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Temperature moderates the infectiousness of two conspecific *Symbiodinium* strains isolated from the same host population

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Summary

Symbioses between cnidarians and symbiotic dinoflagellates (*Symbiodinium*) are ecologically important and physiologically diverse. This diversity contributes to the spatial distribution of specific cnidarian-*Symbiodinium* associations. Physiological variability also exists within *Symbiodinium* species, yet we know little regarding its relevance for the establishment of symbiosis under different environmental conditions. Two putatively conspecific *Symbiodinium* strains (both ITS2-type A4) were isolated from the sea anemone *Exaiptasia pallida* and placed into unialgal culture. Thermal tolerance of these cultures was compared following heating from 26°C to 33.5°C over 18 days. Photosystem II function was negatively impacted by heating in one strain while PSII function in the other showed little response to elevated temperature. Additionally, infection of *Symbiodinium* cells into aposymbiotic anemones was assessed for both strains at 26°C and 30.5°C. The heat-sensitive strain had greater infection success at 26°C, while there was no difference in infection between the two strains at the higher temperature. Results from this work suggest that variability in thermal optima or -tolerance within *Symbiodinium* spp. has relevance for early stages of host-*Symbiodinium* interactions. Thus, varying infectiousness among differentially heat-sensitive *Symbiodinium* strains could provide a mechanism for the emergence of novel and potentially resilient cnidarian-*Symbiodinium* associations in a rapidly warming environment.

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Introduction

Animal-microbe symbioses are significant drivers of biodiversity and evolution (Douglas, 2008; Brucker and Bordenstein, 2012). In marine environments, an important symbiosis involves cnidarians and photosynthetic dinoflagellates (genus *Symbiodinium*) whereby the autotrophic contribution of the dinoflagellates supports some of the animal's energetic needs and contributes to the health of the host-symbiont association, or "holobiont" (Davy *et al.*, 2012). Indeed, this symbiosis underpins the productivity of tropical coral reefs (Muscatine and Porter, 1977; Sheppard *et al.*, 2009). Diversity in cnidarian-dinoflagellate symbioses is high and well-documented at the host-level (Veron and Smith, 2000). Considerable diversity also exists among *Symbiodinium* (LaJeunesse, 2001; LaJeunesse, 2002; Santos *et al.*, 2002; Pochon *et al.*, 2006; Stat *et al.*, 2006; Thornhill *et al.*, 2014), with nine distinct clades (designated A-I) currently recognised (Stat *et al.*, 2006; Pochon *et al.*, 2014). Some of these clades include formally described species (LaJeunesse *et al.*, 2014) as well as numerous non-described "types", identified using genetic markers including the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (LaJeunesse, 2001; 2002) and chloroplast 23S ribosomal DNA (Santos *et al.*, 2002; Sampayo *et al.*, 2009). Analysis of microsatellites (Santos and Coffroth, 2003; Santos *et al.*, 2004; Pettay and Lajeunesse, 2007; Andras *et al.*, 2009; Wham *et al.*, 2011) and chloroplast psbA mini-circle non-coding regions (Moore *et al.*, 2003; Barbrook *et al.*, 2006; LaJeunesse and Thornhill, 2011) has further revealed different *Symbiodinium* multi-locus genotypes or individual "strains" within these species or types.

Shallow tropical marine communities harbour diverse assemblages of *Symbiodinium* (LaJeunesse, 2002; Finney *et al.*, 2010; LaJeunesse *et al.*, 2010a; Tonk *et al.*, 2014). Host-*Symbiodinium* specificity is common, as evidenced by observations of symbiotic association patterns (Thornhill *et al.*, 2009; LaJeunesse *et al.*, 2010a; Thornhill *et al.*, 2013; Thomas *et al.*, 2014) as well as by experimental manipulation. For example, working with larvae from the coral *Fungia scutaria*, Weis *et al.* (2001) and Rodriquez-Lanetty *et al.* (2006) noted faster symbiosis establishment and greater symbiont retention with a

homologous *Symbiodinium* (ITS2-type C1f) than with heterologous types. Other studies have reported similar results for *Acropora* spp. corals (Abrego *et al.*, 2009; Bay *et al.*, 2011; Abrego *et al.*, 2012) and *Montastrea (= Orbicella) faveolata* (Voolstra *et al.*, 2009), and comparable specificity has been demonstrated in sea anemones (Schoenberg and Trench, 1980; Davy *et al.*, 1997; Belda-Baillie *et al.*, 2002; Xiang *et al.*, 2013; Hambleton *et al.*, 2014). While these investigations have improved our understanding of cnidarian-dinoflagellate symbiosis (Davy *et al.*, 2012), we lack a full appreciation of the taxonomic levels at which host-symbiont specificity can operate. Where corals associate with a single *Symbiodinium* species/type, individual hosts are often dominated by a single symbiont strain (Baums *et al.*, 2014; Parkinson and Baums, 2014). Some of these highly specific symbioses (e.g., the coral *Acropora palmata*, and gorgonians *Plexaura kuna* and *Gorgonia ventalina*) involve hosts that acquire their *Symbiodinium* from the environment (i.e., horizontal transmission) (Coffroth *et al.*, 2001; Schwarz *et al.*, 2008; Baird *et al.*, 2009; Andras *et al.*, 2011; Baums *et al.*, 2014). This raises the possibility that the host's symbiont-recognition mechanisms might be sensitive to genetic variability within *Symbiodinium* species. Equally, different *Symbiodinium* strains of the same species might differ in their ability to establish symbiosis with a particular host.

