhill *et al*., 2006*b*; Fig. 1).

Although the potential for horizontal transmission in corals known to transmit symbionts vertically (mixed-modes transmission; Fig. 1) has not received much attention, there is evidence to suggest that it occurs in some corals (Hidaka & Hirose, 2000; Baker, 2001; Coffroth *et al*., 2010; LaJeunesse *et al*., 2010*b*; Pettay *et al*., 2011; Padilla-Gamiño *et al*., 2012; Byler *et al*., 2013; Boulotte *et al*., 2016). Therefore, the potential costs associated with vertical transmission (most prominently, inability to acquire physiologically novel phylotypes) may be mitigated by corals that both inherit and acquire symbionts (Ebert, 2013), but the potential benefits of vertical transmission (coupled host-symbiont fitness and evolutionary success) are not realized by horizontally transmitting corals which lack parental *Symbiodinium* (Fig. 1). If mixed-modes transmission is common among vertically transmitting corals (in the studies cited above, the frequency may be as high as 8/8 species, but see Thornhill *et al*., 2006*a*), it could dramatically alter how we envision the balance of costs and benefits in opposing modes, and our corresponding predictions for the potential to acclimate to climate change.

Transmission mode is classically considered a species-specific trait of corals; however it is also a phylotype-specific trait of *Symbiodinium* as a result of the specificities of hosts and symbionts. Corals acquire *Symbiodinium* through either vertical (*Symbiodinium* cells passed directly to coral offspring by packaging in ova) or horizontal (*Symbiodinium* cells obtained from the environment) transmission (Fig. 1), and this pattern is mirrored in the phylotypes that they host. *Symbiodinium* phylotypes associate almost exclusively with coral hosts that are either vertical or horizontal transmitters, but rarely both (Barneah *et al*., 2004; LaJeunesse *et al*., 2004*a*; van Oppen, 2004; Fabina *et al*., 2012). When phylotypes do associate with both vertical and horizontal transmitters, the distribution of associations is almost always heavily skewed toward one transmission mode (Fabina *et al*., 2012). This pattern of reciprocal host and symbiont specificity should serve to reinforce selection toward specialization in associations between vertically transmitting corals and their vertically transmitted phylotypes.

Although there are distinct and opposing suites of symbiotic features that can be distilled into the binary transmission trait (vertical/horizontal), it remains unclear which may lead to greater resilience to the thermal stress of climate change. It is possible that vertical transmission, with its high fidelity between coral hosts and *Symbiodinium* symbionts that form highly specialized associations, could result in superior matches between host and symbiont physiologies and holobionts that can tolerate a broad range of environmental conditions (Barneah *et al*., 2004; Thornhill *et al*., 2006*a*), which may therefore increase bleaching resistance. Alternatively, horizontal transmission, with its greater potential for assembling a range of physiologically diverse associations and substituting phylotypes in response to stimuli, may result in holobionts that are generally more resistant to thermal stress (Buddemeier & Fautin, 1993; Fautin & Buddemeier, 2004; LaJeunesse, 2005). Furthermore, we do not yet know if many, or perhaps all, vertically transmitting corals can also acquire symbionts from the environment, but if they do there are clear potential benefits for thermal stress resistance. Mixed-modes transmission may serve to rescue otherwise susceptible corals, or alternatively reinforce otherwise resistant corals, depending upon the other attributes of vertically transmitted *Symbiodinium*.

Here we begin to address this issue by evaluating differential thermotolerance and specificity of *Symbiodinium* phylotypes that are vertically or horizontally transmitted. Thermotolerance of *Symbiodinium* symbionts can alter the bleaching thresholds of coral holobionts by 1–2°C (Berkelmans & van Oppen, 2006), which has been demonstrated to alter bleaching susceptibility (e.g. Thornhill *et al*., 2006*b*; LaJeunesse *et al*., 2009; Stat *et al*., 2009; DeSalvo *et al*., 2010; Kemp *et al*., 2014), and vertically transmitted *Symbiodinium* are

hypothesized to tolerate a broad range of environmental conditions (Barneah *et al.*, 2004; Thornhill *et al.*, 2006*a*). However, it is unknown if vertically transmitted phylotypes are generally more thermotolerant than horizontally transmitted phylotypes. Similarly, specificity for *Symbiodinium* symbionts may increase holobiont resistance to bleaching (Putnam *et al.*, 2012) and vertically transmitted *Symbiodinium* phylotypes may be more specific to hosts (LaJeunesse *et al.*, 2004*a*, 2004*b*; LaJeunesse, 2005; Stat *et al.*, 2008*a*; Fabina *et al.*, 2012), but it is unknown if the observed patterns are the result of evolutionary relationships among phylotypes and if host-specific *Symbiodinium* are differentially thermotolerant.

While previous studies have identified high thermotolerance in both horizontally (e.g. D1-4, C40) and vertically (e.g. C15, A4) transmitted phylotypes, what is lacking is a thorough evaluation of the relationship between thermotolerance, specificity and transmission mode using robust sample sizes, phylogenetic correction of statistical analyses and a standardized metric of *Symbiodinium* phylotype-specific thermotolerance. Standard statistical analyses assume independently sampled replicates, but *Symbiodinium* phylotypes violate this assumption in a specific pattern – through shared evolutionary history that is defined by phylogeny (Felsenstein, 1985). Appropriately correcting for phylogeny can reveal significant patterns among species traits that would otherwise be obscured or misleading in simple cross-species comparisons (Harvey, 1996; Freckleton *et al.*, 2002; Revell, 2010). Here we use existing DNA sequence data to create a novel molecular phylogeny of 97 *Symbiodinium* phylotypes onto which we map phylotype-specific transmission, a standardized metric of thermotolerance (Swain *et al.*, 2017*a*), and newly compiled host specificity to examine homoplasy within and relationships between these traits, while simultaneously correcting for evolutionary non-independence of phylotypes (where appropriate). The results provide novel insight into the thermotolerance and specificity of differentially transmitted *Symbiodinium* and their potential for altering bleaching response.

Materials and methods

A molecular phylogeny of *Symbiodinium* was constructed to examine the evolution of, and associations between, phylotype thermotolerance, specificity and transmission. Nucleotide sequences for *Symbiodinium* phylotypes represented in the thermotolerance ranking of Swain *et al.* (2017*a*), for which either (or both) internal transcribed spacer 2 (ITS2) or 28S ribosomal RNA nuclear genes were available from GenBank, were included in the analysis (Table 1). Gene sequences of ITS2 usually include partial sequences of flanking 5.8S and 28S genes, which are generally more conserved in nucleotide identity and sequence length than the hypervariable ITS2 region. These hypervariable regions of ITS2 are used to define *Symbiodinium* phylotypes, but are not often employed in inter-clade phylogenetic inference because of challenging homology assessment. Hypervariable sequences were included in the analyses here by using a staggered alignment, which essentially relies upon conserved regions (5.8S and 28S) to support inferences between clades, and hypervariable regions to support inferences within clades. The phylogeny was generated through maximum-likelihood (ML) inference applied to a concatenated staggered multigene DNA sequence alignment. Thermotolerance, specificity and transmission modes were culled from the literature, mapped onto the phylogeny, and assessed through analysis of homoplasy and phylogenetically corrected logistic and linear regression.