In a heterogeneous environment, symbiotic specificity interacts with host- and *Symbiodinium* ecophysiology to influence the spatial distribution of holobionts (LaJeunesse *et al.*, 2004; Sampayo *et al.*, 2007; Finney *et al.*, 2010; LaJeunesse *et al.*, 2010a; Oliver and Palumbi, 2010; Tonk *et al.*, 2013). The emergence of a particular holobiont could therefore be regarded as a product of the compatibility of the local *Symbiodinium* and their capacity for *in hospite* proliferation/persistence. The latter is almost certainly sensitive to environmental conditions (Mieog *et al.*, 2009; Oliver and Palumbi, 2010), but few studies have investigated abiotic influences on the initial establishment of cnidarian-*Symbiodinium* associations. *Symbiodinium* infection in *F. scutaria* (Schnitzler *et al.*, 2012) and certain *Acropora* spp. (Abrego *et al.*, 2012) can be inhibited by increased temperature. Furthermore, in a competitive infection experiment, relative proportions of a thermally tolerant *Symbiodinium* type increased at higher temperatures (Abrego *et al.*, 2012). Yet, the previous investigation compared *Symbiodinium* from different clades, a degree of genetic divergence comparable to family or order-level diversity in non-symbiotic dinoflagellates (Rowan and Powers, 1992). Given recent reports of physiological variability within individual *Symbiodinium* species/types (Howells *et al.*, 2012; Parkinson and Baums, 2014; Suggett *et al.*, 2015; Parkinson *et al.*, 2016), we sought to investigate whether such fine-scale variability could be relevant for the establishment of symbiosis under different environmental conditions. Here, we describe the isolation, culture, and re-infection of two putatively conspecific *Symbiodinium* strains sourced from single population of the symbiotic sea anemone *Exaiptasia* (= *Aiptasia*) *pallida* (Grajales and Rodriguez, 2014). We demonstrate that the two strains have differing abilities to infect host animals and that their infectiousness is influenced by temperature in a manner that correlates with their thermal tolerances. These findings have implications for our understanding of how fine-scale genetic diversity within *Symbiodinium* species may influence the establishment of cnidarian-*Symbiodinium* associations in a thermally variable environment.

Materials and methods

Establishment of Symbiodinium *isolates*

Specimens of *Exaiptasia pallida* were collected from a single mangrove habitat at Key Largo, FL in August 2014 (maximum spatial separation between individuals < 6 m; FWCC permit no. DD-J2T15642566). Animals were transferred to the laboratory, maintained at 26°C in 15-L flow-through tanks supplied with 1 µm-filtered natural seawater, and fed weekly with freshly hatched *Artemia* nauplii. Photosynthetically active radiation (PAR) was provided by cool white LEDs (12 h: 12 h light: dark cycle, 70 µmol photons m^{-2} s^{-1}, Cree XP-G2; LED Supply, Randolph, VT, USA). *Symbiodinium* cultures were established using cells obtained from two anemones that were randomly selected, pooled and homogenised in 0.22-µm filtered seawater (FSW) using a glass tissue grinder. *Symbiodinium* cells were washed four times by repeated centrifugation (700 × g for 20 min) and resuspension in 10 mL FSW. A 20-µL inoculum of algal suspension was then added to 3 mL sterile (autoclaved) f/2 medium (- Si) (Guillard, 1973) in wells ($n = 20$) in a 24-well plate. The plate was wrapped in laboratory film and placed in an incubator (26°C) under 70 µmol photons m^{-2} s^{-1} (provided by cool white fluorescent tubes, light: dark cycle as above). After 4 weeks, single cells ($n = 60$) were transferred – using an inverted microscope (100× magnification) and a glass Pasteur pipette drawn out in a flame to a fine tip – into 300 µL sterile f/2 medium in individual wells in a sterile 96-well plate. After 6 weeks, ten successfully established cell lines ($n > 1000$ cells as determined with visual counts [100× magnification]) were inoculated into 3 mL sterile f/2 medium in wells in a sterile 24-well plate. After a further 8 weeks, 2-mL aliquots of two of these cultures (designated KLAp1 and KLAp2, respectively) were transferred into 300 mL sterile f/2 medium in autoclaved conical flasks. Cultures were bubbled with sterile (0.22 µm-filtered) activated carbon-treated air and periodically transferred to fresh f/2 medium.

Molecular characterisation of Symbiodinium

DNA was extracted from symbiotic anemones and *Symbiodinium* cultures using a Wizard® Genomic DNA Extraction Kit (Promega Life Sciences, Madison, WI, USA). Initially, a partial sequence of ITS2 nuclear ribosomal DNA was amplified using the PCR primers "ITSintfor2" and "ITSrev" (LaJeunesse and Trench, 2000), and a region of chloroplast 23S rDNA (cp23S) was amplified using the primers "23S1" and "23S2" (Zhang

et al., 2000). Products were purified (ExoSAP-IT, Affymetrix, Santa Clara, CA, USA) and sequenced in both directions (Genewiz, South Plainfield, NJ, USA). Sequences were aligned and consensus sequences extracted using Geneious® software version 8.04 (Geneious Pairwise Alignment, 65% similarity cost matrix, gap opening penalty = 12, gap extension penalty = 3; Biomatters Ltd., Auckland, New Zealand). ITS2-types of cultures KLAp1, KLAp2 and the natural *in hospite Symbiodinium* were determined by comparing the consensus ITS2 sequence to the NCBI database (Geneious® megaBLAST, linear gap opening/extension cost). Chloroplast 23S sequences were compared to the NCBI database (Geneious® megaBLAST, linear gap opening/extension cost) as well as a *Symbiodinium* Clade A cp23S database provided by Dr. Todd C. LaJeunesse.