Sampling strategy and sequence alignment

Symbiodinium phylotypes represented in the thermotolerance ranking of Swain *et al.* (2017*a*) were targeted for inclusion in a staggered multi-gene sequence alignment that would serve as the homology assessment for phylogenetic inference. *Symbiodinium* phylotypes are commonly identified through selective DNA amplification of the ITS2 region of the ribosomal RNA nuclear gene, followed by a unique-sequence separation technique such as Denaturing Gradient Gel Electrophoresis (DGGE) prior to sequencing. Phylotype identity is indicated by alpha-numeric nomenclature that includes a capital letter (referring to the clade) followed by a number that identifies a unique ITS2 DNA sequence. When multiple co-abundant sequences are simultaneously detected, a hyphenated lower-case letter or pair of letters (or number, as is the practice with clade-D phylotypes) is appended to the alpha-numeric designation to indicate the presence of multiple unique sequences (LaJeunesse *et al.*, 2010*a*). Each hyphenation indicates an additional closely related co-abundant sequence, listed from ancestral to divergent (e.g. D1-4, D1-4-6; LaJeunesse & Thornhill, 2011), but the entire designation (regardless of the number of hyphenations) is considered a single distinct phylotype which is treated similarly to a species. We favoured ITS2 phylotypes rather than taxonomic binomials (but see AlgaeBase (Guiry & Guiry, 2018) for named taxa or Jeong *et al.* (2014) for phylotype to taxon conversions) to maintain homogeneity among designations.

Of the 110 phylotypes ranked in Swain *et al.* (2017*a*), GenBank (http://www.ncbi.nlm.nih.gov/nuccore/) contained ITS2 sequences (including partial 5.8S and 28S) for 95 phylotypes, and 28S sequences for 34 phylotypes, for a combined total of 97 *Symbiodinium* phylotypes representing clades A, B, C, D, E and F. Phylotypes that are comprised of co-abundant unique ITS2 sequences

Table 1. *Symbiodinium* phylotypes and their GenBank accessions, thermotolerance scores (R_k), per cent of hosts that vertically transmit symbionts, host species richness and ocean basin of origin.

Phylotype	ITS2	28S	Score (R_k)	Coral host richness	% vertical transmission	Ocean basin
A1	EU449049	KM972549	32.07	18	0.33	Indo-Pacific
A13	JN558096	JN558096	20.22	3	0.33	Atlantic
A2	AF333506	KF740671	23.02	1	0.00	Atlantic
A20	KR022079	–	59.02	–	–	Atlantic
A3	EU074857	KF364601	35.86	11	0.45	Pan-tropical
A4	EU449050	KF364602	45.95	5	1.00	Atlantic
A4a	EU449052	–	36.86	1	1.00	Atlantic
B1	EU074864	KT149349	23.63	23	0.47	Atlantic
B10	AF499787	–	10.62	3	0.00	Atlantic
B17	AY258470	–	17.25	2	0.00	Atlantic
B18	AY258471	–	15.80	2	0.00	Indo-Pacific
B1j	GU907637	–	8.85	2	0.00	Atlantic
B2	JN558060	JN558060	34.94	3	0.00	Atlantic
B40	EU099825	–	23.71	2	0.00	Indo-Pacific
B5	AF499781	–	26.56	2	1.00	Atlantic
C1	EU074876	AY239384	21.72	146	0.14	Pan-tropical
C101	GU111881	–	13.17	26	0.00	Indo-Pacific
C101a	GU111882	–	4.39	2	0.00	Indo-Pacific
C110	GU111892	–	4.39	2	1.00	Indo-Pacific
C114	GU111897	–	21.95	1	1.00	Indo-Pacific
C116	GU111899	–	71.12	1	0.00	Indo-Pacific
C119	GU111900	–	39.51	1	0.00	Indo-Pacific
C12	AF499801	–	31.87	4	0.00	Atlantic
C120	HM185737	–	7.90	1	1.00	Indo-Pacific
C120a	HM185738	–	5.27	1	1.00	Indo-Pacific
C123	EU099826	–	55.32	1	1.00	Indo-Pacific
C124	EU099827	–	23.71	1	1.00	Indo-Pacific
C125	EU597011	–	39.51	1	1.00	Indo-Pacific
C126	EU597012	–	39.51	1	1.00	Indo-Pacific
C127	HQ328056	–	7.90	1	0.00	Indo-Pacific
C128	EU099821	–	23.71	1	1.00	Indo-Pacific
C129	HQ385805	–	39.51	1	0.00	Indo-Pacific
C130	HQ385807	–	55.32	1	0.00	Indo-Pacific
C15	AY239369	KF740678	37.59	49	0.85	Indo-Pacific
C15.21	KC597683	–	53.12	1	1.00	Indo-Pacific
C15.23	KC597697	–	53.12	2	1.00	Indo-Pacific
C15.25	KC597685	–	53.12	2	1.00	Indo-Pacific
C161	KJ184183	–	7.90	1	1.00	Indo-Pacific
C17.2	FJ461513	–	10.62	2	1.00	Indo-Pacific
C1b-c	AY239364	KF740674	19.11	4	1.00	Indo-Pacific
C1c	AY239364	KF740674	35.71	15	0.50	Indo-Pacific
C1d-t	GU111867	–	4.39	3	1.00	Indo-Pacific
C1gg	HQ328057	–	31.61	1	0.00	Indo-Pacific
C1h	AY258473	–	55.32	5	0.80	Indo-Pacific
C1hh	HQ328055	–	7.90	1	0.00	Indo-Pacific
C1m-aa	HM185739	–	5.27	1	1.00	Indo-Pacific
C21	AY239372	KF740679	40.25	33	0.24	Indo-Pacific
C26	AY239378	–	63.22	2	1.00	Indo-Pacific
C26a	JQ003841	–	4.39	5	1.00	Indo-Pacific
C26bb	KJ184189	–	71.12	1	1.00	Indo-Pacific
C3	GU111863	AY239386	25.98	146	0.18	Pan-tropical
C31	JQ003850	–	25.20	9	1.00	Indo-Pacific
C33	FJ529633	FJ529532	37.03	1	1.00	Indo-Pacific
C35a	FJ529582	FJ529529	35.56	1	1.00	Indo-Pacific
C3-ff	HM185740	–	2.63	1	1.00	Indo-Pacific
C3h	AY239365	–	39.51	30	0.00	Indo-Pacific
C3jj	HQ385809	–	63.22	1	0.00	Indo-Pacific
C3n	EU449106	–	39.51	1	1.00	Indo-Pacific
C3n-t	FJ529594	FJ529530	19.83	1	1.00	Indo-Pacific
C3-o	GU907653	–	44.27	1	0.00	Atlantic
C3-p	GU907654	–	8.85	1	0.00	Atlantic
C3u	GU111879	KF740676	21.95	87	0.00	Indo-Pacific
C3z	GU111884	–	8.78	48	0.05	Indo-Pacific
C4	AF333518	–	22.97	3	1.00	Atlantic
C40	AY258485	KF740681	55.32	16	0.00	Indo-Pacific
C42	AY258487	–	7.90	5	1.00	Indo-Pacific
C42a	FJ529634	FJ529525	25.90	3	1.00	Indo-Pacific
C42a-b	AY765403	–	18.52	1	1.00	Indo-Pacific
C47	AY589752	–	11.49	2	1.00	Atlantic
C66	AY589771	–	29.21	1	1.00	Indo-Pacific
C7	AF499797	–	10.36	8	0.33	Pan-tropical
C78	EU807999	–	31.30	1	1.00	Indo-Pacific
C79	FJ529581	FJ529528	17.78	1	1.00	Indo-Pacific
C7a	–	KF740677	8.85	3	0.00	Atlantic
C7d	HQ385808	–	7.90	2	0.00	Indo-Pacific
C8	AY239367	–	33.10	1	1.00	Indo-Pacific

(*Continued*)

Table 1. (Continued).

Phylotype	ITS2	28S	Score (R_k)	Coral host richness	% vertical transmission	Ocean basin
C81	GU907657	–	8.85	4	0.33	Atlantic
C8a	FJ529612	FJ529526	71.12	1	1.00	Indo-Pacific
C8b	AY258475	–	23.71	1	1.00	Indo-Pacific
C94a	GU111877	–	39.51	6	0.00	Pan-tropical
D1	JN558077	JN558077	43.82	40	0.39	Pan-tropical
D1-11	EU812740	–	39.51	1	1.00	Indo-Pacific
D12-13	KJ019892	KF740687	26.56	1	0.00	Indo-Pacific
D13	KJ019892	KF740687	35.41	1	0.00	Indo-Pacific
D1-4	EU074894	KF740689	53.12	96	0.20	Pan-tropical
D1-4-10	EU812741	–	39.51	5	0.00	Indo-Pacific
D1-4-6	EU812742	–	30.73	2	1.00	Indo-Pacific
D15	KJ019893	KF740688	13.28	1	0.00	Indo-Pacific
D1a-c	–	EF372071	37.03	1	0.00	Indo-Pacific
D4-5	EU812743	–	4.39	1	0.00	Indo-Pacific
D5	EU449054	–	17.56	12	0.83	Indo-Pacific
D8	KJ019890	KF740686	35.41	1	0.00	Indo-Pacific
D8-12	KJ019891	–	30.99	1	0.00	Indo-Pacific
E1	JN558086	JN558086	12.54	–	–	Indo-Pacific
F1	JN558068	JN558068	39.84	1	1.00	Indo-Pacific
F2	JQ247043	KF740673	70.83	1	0.00	Atlantic
FDia11	AF333517	–	38.36	1	1.00	Indo-Pacific

cannot simply be simultaneously assigned to the same terminal taxon in the phylogenetic tree, therefore, for the purposes of phylogenetic inference and analysis of phylogenetic patterns, we used the most divergent co-abundant ITS2 sequence listed in the phylotype designation to represent that phylotype in the analysis (e.g. the ITS2 sequence of D13 was used to represent phylotype D12–13 in the phylogenetic inference). Of the phylotypes associated with scleractinian corals (95 of 97), 80% originate from the Indo-Pacific. Outgroup taxa included two species of *Gymnodinium* that had available sequence for ITS2 and 28S in GenBank (DQ779989, JN558105), and were chosen following the outgroup selection of Pochon *et al.* (2014).

A staggered alignment was assembled from the concatenated ITS2 and 28S sequences, which were automatically aligned and manually staggered. A staggered alignment is a form of phylogeny-informed alignment that contains regions of universally aligned sequences representing all taxa, and regions of locally aligned sequences representing only closely related taxa which are then offset, or staggered, relative to other sections of the local alignments to avoid making inferences across alignment positions that are not demonstrably homologous (Morrison, 2006; Morrison *et al.*, 2015; Swain, 2018). Staggered alignments simultaneously assess homology of complex evolutionary events across divergent lineages without discarding raw sequence data, and have been demonstrated to result in phylogenetic inferences that are superior to the common practice of excluding divergent regions of the alignment (e.g. Swain, 2018). Although concatenation of multiple genes carries the possibility of statistical inconsistency because of discordance in gene evolution (which can be addressed through coalescent model-based methods: Degnan & Rosenberg, 2006; Rosenberg, 2013), datasets with incomplete taxon sampling for each gene (as is the case here) are not fully assessable and are predicted to be inferred with greater confidence using concatenation methods (Edwards, 2009). Sequences were compiled in Bioedit 7.2.5 (Hall, 1999) and a first approximation alignment was generated using the ClustalW accessory application within Bioedit. This automated first approximation was sufficient for aligning 5.8S and 28S genes, but was unable to identify potentially homologous sequences within ITS2. The ITS2 region is hypervariable (highly divergent in both nucleotide identity and sequence length), and while alignment within clades is relatively simple, alignment across clades is not possible based on sequence similarity alone. Using the manual editing functions in Bioedit, we staggered the hypervariable regions of ITS2 into locally aligned regions (i.e. aligned only within clades) and universally aligned regions (i.e. aligned across all clades). All decisions that were made in constructing the staggered alignment are completely documented in the TreeBase accession: S20211 (https://treebase.org/).

Model parameters and phylogenetic inference

The staggered alignment was partitioned for model-fitting and ML phylogenetic inference. Partitioning traced the boundaries of ribosomal subunits and staggered hypervariable regions (following the recommendations of Li *et al.*, 2008) into three partitions: 5.8S and conserved regions of ITS2, hypervariable regions of ITS2 and 28S (Table 2). Base frequencies, nucleotide transition rates and gamma parameter of each independent partition were estimated from the empirical data simultaneous with phylogenetic inference. These partitions were simultaneously applied in evolutionary parameter-fitting of the General Time Reversible (GTR) model with gamma (+C) and in ML phylogenetic inference using RAxML v8.2.8 (Stamatakis, 2014) in the CIPRES Science Gateway v3.3 (Miller *et al.*, 2010).

Table 2. Partition definitions and per-partition parameter estimates used to model sequence evolution in the phylogenetic inference.