Further molecular analysis involved partially sequencing the non-coding region of the chloroplast psbA mini-circle ($psbA^{ncr}$) following PCR amplification using the primers "psbAFor_1" and "psbARev_1" (LaJeunesse and Thornhill, 2011). Products were visualised using agarose gel electrophoresis (Supporting Information Fig. S1). The 2500-bp amplicon was excised and the DNA extracted/purified using Qiagen's Qiaquick® Gel Extraction Kit (Qiagen, Hilden, Germany) and ligated into a pCR2.1 plasmid vector using a TOPO TA Cloning® kit (Life Technologies, see above). Plasmids were transformed using One Shot® Mach1™ T1 phage-resistant chemically competent *Escherichia coli* (ThermoFisher [Life Technologies], Waltham, MA, USA) and cells were plated onto Luria-Bertani (LB)-agar containing 50 μg/mL kanamycin. After growth overnight at 37°C, colonies indicating successful uptake of the $psbA^{ncr}$ insert (blue/white screening, $n \geq 10$ per sample) were inoculated into 4 mL LB medium and grown on a shaking platform (200 rpm) overnight at 37°C. Plasmid DNA was extracted using the PureLink® Plasmid MiniPrep kit (ThermoFisher [Invitrogen], Waltham, MA, USA), and the presence of the insert in the purified plasmid confirmed by a) digestion with EcoR1 restriction enzyme (Life Technologies, see above), and b) PCR amplification of $psbA^{ncr}$ DNA using the purified plasmid as a template.

Cloned $psbA^{ncr}$ fragments were sequenced ($n \geq 5$ clones for each *Symbiodinium* culture, $n = 3$ clones for each re-infected anemone group [see below]) using M13R "reverse" and M13F(−21) "forward" primers. Using the reverse sequences as templates, an additional internal sequencing primer ("psbARev_1Int_2": 5′-CAG GCC GAC TAT TTC CTA-3′) was designed using an online tool (PrimerQuest™, Integrated DNA Technologies, IA, USA). M13R- and psbARev_1Int_2-derived sequences were aligned and a consensus reverse sequence extracted for each clone individually. To gauge within-culture variability in the $psbA^{ncr}$ sequence and to generate consensus sequences for each *Symbiodinium* culture, forward and reverse sequences from individual clones were respectively aligned and compared using a ClustalW Multiple Alignment (Geneious® [see above]; cost matrix = IUB, gap open cost = 20, gap extension cost = 6.66). Consensus sequences were compared between cultures using a Geneious Pairwise Alignment (see above).

In vitro *growth rates and photo-physiology of* Symbiodinium *cultures prior to and following heating*

Symbiodinium cultures ($n = 6$ flasks each for KLAp1 and KLAp2) were established in 350 mL f/2 medium (-Si) and maintained in two incubators (three flasks of each culture per incubator) at 26°C under the irradiance conditions described above. Cultures were diluted weekly with fresh f/2 medium for at least 6 weeks in order to maintain logarithmic growth and were bubbled with sterile, activated carbon-filtered air. Cell densities were quantified every 72 hours using an Improved Neubauer haemocytometer and fluorescence microscopy of algal chlorophyll *a* (chl *a*) fluorescence (EVOS® system, Life Technologies, see above excitation: 628 ± 20 nm, emission: 692 ± 20 nm). Field of view was determined using EVOS® operating software (4× objective), and cells were counted using ImageJ (NIH, Bethesda, MD, USA). At least 6 independent images were analyzed for each sample.

Cultures were diluted with fresh f/2 medium to a density of 5 × 10^4 cells mL^{-1} 48 hours prior to heating. Following Day-0 sampling, the temperature of one incubator was increased by 0.7°C d^{-1} to 32°C, maintained at 32°C for 6 days, and then heated by 0.5°C d^{-1} to 33.5°C. These heating rates are slightly less than the maximal rates experienced by *in hospite Symbiodinium* during a thermal stress event in the field (Middlebrook and Anthony, 2010). Peak temperatures were selected in order to more precisely determine maximum thermal thresholds for the two strains. Irradiance remained unchanged and flasks were rotated within incubators every 48 h. Cell densities were determined every 72 hours, and cultures were diluted with fresh, sterile f/2 medium at appropriate intervals in order to maintain logarithmic growth. Concurrent with cell density analysis, 3 mL of each culture were removed and dark-adapted for 20 min. Photosystem II (PSII) function was quantified using single-turnover fast-repetition rate fluorometry (FastACT, Chelsea Instruments, Surrey, UK). PSII fluorescence was induced using 100 flashlets (each 1-μs duration) at 1-μs intervals and PSII relaxation was determined at 49-μs intervals over the following 2.5 ms. This excitation/relaxation cycle was repeated 25 times for each measurement of maximum- or effective quantum yield (F_v/F_m and F_q'/F_m', respectively), functional PSII cross-section (σ_{PSII}), and PSII relaxation kinetics (τ_{PSII}). A rapid-light-curve was then initiated, with increasing PAR from 0 to 1629 μmol photons m^{-2} s^{-1} over 16 steps. Each PAR level was maintained for 20 seconds before F_q'/F_m' was recorded. Electron transport rate (ETR_{PSII}) was estimated as PAR × σ_{PSII} × F_q'/F_m' × 21.683 (Suggett *et al.*, 2006) with a 10^{-18}-factor conversion applied to account for differences in PAR and σ_{PSII} units. Maximum ETR (ETR^{max}_{PSII}) was estimated using the parabolic equations of Platt *et al.* (1980) in the R package "FRRF" (Silsbe and Kromkamp, 2012).

Effect of temperature on symbiosis establishment

Aposymbiotic anemones were obtained using a menthol treatment (Matthews *et al.*, 2015), but with dark-incubation used between recovery periods. Aposymbiosis was confirmed by the absence of *Symbiodinium* chl *a* fluorescence when anemones were visualized with a fluorescence microscope (EVOS® system, see above). Aposymbiotic anemones were transferred to a 30-L tank containing UV-sterilized artificial seawater (salinity 34 ppt; Instant Ocean®, Blacksburg, VA, USA), maintained at 26°C in the dark, and fed weekly with freshly hatched *Artemia* nauplii.