		Base frequencies				Substitution rates (G–T = 1)					Rate heterogeneity
Partition	Concatenated alignment positions	A	C	G	T	A–C	A–G	A–T	C–G	C–T	Gamma shape
5.8S + conserved ITS2	1–74, 582–593, 800–807	0.1767	0.2261	0.2585	0.3384	1.2172	5.3180	0.1958	1.4654	3.9686	0.7801
hyper. ITS2	75–581, 594–799, 808–1253	0.1933	0.2588	0.2400	0.3077	0.6264	2.8619	1.0377	0.6650	2.6178	2.6655
28S	1254–1920	0.2590	0.2041	0.2883	0.2484	0.6632	2.2786	0.5175	0.6630	5.9719	0.5512

However, branch length optimization was linked across partitions during phylogenetic inference due to incomplete per-partition taxon sampling. Nonparametric bootstrap support was estimated using GTR and a categorical per-site rate heterogeneity approximation from 1000 pseudoreplicates in RAxML (Stamatakis, 2014).

Character collection and phylogenetic analyses

Symbiodinium thermotolerance, specificities, transmission modes and sampling locations were culled from the literature. Thermotolerance values, reported as a relative thermotolerance consensus score (R_k) ranging from 0–100 (thermosensitive to thermotolerant) by Swain *et al.* (2017*a*), represent a consensus of reported relative thermotolerance of specific *Symbiodinium* ITS2 phylotypes (Table 1). Transmission modes of *Symbiodinium* phylotypes were assigned by the transmission mode of their associated host corals under non-bleaching conditions. Using unique associations with coral species (Table S1), which also describes specificity (Table 1), culled from the literature and paired with coral species-specific binary transmission mode (Table S1) detailed in the CoralTraits database (Madin *et al.*, 2016; https://coraltraits.org/) and Keith *et al.* (2013), we assigned each *Symbiodinium* phylotype a transmission mode as the per cent of unique associations with vertically transmitting coral hosts (Table 1).

Phylotype specificity for coral hosts was further assessed with an additional five subsets of the complete dataset in order to gauge the stability of our conclusions in the face of sampling bias. Because the method of sampling *Symbiodinium* phylotypes invariably focuses on specific coral hosts rather than specific phylotypes, additional sampling of the coral does not increase our certainty that we are adequately describing the full diversity of hosts for each phylotype; it simply increases our certainty in the existence of the association with that specific host. As we are probably underestimating the diversity of corals associated with each phylotype when there are few host species reported, we systematically reevaluated correlations with coral host richness on datasets containing only symbionts with >1 (n=48), >2 (n=34), >3 (n=27), >4 (n=24) and >5 (n=19) host coral species.

Symbiodinium phylotype characters were mapped onto the molecular phylogeny for assessment of homoplasy (a measure of character evolution which describes the presence of the character among shared ancestors), and the relationships between thermotolerance, specificity, transmission mode and ocean basin. Standard statistical analyses do not properly account for information loss caused by similarities among relatives, violating the assumption of sample replicate independence, and as a result increase type I error rates and the probability of incorrectly identifying statistical significance (Felsenstein, 1985). Character by phylotype matrices were assembled from the continuous thermotolerance, host richness and transmission data (Table 1), and an identical dataset was categorized into bins. Phylotype thermotolerance was binned as low (R_k <17), intermediate (17 <R_k <33) and high (R_k >33) following Swain *et al.* (2017*a*). Phylotype specificity to coral hosts was classified as a specific (<9 unique hosts) or generalist association (> 9 hosts) following Fabina *et al.* (2012). Phylotype mode of transmission was binned as vertical (≥50% of unique associations with vertically transmitting coral hosts) or horizontal (>50% of unique associations with horizontally transmitting coral hosts). Phylotype prevalence was binned as found mostly in the Atlantic (>50% of unique associations identified from the Atlantic) or Indo-Pacific ocean basins (>50% of unique associations identified from the Indo-Pacific).

Categorical and continuous matrices were mapped on the molecular phylogeny with Mesquite 2.75 (Maddison & Maddison, 2011) for regression analyses and were visualized with Evolview (Zhang *et al.*, 2012). Continuous thermotolerance and specificity were assessed with Phylogenetic Independent Contrasts (PIC) analysis within the Phenotypic Diversity Analysis Programs (PDAP:PDTREE; Midford *et al.*, 2010) module of Mesquite, and the continuous values for thermotolerance and specificity were paired with categorical values of transmission and ocean basin in phylogenetically corrected logistic regressions (Ives & Garland, 2010) performed in Phyloglm v2.4 (Phylogenetic Generalized Linear Model; Ho & Ane,

2014) in R. The PIC between thermotolerance and specificity, and the logistic regression between transmission and specificity, were repeated on each of the data subsets (containing only symbionts with >1, >2, >3, >4, and >5 host coral species) derived to assess sampling bias. Additionally, associations between transmission mode and ocean basin were assessed with the Pagel-94 discrete correlation (assesses association of two binary variables while correcting for phylogeny; Pagel, 1994) analysis in Mesquite. The categorical data were also used for homoplasy assessment through the Retention Index (RI) in Mesquite. The Retention Index is a measure of character evolution shared between an ancestor and their evolutionary descendants, determined as the fraction of apparent synapomorphy that is retained after mapping a character to the phylogeny and ranges from 0 to 1 – highly homoplasious, i.e. independently evolved, characters have low RI values (Farris, 1989).

Phylogenetic Independent Contrasts corrects a standard linear regression analysis for non-independence due to phylogeny (Midford *et al.*, 2010). However, inappropriately applying phylogenetic correction to analyses of data that lack phylogenetic signal is likely to result in poor statistical performance and uncorrected regression will return a more robust result (Revell, 2010). *A priori* assessment of phylogenetic signal for the PIC analysis was assessed by maximum likelihood model fitting (including a model of no phylogenetic signal; Oakley *et al.*, 2005) of the continuous thermotolerance and specificity ordinary least squares (OLS) regression residuals mapped on the phylogeny using the Continuous-character Model Evaluation and Testing (CoMET) module (Lee *et al.*, 2006) in Mesquite.

Phylogenetic logistic regression uses a binary logistic model to estimate the probability of a binary response (in this case, transmission mode and ocean basin) based on a continuous variable (here, thermotolerance and host richness) and was chosen to assess transmission because 83% of phylotypes were found to exclusively associate with either vertically or horizontally transmitting corals, and therefore transmission mode is essentially a binary character in our dataset, as in previous research (LaJeunesse *et al.*, 2004*a*; Fabina *et al.*, 2012). This analysis estimated the probability that vertically transmitted phylotypes have higher thermotolerance and associate with fewer hosts (lower host richness). The phylogenetic logistic regression used here maximizes the penalized likelihood of the logistic regression (MPLE) using Firth's correction as the penalty (Ives & Garland, 2010; Ho & Ane, 2014) and 100 fitted replicates of parametric bootstrapping. This version of the phylogenetic logistic regression does not require an *a priori* assessment of significant phylogenetic signal in the non-corrected regression residuals to justify the application of phylogenetic comparative methods, but instead empirically determines the magnitude of phylogenetic signal directly from the data (α; alpha increases with the rate of transitions, therefore high alpha values indicate low phylogenetic correlations among species) and applies the appropriate correction in the statistical model, thereby estimating the strength of phylogenetic signal in the residuals simultaneously with the logistic regression (Ives & Garland, 2010).