Prior to re-infection, 45 small aposymbiotic anemones (2–3 mm oral disk) were placed into 10 mL sterile FSW in individual wells on sterile 6-well plates and transferred to a dark 26°C incubator. Water changes were conducted daily for three weeks and anemones were fed weekly with freshly hatched *Artemia* nauplii, and then starved for 1 week prior to exposure to *Symbiodinium* cultures. Anemones were then illuminated (PAR as for symbiotic populations, see above) and, after 48 hours, 24 animals were randomly exposed to a 1000-cells mL^{-1} suspension of either KLAp1 ($n=12$) or KLAp2 ($n=12$) *Symbiodinium*. The remaining animals were not exposed to any *Symbiodinium*. To facilitate continuous host-infection, *Symbiodinium* suspensions in FSW were replaced daily over the following 7 days. From Day 7 until the end of the experiment (Day 21), anemones received only FSW, thus enabling estimation of *in hospite* algal proliferation. This re-infection procedure was repeated using aposymbiotic anemones that had been acclimated for 3 weeks to a temperature of 30.5°C, with KLAp1- and KLAp2 cultures heated from 26°C to 30.5°C (0.5°C d^{-1}) over the 9 days preceding exposure to anemones.

Establishment of symbiosis was defined as the appearance of *Symbiodinium* within anemone tissues. This was quantified non-destructively at $t=0$, 2, 4, 7, 14, and 21 days after initial exposure to *Symbiodinium*, and by sacrificing anemones on Day 21 and counting the number of *Symbiodinium* cells within animal homogenates. Each anemone was removed, washed twice with FSW and then placed into a glass-bottom petri-dish (MatTek, Ashland, MA, USA) with a few drops of FSW. A second cover slip was placed over the anemone in order to reduce movement. The ~1-mm gap between cover slips prevented excessive squashing so anemones were not harmed by the imaging process. Animals were visualised with a fluorescence microscope (EVOS® system, 4x objective magnification) with the focal plane adjusted under phase-contrast settings to ensure that tentacle gastrodermis was in focus. *Symbiodinium* chl *a* fluorescence was induced and detected as described above, and a quantitative monochrome image was recorded (Exposure: 150 ms, Gain: 6.2, Lamp Intensity: 19). This process was repeated for 4–6 tentacles of each anemone. Objective magnification and working distance were kept constant between samples in order to standardise the depth of field. Regions-of-interest (ROI) were consistently defined using ImageJ software (see above) as the internal area of an in-focus tentacle. *Symbiodinium* cell density was quantified as the mean fluorescence intensity (MFI) of each ROI. After 21 days, and due to the small size of the animals, anemones were randomly pooled within each culture- and temperature group ($n \geq 3$) and transferred to individual 2-mL screw-cap vials. They were then homogenised in 300 μL 25 mM Tris, pH 7.8, 1 mM EDTA, 10% [w/v] glycerol using a single 5 mm-diameter stainless steel bead shaken for 30 seconds at 5 m s^{-1} (Fast-Prep®, MP Bio, Santa Ana, CA, USA). One-hundred microliters were removed for *Symbiodinium* quantification (fixed with 5 μL 8% [w/v] glutaraldehyde and stored at 4°C) and the remainder was centrifuged at 16 000 x g for 20 min and frozen at −20°C. Protein content of the supernatant was calculated using a Bradford assay. *Symbiodinium* number was determined using a haemocytometer (see above) and normalised to anemone protein content. *Symbiodinium* DNA was extracted from the frozen pellets ($n=3$ per strain) using the methods described above, and we amplified, cloned and partially sequenced the $psbA^{ncr}$ from both populations ($n=2$ clones per anemone). In all cases, re-infected anemones contained the appropriate *Symbiodinium* strain (see Supporting Information).

Statistical analysis

All variables were tested for normality and homoscedasticity prior to parametric analysis and were log_{10}-transformed where necessary. Thermal responses of F_v/F_m, ρ_{PSII}, τ_{PSII}, and ETR_{PSII}^{max} were compared using a linear mixed-effects model [R package "nlme" (Pinheiro *et al.*, 2016)]. "Culture flask" was defined as the subject, "Temperature" and "Strain" as between-subjects factors and "Day" as a within-subjects factor. Full-factorial models were constructed by sequentially adding main-effect and interaction terms, and Akaike Information Criteria were compared to select the best model. F-statistics were obtained using the anova() function, and pairwise *post hoc* analysis was conducted using the glht() function [R package "multcomp" (Hothorn *et al.*, 2016)] and Tukey's correction for multiple comparisons. Pre-treatment (Day 0) chl *a* contents, respiration and net photosynthetic rates, and estimates of cell size (see Supporting Information) were compared between cultures using Student's *t*-tests.

Anemone tissue chl *a* data were strongly heteroscedastic (Levene's Test, $P<1 \times 10^{-16}$), and were analyzed using non-parametric tests within the R package "nparLD," designed for longitudinal data from multi-factorial experiments (Noguchi *et al.*, 2012). Mean tentacle MFI values for uninfected controls (at each time-point and for each temperature group) were subtracted from those of the re-infected anemones. Missing values (due to anemone mortality, generally <20% of each group) were replaced with the mean of the remaining replicates. Effects of "Strain," "Temperature," "Day," and full-factorial interactions were tested for using the f2.ld.f1() function. Pair-wise *post hoc* comparisons were made between groups of anemones exposed to different *Symbiodinium* cultures at each level of "Day x Temperature" using anon-parametric permutation analysis [ezPerm() function, $n=1000$ replications, R package "ez" (Lawrence, 2015)] and a Bonferroni correction. Final-day *Symbiodinium* densities (per mg host protein) were compared using two-way ANOVA ("Temperature" and "Strain" as fixed factors) and Tukey's *post hoc* test.