Results

Novel phylogenetic hypothesis of Symbiodinium

The staggered alignment contains three locally aligned regions within ITS2, bordered by four short universally aligned regions, and is capped at either end by the universal alignments of 5.8S and 28S (see TreeBase study accession: S20211). The process of staggering hypervariable regions of the alignment extended the overall length of the matrix to >1.9 × 10^5 positions (99 rows by 1920 columns). Partitioning of the data matrix resulted in three distinct models of nucleotide evolution, detailed in Table 2, which were applied to ML inference of the best tree topology and bootstrap support.

A search for the optimal ML phylogeny using the partitioned data resulted in a best tree with a likelihood score of –8282.33. This analysis recovered highly supported monophylies (bootstrap values of 97–100) of *Symbiodinium* phylotypes representing the same lettered clades (A–F), except for E which is represented by a single phylotype (Fig. 2). The previously reported nested topology (A,(E,(D,(B,(C, F))))) of *Symbiodinium* lettered clades (Pochon *et al.*, 2014) was also recovered here and was highly supported (bootstrap values of 85–100; Fig. 2). Within lettered clades, relationships between phylotypes are not as distinct nor inferred with the same consistently high level of confidence, with branch lengths 1–3 orders of magnitude shorter than between lettered clades and support values highly variable (bootstrap values of 1–100; Fig. 2).

Relating symbiont transmission, host richness and thermotolerance across the Symbiodinium *phylogenetic tree*

Compiling symbiont transmission and specificity data for corals associated with each phylotype represented in the phylogeny identified associations for all except A20 and E1, which were not identified as associated with corals (Table 1). We identified 952 unique coral–phylotype combinations from 68 publications (Table S1). Phylotypes were almost exclusively associated with corals that used vertical (44 out of 95 phylotypes) or horizontal (35 out of 95) transmission, but rarely both (16 out of 95). When phylotypes were associated with corals that

used both vertical and horizontal transmission, almost all (13 out of 16) associated at >50% with horizontally transmitting corals; resulting in a nearly balanced (47 vertically and 48 horizontally transmitted phylotypes) assignment of transmission modes for the homoplasy and regression analyses (Table 1). Host richness of phylotypes (i.e. how many coral hosts a phylotype associates with) targeted in these analyses ranged from 1–146 known host coral species (Table 1). Sampling locations fell into a binary character definable by ocean basins that were exclusively Atlantic (19 out of 97 phylotypes) or Indo-Pacific (71 out of 97), with pan-tropical phylotypes (7 out of 97) mostly associated with Indo-Pacific (5 out of 7) corals (Table 1, Fig. 2, Supplementary table S1).

Mapping symbiosis characters onto the phylogeny revealed the rich homoplasy (independent origins of shared character states) of thermotolerance and specificity, and a concentration of vertical transmission (Fig. 2). While nearly all lettered clades encompassed representatives that use either transmission mode and displayed a broad range of specificities and thermotolerances, specificity (RI = 0.06) and thermotolerance (RI = 0.29) were much more homoplastic traits than mode of transmission (RI = 0.61), and most vertical transmission occurred in the highly derived C/F monophyly (Fig. 2). More than 77% (21 out of 27) of non-C/F phylotypes were predominantly transmitted horizontally.

Phylogenetically corrected logistic regressions revealed significantly higher specificities (phylogenetic logistic regression coefficient = −0.03, $P = 0.036$, df = 94, α = 100.54, Table 3A) and thermotolerances (0.03, $P = 0.007$, df = 94, α = 2.95, Table 3B) among vertically transmitted phylotypes. This relationship between transmission and specificity was robust to any sampling biases of our dataset. It is probable that the diversity of corals associated with each phylotype is underestimated when there are few host species reported, and we therefore reassessed the relationship between transmission and specificity on subsets of the data that were increasingly exclusive of phylotypes with few reported host species. All subsets (only phylotypes associated with >0 (n=95), >1 (n=48), >2 (n=34), >3 (n=27), >4 (n=24) coral hosts) returned the same significant pattern (Table S2), except for the smallest subsample (only phylotypes with hosts numbering >5, n=19).

These phylogenetic logistic regression coefficients are translatable (by exponentiation) to the change in probability that a phylotype is vertically transmitted with each single-unit change in the continuous variable (increase in coral specificity or thermotolerance), after correction for phylogenetic relatedness among phylotypes. This means that phylotypes are 3% (exp(−0.03) = 0.97) less likely to be vertically transmitted with each additional associated coral host (a change of 438% probability over the full range of specificity; 1–146 hosts) and are 3% (exp(0.03) = 1.03) more likely to be vertically transmitted with each additional R_k-score increase (a change of 206% probability over the full range of thermotolerance; 2.63–71.12). Standard logistic regression (uncorrected for phylogeny) did not identify a significant relationship between thermotolerance and transmission mode (logistic regression coefficient = 0.003, $P = 0.83$, df = 94) and overestimated the rate of decrease in the probability of vertical transmission with increasing numbers of host species (−0.06, $P = 0.04$, df = 94), demonstrating the misleading effects of not considering phylogenetic non-independence in statistical analyses.

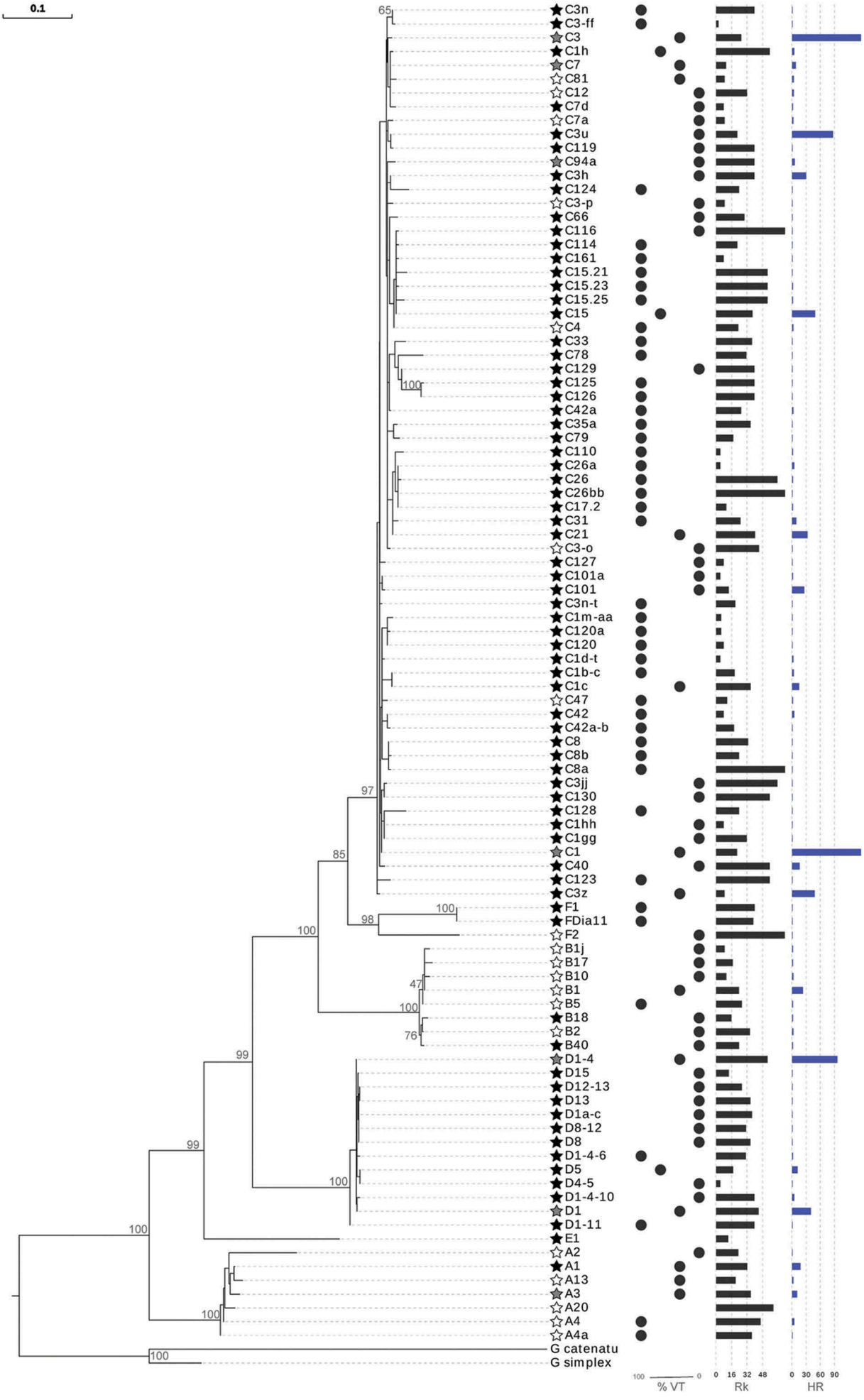
Given the significant relationship between phylotype transmission, thermotolerance and specificity, it is possible that a relationship between thermotolerance and specificity is driving the observation. However, phylogenetically corrected and uncorrected regression analyses of thermotolerance and host richness at nearly all exclusion sets (containing only phylotypes with >0, >1, >2, >3, >4 and >5 host coral species respectively) were positive and non-significant among all uncorrected regression analyses (regression coefficients = −0.015–0.056, $P = 0.44$–0.93, df = 18–94). This trend is the opposite relationship of what is expected if a correlation between thermotolerance and specificity were driving the observation of increased thermotolerance and specificity among vertically transmitted phylotypes. Additionally, there was no significant phylogenetic signal in the OLS regression residuals among any of the exclusion sets (ML best-fit model is non-phylogenetic), and therefore the OLS regression results are the statistically appropriate assessment of these data and the positive trends observed between thermotolerance and specificity are not significant.

Ocean basin origin mapped on the *Symbiodinium* phylogeny (Fig. 2) retained half of its apparent synapomorphy (RI = 0.5) and the patterns observed indicated that while the targeted Indo-Pacific phylotypes are more likely to be vertically transmitted (Page 1-94 discrete correlation, Δlog likelihood = 4.86, $P < 0.02$), they are less specific to hosts (logistic regression coefficient = 0.02, $P = 0.041$, df = 94, α = 10.45, Table 3C) and are not differentially thermotolerant (logistic regression coefficient = 0.02, $P = 0.107$, df = 94, α = 2.93, Table 3D). Standard logistic regression (uncorrected for phylogeny) did not identify a significant relationship between thermotolerance and ocean basin (logistic regression coefficient = 0.017, $P = 0.26$, df = 94) or host species richness and ocean basin (0.025, $P = 0.31$, n = 94).

Discussion

Novel phylogenetic tree reveals multiple independent evolutionary origins of thermotolerance, specificity and transmission mode

Assessment of trait evolution on the *Symbiodinium* phylogeny revealed a broad range of homoplasy values,


0.1
C3n
C3-ff
C3
C1h
C7
C81
C12
C7d
C7a
C3u
C119
C94a
C3h
C124
C3-p
C66
C116
C114
C161
C15.21
C15.23
C15.25
C15
C4
C33
C78
C129
C125
C126
C42a
C35a
C79
C110
C26a
C26
C26bb
C17.2
C31
C21
C3-o
C127
C101a
C101
C3n-t
C1m-aa
C120a
C120
C1d-t
C1b-c
C1c
C47
C42
C42a-b
C8
C8b
C8a
C3jj
C130
C128
C1hh
C1gg
C1
C40
C123
C3z
F1
FDia11
F2
B1j
B17
B10
B1
B5
B18
B2
B40
D1-4
D15
D12-13
D13
D1a-c
D8-12
D8
D1-4-6
D5
D4-5
D1-4-10
D1
D1-11
E1
A2
A1
A13
A3
A20
A4
A4a
G catenatu
G simplex
65
100
97
85
100
98
100
47
100
76
99
99
100
100
100
100
100
% VT
Rk
HR
0 16 32 48
0 30 60 90

(Continued)

allowing us to distinguish independent evolution of traits as a result of convergence (high homoplasy), from trait inheritance through a common ancestor (low homoplasy). The number of coral hosts a phylotype associates with (i.e. specificity) is nearly perfectly homoplastic (RI = 0.06) and cannot be inferred from the evolutionary relationships between phylotypes. Thermotolerance has classically been considered a trait associated with clade membership, and although this generalization has been previously discredited (e.g. Tchernov *et al.* 2004; Swain *et al.* 2017*a*), it continues to be cited in the current coral literature. Here we demonstrated again that the connection between phylotype relatedness and similarity in thermotolerance is weak (RI = 0.29), and that clade membership or phylogenetic proximity should not be used to predict unassessed thermotolerance. Both thermotolerance and specificity are homoplastic, suggesting they were independently derived multiple times across the evolutionary history of *Symbiodinium*. Transmission was the least homoplastic trait assessed (RI = 0.61), with obvious concentrations of vertical transmission among phylotypes within the C/F monophyly, which follows a similar pattern of transmission evolution observed among corals with concentrations within specific groups (Baird *et al.*, 2009), but multiple apparently independent origins of the trait (Fig. 2). The unequal homoplasy of these characters opens up the possibility of detecting differential specificity or thermotolerance between vertically and horizontally transmitted symbionts, because the patterns of character state change observed in these traits cannot be entirely explained by their shared evolutionary history. If all character state changes were synapomorphic (trait present in common ancestor and shared exclusively by evolutionary descendants), the patterns of state change might be inseparable from evolutionary history.