Results and discussion

Isolation of two putatively conspecific Symbiodinium *strains with different thermal sensitivities*

Partial ITS2 and cp23S sequences of KLAp1 and KLAp2 *Symbiodinium* were identical to those of the *in hospite* symbiont population. MegaBLAST alignment with the NCBI NR database identified the ITS2 type as A4 (99.8% identity with "Symbiodinium sp. A4 isolate GK5" [Acc. No. KR002395], E = 5.26 x 10^{-141}). This finding is consistent with previous analyses of *E. pallida*, which hosts *S. minutum* (ITS2, B1) at most locations, but also associates with *Symbiodinium* Clades A and C in the Florida Keys (Thornhill *et al.*, 2013; Grajales *et al.*, 2015). However, we have a

Nucleotide alignment of 2 sequences:
KLAp1_psbAncr-rev (consensus), KLAp2_psbAncr-rev (consensus)

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Identities = 791/1034 (76%),
Positives = 791/1034 (76%), Gaps = 94/1034 (9%)

KLAp1_psbAncr-rev (consensus) (reversed)  1016 AAAGCTGGGCCCGAAGGGCC---CA-GCT--TTTTTGGGTAGAGCGGCCCCGAAGGGCCG  963
                                               A A  TG  C   AA G CC   CA  CT  TTTTT G   G  C  CCC  AA G   G
KLAp2_psbAncr-rev (consensus) (reversed)   958 ATATTTGTACTAAAATGACCTGTCACACTCGTTTTTTGCACGTTCTCCCCGAAAAGTAAG  899

KLAp1_psbAncr-rev (consensus) (reversed)   962 CGATACTTTTATTTTAAAGAGGGCCCGAAGGGCCCGATCGCATAAACTGGCCCTTCGGGC  903
                                               C         ATTTTA     G  CCG  G GCC G  C  A A  CT G   TTCGGG
KLAp2_psbAncr-rev (consensus) (reversed)   898 C---------ATTTTA-----GCACCG--GTGCCGGTGCTAAAATGCTTGTTTTTCGGG-  856

KLAp1_psbAncr-rev (consensus) (reversed)   902 CAGTTTATGCTCTATAAAACCGGCCCGAAGGGCCGGTAAGTAGCAATAAATATCAGTTCA  843
                                                AG    TGC   A AAAAC     CG AG  C GGT   T  CAA  AA A  AG  CA
KLAp2_psbAncr-rev (consensus) (reversed)   855 GAGAACGTGC---AAAAAACT----CGTAGCACGGGTGTTTTTCAACTAAAA--AGGCCA  805

KLAp1_psbAncr-rev (consensus) (reversed)   842 TAACT---GGCCGAAGGCCAGTTAT-GAAACTGATATTTATTGCTCTACGCGCGCGCGAG  787
                                                AA T   GGCCGAAGGCC G  AT G A CT  T TTT TT  T T  G        AG
KLAp2_psbAncr-rev (consensus) (reversed)   804 CAATTTCAGGCCGAAGGCCTGAAATTGGAGCTTGTGTTT-TTATTTTGAGT-------AG  753

KLAp1_psbAncr-rev (consensus) (reversed)   786 GCCCGAAGGGCCGGCCAGCTGAAAATCAGGCCTTCCTGCTGATTTAAGCTAAAAACACCC  727
                                               GCCCGAAGGGCC    A C  A  ATC  GC T    GC GA   A GC    A CA  C
KLAp2_psbAncr-rev (consensus) (reversed)   752 GCCCGAAGGGCCTCATATCGAAGCATC--GCATGATGGCCGA---AGGC---CATCATGC  701

KLAp1_psbAncr-rev (consensus) (reversed)   726 TCGTGAAGGCTTCGCCTTCACGAGGGTGTTTTTTAGCTTGAATCA---GCAGGAAAATCC  670
                                               TC T    GCT  GCC     GA GG        AGCT GAA  A    CA GA AA  C
KLAp2_psbAncr-rev (consensus) (reversed)   700 TCTTACTCGCTG-GCC-----GAAGGCC------AGCTCGAAATAAATCCAAGAGAAGGC  653

KLAp1_psbAncr-rev (consensus) (reversed)   669 GGAAGTATTTTCCCCTGGCTGGCCTTCGGCCAGCCAGGGGAATTGAAGCTCGTGCTACAC  610
                                                GA  T     CC  T  CTGGCC   GGCCAG        ATTGA GCTCGTG  A  C
KLAp2_psbAncr-rev (consensus) (reversed)   652 CGA--TTCGAGCCAATATCTGGCCGAAGGCCAGAT------ATTGACGCTCGTGAAAT-C  602

KLAp1_psbAncr-rev (consensus) (reversed)   609 GCTCGTGAAGGCCATTTTAGTCCAAATATCAGGCC-TTCGGCCTGAT-ATTT-GT--ACT  555
                                               G   GTGAA  C A T       AAATA   GGCC TTCGG C  AT ATTT GT  ACT
KLAp2_psbAncr-rev (consensus) (reversed)   601 G---GTGAAATCAACT-------AAATAATGGGCCCTTCGGGCCCATTATTTAGTTGACT  552
                                                     Variable region
KLAp1_psbAncr-rev (consensus) (reversed)   554 AAAATGACCTGTCACACTCGTTTTTTGCACGTTCTCCCCGAAAAGTAAGCATTTTAGCAC  495
                                                   T ACCTGTC CA   GTTTTTTGCACGTTCTCCCCGAAAAGTAAGCATTTTAGCAC
KLAp2_psbAncr-rev (consensus) (reversed)   551 ---TTCACCTGTCTCATAAGTTTTTTGCACGTTCTCCCCGAAAAGTAAGCATTTTAGCAC  495

KLAp1_psbAncr-rev (consensus) (reversed)   494 CGGTGCCGGTGCTAAAATGCTTGTTTTTCGGGGAGAACGTGCAAAAAACTCGTGAGACAG  435
                                               CGGTGCCGGTGCTAAAATGCTTGTTTTTCGGGGAGAACGTGCAAAAAACTCGTGAGACAG
KLAp2_psbAncr-rev (consensus) (reversed)   494 CGGTGCCGGTGCTAAAATGCTTGTTTTTCGGGGAGAACGTGCAAAAAACTCGTGAGACAG  435