Patterns in transmission modes among phylotypes and corals

Observed reciprocal specificity of hosts and symbionts for partners with the same transmission mode, reported here and elsewhere (Barneah *et al.*, 2004; LaJeunesse *et al.*, 2004*a*; van Oppen, 2004; Fabina *et al.*, 2012), suggests that differential selection pressures experienced in each mode (Leigh, 2010) would be reinforced by the linkage of modes between transmitters (hosts) and the transmitted (symbionts). However, differential selection and reinforcement could be undermined by mixed-modes transmission, even if it is rare or transient (Ebert, 2013). The few examples of vertical transmitting coral species that are known to be capable of also acquiring symbionts from the environment may be representative of a larger trend of mixed-modes transmission among corals that vertically transmit their *Symbiodinium*. One possible indication that this may be more common, is that the *Symbiodinium* phylotypes assessed here, that have been observed to associate with both vertically and horizontally transmitting corals, are almost exclusively phylotypes that are most commonly found in horizontally transmitting corals (Supplementary table S1); meaning that these are predominately horizontally transmitted phylotypes which are also found in a few vertically transmitting corals, i.e. vertically transmitting corals sampling phylotypes from the horizontally transmitted phylotype pool. Knowledge of how common these associations are in the field, and discovering if vertical transmitting corals that participate in these associations can also acquire symbionts from the environment, should be a focus of future research.

Potential benefits of mixed-modes transmission for resilience to thermal stress could be further enhanced by bleaching resistance traits that are associated with vertical transmission

Here we demonstrated that vertically transmitted phylotypes are both more thermotolerant and specific, but that specificity does not predict thermotolerance. Phylotype thermotolerance has long been thought to affect the resilience of holobionts to thermal stress (Berkelmans & van Oppen, 2006; Thornhill *et al.*, 2006*b*; LaJeunesse *et al.*, 2009; Stat *et al.*, 2009; DeSalvo *et al.*, 2010; Kemp *et al.*, 2014) and the higher thermotolerance of vertically transmitted phylotypes should be advantageous for enhanced bleaching resistance, although there may be an associated physiological trade-off with resource provisioning that makes thermotolerant *Symbiodinium* sub-optimal under other conditions (Jones & Berkelmans, 2010, 2012). Additionally, increased specificity for symbionts has

Fig. 2. Continued
Maximum likelihood phylogeny of *Symbiodinium* based on concatenated nuclear 5.8S, ITS2, and 28S ribosomal RNA nucleotide sequences in a staggered alignment. Multi-sequence phylotypes represented in the phylogenetic inference by the most divergent ITS2 sequence listed in the phylotype designation. Branch lengths represent the number of nucleotide substitutions per site. Support indicated (for values > 50) by 1000 pseudoreplicate maximum likelihood bootstrap values. Stars represent the ocean basin of origin (black = Indo-Pacific, white = Atlantic, grey = Pan-tropical). Dots represent four categories of the per cent of unique associations with corals that vertically transmit (%VT) their symbionts (proceeding left to right: 100, 99–50, 49–1, 0%). Left bars represent thermotolerance scores (R_k), with increasing values representing increasing thermotolerance. Right bars represent coral host richness.

Table 3. Phylogenetic logistic-regression tables for transmission mode against (A) host species richness and (B) thermotolerance, and for ocean basin against (C) host species richness and (D) thermotolerance.

	Estimate	SE	z value	*p* value	Lower boot CI	Upper boot CI
A. Host species richness (continuous) × vertical transmission (discrete), α = 100.54.						
Intercept	0.5003	0.3606	1.3872	0.1653	0.3091	2.160
Vertical transmission	−0.0338	0.0161	−2.0942	**0.0362**	−0.0731	−0.0196
B. Thermotolerance (continuous) × vertical transmission (discrete), α = 2.95.						
Intercept	−0.0923	0.8459	−0.1091	0.9131	−0.0938	−0.0186
Vertical transmission	0.0270	0.0099	2.7159	**0.0066**	0.0080	0.0537
C. Host species richness (continuous) × ocean basin (discrete), α = 10.45.						
Intercept	−0.5397	0.7268	−0.7426	0.4577	−1.3913	1.1556
Pacific	0.0196	0.0096	2.0459	**0.0407**	0.0015	0.0228
D. Thermotolerance (continuous) × ocean basin (discrete), α = 2.93.						
Intercept	0.8470	1.038	0.8153	0.4148	0.8469	1.4463
Pacific	0.0160	0.0099	1.6111	0.1071	0.0000	0.0580

Values reported include standard error (SE), logistic regression correlation coefficient (z value), significance (P value), and the lower and upper bootstrap confidence intervals. α indicates the magnitude of phylogenetic signal empirically determined from the data. Significant P values are in bold.

recently been shown, in *Acropora, Pocillipora* and *Porites*, to also be associated with increased holobiont resistance to bleaching (Putnam *et al.*, 2012) and may therefore display a similar pattern among the vertically transmitted phylotypes that we have demonstrated to be more specific to hosts. Although increased specificity among vertically transmitted phylotypes has been previously detected (LaJeunesse *et al.*, 2004*a*, *b*; LaJeunesse, 2005; Stat *et al.*, 2008*a*; Fabina *et al.*, 2012), this study is the first to analyse specificity and transmission in the context of evolutionary relationships and correct for non-independence among phylotypes. While vertically transmitted phylotypes are both more specific and more thermotolerant, specificity and thermotolerance are not correlated, and therefore, if specificity is related to holobiont bleaching resistance (Putnam *et al.*, 2012) it cannot be caused by phylotype thermotolerance alone.

Are vertically transmitting corals more resistant to bleaching stress?

Higher thermotolerance and specificity of vertically transmitted phylotypes and their fidelity to vertically transmitting hosts, combined with our emerging understanding of the prevalence of mixed-modes in vertically transmitting corals, supports the hypothesis that vertically transmitting corals may be able to support symbioses that are more robust to climate change than their horizontally transmitting relatives. Beyond the previously hypothesized benefits of vertical transmission (Sachs & Wilcox, 2006; Leigh, 2010; Ebert, 2013), this confluence of potential resistance-building traits among vertically transmitting corals and their vertically transmitted *Symbiodinium* suggests that we should see significant increases in bleaching resistance among these holobionts. How this pattern will shape coral assemblages is unclear, as modelling of assemblage stability suggests that selection for thermal tolerant symbionts, which may be better at providing thermotolerance than nutritional benefit, will result in less stability within coral assemblages (Fabina *et al.*, 2013).

Previous efforts to assess the relationship between transmission-mode of corals and their resistance to thermal stress have had mixed results which trend toward a pattern of increased bleaching resistance among vertically transmitting corals (e.g. Stat *et al.*, 2009; Kenkel & Bay, 2016) and tolerance of a broader range of environmental conditions (Barneah *et al.*, 2004; Thornhill *et al.*, 2006*a*). However, the small numbers of species or species-pairs in these studies may not be reflective of the general pattern. With multiple independent evolutionary origins of both vertical transmission (Baird *et al.*, 2009) and bleaching resistance (Marcelino *et al.*, 2013), there should be ample opportunity to detect phylogenetically corrected associations between transmission and bleaching response within a more comprehensive sampling of the diversity of corals.