KLAp1_psbAncr-rev (consensus) (reversed)   434 GTTAATTTGTACTAAAATGGTGTTATTTTTAGGAAATAGTCGGCCTGCGGCCTCCAAATT  375
                                               GTTAATTTGTACTAAAATGGTGTTATTTTTAGGAAATAGTCGGCCTGCGGCCTCCAAATT
KLAp2_psbAncr-rev (consensus) (reversed)   434 GTTAATTTGTACTAAAATGGTGTTATTTTTAGGAAATAGTCGGCCTGCGGCCTCCAAATT  375

KLAp1_psbAncr-rev (consensus) (reversed)   374 CGTGGCCTTCGGCCACTCATATCCAAGGTTCGCAGCCCGAAGGCTGCTCTTACTCGCCCT  315
                                               CGTGGCCTTCGGCCACTCATATCCAAGGTTCGCAGCCCGAAGGCTGCTCTTACTCGCCCT
KLAp2_psbAncr-rev (consensus) (reversed)   374 CGTGGCCTTCGGCCACTCATATCCAAGGTTCGCAGCCCGAAGGCTGCTCTTACTCGCCCT  315

KLAp1_psbAncr-rev (consensus) (reversed)   314 TCGGGCTCGAAATAAATCCAAAGAGTTTATGCTAGATCTAATGGAAAGTTGTGTGCATTT  255
                                               TCGGGCTCGAAATAAATCCAAAGAGTTTATGCTAGATCTAATGGAAAGTTGTGTGCATTT
KLAp2_psbAncr-rev (consensus) (reversed)   314 TCGGGCTCGAAATAAATCCAAAGAGTTTATGCTAGATCTAATGGAAAGTTGTGTGCATTT  255

KLAp1_psbAncr-rev (consensus) (reversed)   254 CTTTCATGCATGACTTCCATTCCTAGGTCAGCACGATTAAGAATATCTGCCCAACTTAAA  195
                                               CTTTCATGCATGACTTCCATTCCTAGGTCAGCACGATTAAGAATATCTGCCCAACTTAAA
KLAp2_psbAncr-rev (consensus) (reversed)   254 CTTTCATGCATGACTTCCATTCCTAGGTCAGCACGATTAAGAATATCTGCCCAACTTAAA  195

KLAp1_psbAncr-rev (consensus) (reversed)   194 ATAAGATGGCCACTGGAATCTAGGATGGATTGGTTGAAGTTTAAACCATTTAAGTTGAAA  135
                                               ATAAGATGGCCACTGGAATCTAGGATGGATTGGTTGAAGTTTAAACCATTTAAGTTGAAA
KLAp2_psbAncr-rev (consensus) (reversed)   194 ATAAGATGGCCACTGGAATCTAGGATGGATTGGTTGAAGTTTAAACCATTTAAGTTGAAA  135

KLAp1_psbAncr-rev (consensus) (reversed)   134 GCCATTGTACTTACTCCAAGTGCTGTAAACCAAATCCCAATAACTGGCCAAGCTGCTAAG   75
                                               GCCATTGTACTTACTCCAAGTGCTGTAAACCAAATCCCAATAACTGGCCAAGCTGCTAAG
KLAp2_psbAncr-rev (consensus) (reversed)   134 GCCATTGTACTTACTCCAAGTGCTGTAAACCAAATCCCAATAACTGGCCAAGCTGCTAAG   75

KLAp1_psbAncr-rev (consensus) (reversed)    74 AAGAAGTGTAAACTACGAGAGTTATTAAAGGAAGCATATTGAAAAATTAGTCTACCAAAA   15
                                               AAGAAGTGTAAACTACGAGAGTTATTAAAGGAAGCATATTGAAAAATTAGTCTACCAAAA
KLAp2_psbAncr-rev (consensus) (reversed)    74 AAGAAGTGTAAACTACGAGAGTTATTAAAGGAAGCATATTGAAAAATTAGTCTACCAAAA   15
                                                GenBank Acc #AB456560 (PSII D1protein)
KLAp1_psbAncr-rev (consensus) (reversed)    14 TAACCATGAGCTGC    1
                                               TAACCATGAGCTGC
KLAp2_psbAncr-rev (consensus) (reversed)    14 TAACCATGAGCTGC    1
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Fig. 1. Pair-wise alignment of consensus sequences generated from psbA$_{ncr}$-encoding plasmids after sequencing using M13R and psbA_Rev1_Int_2 primers (see text for details).
Location of the insert in M13R-derived sequences was identified using the TOPO-TA cloning site, and M13R and psbA_Rev1_Int2 sequences were aligned/concatenated for each of at least 5 clones per *Symbiodinium* culture. The best-match coding sequence in the NCBI database (megaBLAST) is indicated in addition to the variable non-coding region.

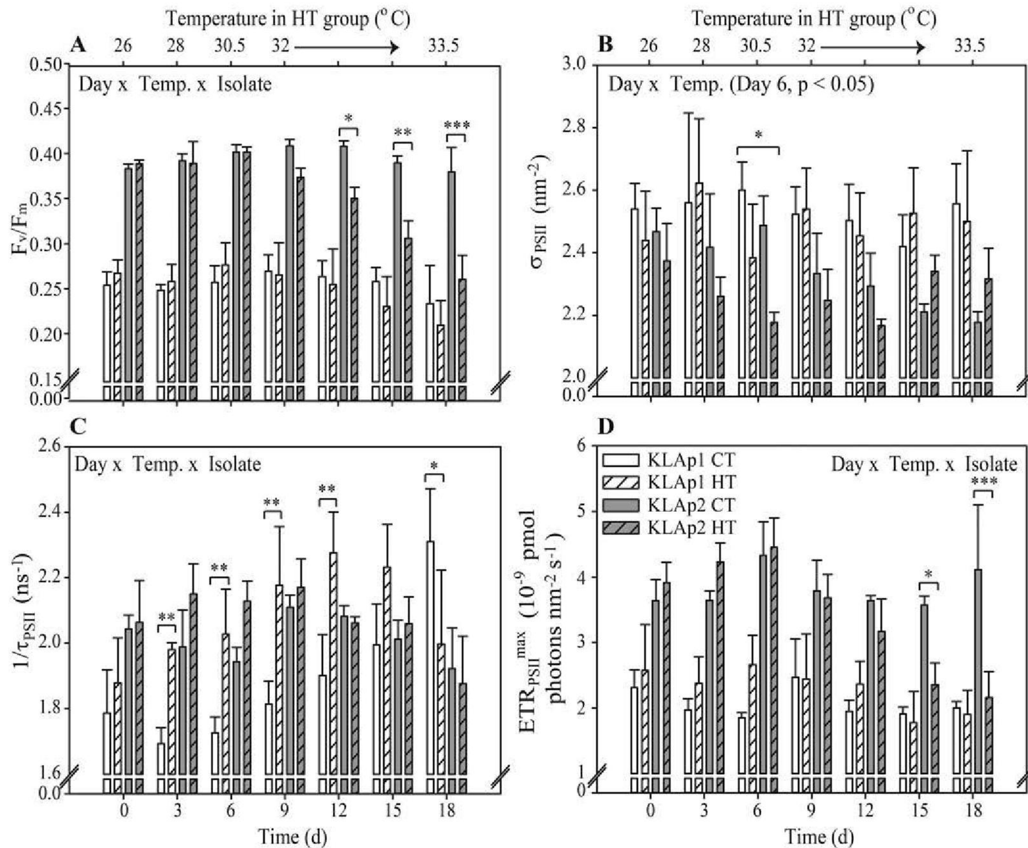

Fig. 2. Responses of (panel A) maximum quantum yield of PSII (F_v/F_m), (B) PSII effective cross-section (σ_{PSII}), (C) PSII turnover kinetics ($1/\tau_{PSII}$), and (D) maximum electron transport rate (ETR^{max}) of *Symbiodinium* cultures KLAp1 and KLAp2 heated from 26°C to 33.5°C (or maintained at 26°C) over a period of 18 days. The highest-level significant interaction term (Linear Mixed-Effects Model, maximum likelihood method) is indicated for each panel. Where a three-way interaction (Day x Temperature x Strain) is reported, asterisks denote significance between treatments (heated [HT] or control [CT]) within each culture. Where a two-way interaction is reported, asterisks denote the effect of "Temperature" at a certain time-point regardless of strain ($n = 3$ flasks per culture per treatment; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

poor understanding of the genetic diversity within these ITS2-type A4 *Symbiodinium*. The cp23S consensus sequence described here aligned with a "FLAp#4" ("Clade A4") isolate described by Santos *et al.* (2002) and, interestingly, differed from that of the ITS2-type A4 symbionts of the Caribbean coral *Porites astreoides* (Supporting Information Fig. S2). While further work is needed to fully characterise the possible genetic differentiation between these two A4 strains, since *P. astreoides* reproduces by brooding, maternal (vertical) transmission of *Symbiodinium* in this coral species could promote significant genetic differentiation within this ITS2 type (Thornhill *et al.*, 2006).

After noting that KLAp1 and KLAp2 cultures adhered to culture vessels differently (Supporting Information Fig. S3) we characterised them further using the variable chloroplast $psbA^{ncr}$ locus (LaJeunesse and Thornhill, 2011). Analysis of this locus has been a useful tool for discerning closely related *Symbiodinium* types within Clades C and D (Moore *et al.*, 2003; LaJeunesse and Thornhill, 2011; Hume *et al.*, 2016), but to our knowledge it has yet to be employed to investigate fine-scale diversity in Clade A *Symbiodinium*. Here, within-culture similarity among $psbA^{ncr}$ clones was high: > 99.5% for "forward" sequences (KLAp1: 800 bp, $n = 5$ clones; KLAp2: 700 bp, $n = 6$ clones). "Reverse" sequences also displayed high within-culture similarity: > 98.5% for KLAp1 (~1000 bp, $n = 5$ clones) and> 99% for KLAp2 (~1000 bp, $n = 6$ clones). Variability in forward consensus sequences between KLAp1 and KLAp2 was < 0.5%, and alignment of reverse consensus sequences revealed comparable similarity over the initial 530 bp. However, there was significant divergence over the following 500 bp such that overall reverse sequence similarity between KLAp1 and KLAp2 was 76% (Fig. 1). Henceforth, we refer to KLAp1 and KLAp2 as distinct strains of the *Symbiodinium* A4 found naturally within *E. pallida* at Key Largo, FL. Partial ITS2, cp23S, and psbA sequences are provided in the accompanying Supporting Information and all cp23S and $psbA^{ncr}$ sequences

Table 1. Statistical analysis (linear mixed effects model) of the photo-physiology and population growth rates of two *Symbiodinium* A4 strains from *Exaiptasia pallida* grown at 26°C and experimentally heated to 33.5°C over 18 days.