Limitations and general considerations

Symbiodinium phylotypes targeted in this analysis represent a subsample of the known diversity and were not randomly chosen, therefore there are sampling biases that should be noted. Phylotypes were selected because of the existence of experimental or observational data on thermotolerance (Swain *et al.*, 2017*a*), and therefore represent phylotypes that are generally associated with corals, or are culturable, and were originally targeted for diverse experimental and biological expediencies. There are many other phylotypes associated with corals for which we have no knowledge of their relative thermotolerance, or are associated with other hosts (such as Foraminifera, Mollusca, Platyhelminthes and Porifera) or free-living (see review in Pochon *et al.*, 2014). These phylotypes are either interspersed among the phylotypes targeted in this

analysis (in a hypothetical complete phylogeny) or grouped in subclades of phylotypes separate from the coral symbionts (Pochon *et al.*, 2014). A robust comprehensive *Symbiodinium* phylogeny containing all known phylotypes and named species would be a valuable tool to further our understanding of the evolution of symbioses and symbiosis-related traits in this group.

Sampling based on availability of thermotolerance data also resulted in oversampling of some clades and ocean basins. There is an overrepresentation of clade C phylotypes (although Clade C is highly diverse), an absence of clades H and I and 76 out of 95 coral-specific phylotypes in the phylogeny originate from, or are predominately represented in, the Indo-Pacific (Table 1, Fig. 2, Table S1). While Indo-Pacific phylotypes included in these analyses are more likely to be vertically transmitted, they are also less specific to hosts and are not differentially thermotolerant, and therefore differences between ocean basins are not driving the patterns observed here. However, once more data are available to increase sample sizes, it may be useful to independently analyse these largely non-overlapping phylotype sets by ocean basin, as they have been on separate evolutionary trajectories for at least 3 million years and may be under distinct selection pressures (LaJeunesse *et al.*, 2003; O'Dea *et al.*, 2016).

Collection of host species identities was focused on coral species–*Symbiodinium* phylotype associations observed under non-bleaching conditions, and could therefore underestimate the diversity of associations that are both possible and have been observed. This approach establishes normal association patterns, but probably under-samples low-density 'background' or transient associations that may only be detectable during bleaching episodes or recovery periods, and also ignores associations that were not simultaneously identified to the coral species and *Symbiodinium* ITS2 phylotype levels. Although there are studies which have followed *Symbiodinium* phylotype associations throughout bleaching and recovery and identified changes in symbiont identities (e.g. Thornhill *et al.*, 2006*b*), or lack thereof (e.g. Thornhill *et al.*, 2006*a*), among a limited number of coral species, it would be useful to compile those data and analyse them using phylogenetic comparative methods as an expansion of the results reported here.

The phylogeny inferred here from 5.8S-ITS2-28S data (Fig. 2) closely mirrors clade-level relationships detailed in the six-gene, three-genomic-compartment analysis of Pochon *et al.* (2014). This result was anticipated because of the use of 28S sequence data. As Pochon *et al.* (2014) demonstrated, a phylogeny inferred from 28S alone is congruent with the full six-gene analysis. The use of 5.8S-ITS2 to explore intra-clade relationships was simply a matter of necessity: 28S is insufficiently variable to distinguish between phylotypes, and 5.8S-ITS2 is the only gene segment available for nearly all phylotypes because it is used to define *Symbiodinium* identity and taxonomy. As other high resolution genes (e.g. psbA minicircle; LaJeunesse & Thornhill, 2011) become increasingly available for *Symbiodinium* it should be possible to further resolve the intra-clade relationships among phylotypes with greater confidence.

Here we used the most divergent co-abundant ITS2 sequence listed by the phylotype designation to represent that phylotype in the analysis. This approach maximizes phylogenetic resolution by maximizing branch lengths and support values between closely related phylotypes, but also makes a phylotype with multiple co-abundant sequences indistinguishable from the single-copy phylotype that is comprised solely of that most divergent ITS2 sequence (e.g. phylotype pairs D13 and D12-13, and C1c and C1b-c have branch lengths of 0 separating them). It could be argued that using the most ancestral co-abundant ITS2 sequence listed in the phylotype designation, or using a consensus ITS2 sequence with dissimilarities represented by uncertainty codes (although that sequence does not naturally exist), are equally defensible approaches. However, because of the similarity between sequences within a phylotype, all of these approaches should result in nearly identical tree topologies and should not significantly alter the results presented here. Use of higher resolution markers (e.g. psbA minicircle; LaJeunesse & Thornhill, 2011) may facilitate phylogenetic reconstruction if they recover single unique DNA sequences per phylotype.

Using the identical data in standard (non-phylogenetically corrected) statistical analyses overestimates the probability of vertical transmission with decreasing specificity (uncorrected = 6%, phylogenetically corrected = 3%) and cannot identify the significant relationship between thermotolerance and transmission, highlighting the importance of phylogenetic comparative methods for identifying and properly assessing the strength and significance of correlations between traits in inter-species analyses. Regressions with small α values (i.e. strong phylogenetic correlations among species) reflect important phylogenetic signal in the regression residuals that, if not properly accounted for, are known to result in misleading conclusions (Revell, 2010). In the data and analyses presented here, only those logistic regressions with small α values produced dramatically different results that would have misled their interpretation. Alternatively, analysing data that lack significant phylogenetic signal in the regression residuals using phylogenetic comparative methods can also result in misleading conclusions (Revell, 2010) and this would have been the case with our data. Standard OLS regressions returned mostly positive, but not significant, relationships between thermotolerance and specificity, and because of the lack of significant phylogenetic signal in the regression residuals, the correct interpretation is 'no

relationship'. However, if phylogenetic correction was inappropriately applied, the PIC analysis would return significant positive relationships from four of six exclusion sets, leading to the incorrect conclusion of a significant relationship between phylotype thermotolerance and specificity. These considerations highlight the importance of identifying the conditions (lack of phylogenetic signal) under which phylogenetic correction is adverse to robust statistical performance.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://10.1080/09670262.2018.1466200

Supplementary text S1. Reference list for Table S1.

Supplementary table S1. Literature records of *Symbiodinium* phylotype/coral species associations.

Supplementary table S2. Phylogenetic logistic-regression tables for transmission mode against host species richness for the (A) complete, (B) >1 host coral species, (C) >2 host coral species, (D) >3 host coral species, (E) >4 host coral species and (F) >5 host coral species datasets. Values reported include standard error (SE), logistic regression correlation coefficient (z value), significance (*P* value), and the lower and upper bootstrap confidence intervals. α indicates the magnitude of phylogenetic signal empirically determined from the data. Significant *P* values are in bold.

Author contributions

L. Marcelino & T. Swain conceived the project and drafted the manuscript; T. Swain collected and compiled the data, and together with M. Westneat conducted the phylogenetic analyses; L. Marcelino and V. Backman provided material support and funding for this project. All authors commented on and contributed to the final manuscript.

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