Variable	Effect	Statistic$_{(df)}$	*P*-value
F_v/F_m (dimensionless)	Day	$22.046_{(6,48)}$	< 0.0001
	Strain	$218.871_{(1,8)}$	< 0.0001
	Temp.	$7.656_{(1,8)}$	0.024
	Day × Strain	$2.732_{(6,48)}$	0.044
	Day × Temp.	$11.697_{(6,48)}$	< 0.0001
	Strain × Temp.	$5.613_{(1,8)}$	0.045
	Day × Strain × Temp.	$2.565_{(6,48)}$	0.031
σ_{PSII} (nm^{-2})	Day	$1.380_{(6,54)}$	0.239
	Strain	$34.577_{(1,9)}$	0.0002
	Temp.	$2.241_{(1,9)}$	0.169
	Day × Strain	$1.100_{(6,54)}$	0.374
	Day × Temp.	$2.988_{(6,54)}$	0.014
τ_{PSII} (ns)	Day	$5.172_{(6,48)}$	0.0004
	Strain	$4.026_{(1,8)}$	0.080
	Temp.	$11.429_{(1,8)}$	0.010
	Day × Strain	$10.437_{(6,48)}$	< 0.0001
	Day × Temp.	$6.973_{(6,48)}$	< 0.0001
	Strain × Temp.	$4.006_{(1,8)}$	0.08
	Day × Strain × Temp.	$2.944_{(6,48)}$	0.016
ETR^{max}_{PSII} (10^{-9} pmol photons nm^{-2} s^{-1})	Day	$10.309_{(6,48)}$	< 0.0001
	Strain	$97.374_{(1,8)}$	< 0.0001
	Temp.	$0.295_{(1,8)}$	0.602
	Day × Strain	$3.200_{(6,48)}$	0.010
	Day × Temp.	$7.631_{(6,48)}$	< 0.0001
	Strain × Temp.	$4.687_{(1,8)}$	0.062
	Day × Strain × Temp.	$2.994_{(6,48)}$	0.015
Growth rate (cells day^{-1})	Day	$14.410_{(6,54)}$	< 0.0001
	Strain	$86.664_{(1,8)}$	< 0.0001
	Temp.	$6.660_{(1,8)}$	0.033
	Day × Strain	$6.166_{(6,54)}$	0.0001
	Day × Temp.	$12.885_{(6,54)}$	< 0.0001
	Strain × Temp.	$19.254_{(1,8)}$	0.002

referenced in this article are publicly available (GenBank accession numbers KX812406-39 and KX364155-6, respectively).

Several physiological differences were noted between the two cultures. Growth rates of KLAp1 at 26°C were lower than those of KLAp2 (0.043 ± 0.009 day^{-1} [SD] and 0.136 ± 0.016 day^{-1} [SD], respectively), a difference comparable to that observed between two *Symbiodinium psygmophilum* (ITS2, B2) cultures (Parkinson and Baums, 2014). Likewise, maximum quantum yield of PSII (F_v/F_m) in KLAp1 was significantly lower at ambient temperature (26°C) than that of KLAp2 (Fig. 2a). While this is the first time such photo-physiological differences have been noted within *Symbiodinium* A4, Suggett and co-workers (2015) described significant between-culture variability in *Symbiodinium tridacnidorum* (ITS2, A3; Lee *et al*., 2015) and *Symbiodinium minutum* (ITS2, B1). The combination of differing growth rates and PSII quantum yields, as well as the differences in cellular chl *a* contents, apparent cell size, and respiratory/photosynthetic O_2 fluxes (Supporting Information Fig. S3), suggests a level of intraspecific physiological divergence similar to that noted in other marine microalgae (Marshal and Newman, 2002; Martinez *et al*., 2010).

PSII function in the two strains was differentially sensitive to elevated temperature (Table 1, Fig. 2). Specifically, F_v/F_m of KLAp2 declined following heating to 32°C, while KLAp1 was unaffected by heating (Fig. 2a). Changes in the effective absorption cross-section of PSII (σ_{PSII}) were less clear; a slight negative effect of temperature was noted, but this did not differ between the two strains (Table 1; Fig. 2b). Thermal sensitivity of reaction centre re-oxidation rates (τ_{PSII}, plotted in as $1/\tau_{PSII}$ in Fig. 2c) was different for the two cultures (Table 1). Increased temperature had no effect on $1/\tau_{PSII}$ in KLAp2, but resulted in heightened $1/\tau_{PSII}$ in KLAp1. Further heating to 33.5°C caused an apparent reduction in $1/\tau_{PSII}$ in KLAp1, but this was partly due to changes in the control group. Effects of temperature on the two strains' maximum electron transport rates (ETR^{max}_{PSII}) mirrored their respective thermal sensitivities (Table 1, Fig. 2d); heating caused significant declines in ETR^{max}_{PSII} of KLAp2, but no detectable impact on KLAp1. In summary,

Table 2. Results of non-parametric analysis (f2.ld.f1() model in R package "nparLD", see text) of changes in *Exaiptasia pallida* tentacle chlorophyll *a* fluorescence following exposure to two different *Symbiodinium* A4 strains at two temperatures (26°C and 30.5°C).

Effect	Wald-type statistic (df)	$P_{(Wald)}$	ANOVA-type statistic (df)	$P_{(ANOVA)}$
Day	1117.695 (5)	< 0.0001	224.26 (3.749)	< 0.0001
Strain	6.694 (1)	0.010	6.694 (1)	0.010
Temp.	0.963 (1)	0.33	0.963 (1)	0.33
Day x Strain	0.810 (5)	0.976	0.184 (3.749)	0.939
Day x Temp.	24.865 (5)	0.0001	5.032 (3.749)	0.0007
Strain x Temp.	14.663 (1)	0.0001	14.663 (1)	0.0001
Day x Strain x Temp.	25.051 (5)	0.0001	3.174 (3.749)	0.015

light energy capture and transport through PSII in KLAp1, while less efficient than that of KLAp2, was fairly resilient to heating, while photochemistry in KLAp2 was more negatively impacted by increased temperatures. To our knowledge, the only other empirical demonstration of intraspecific variability in *Symbiodinium* thermal tolerance *in vitro* was between two *Symbiodinium goreauii* (ITS2, C1) cultures established using cells from different latitudes (Howells *et al*., 2012). Pettay and LaJeunesse (2013) also detected an environmental signal in the population structure of *Symbiodinium glynni* (ITS2 D1) in the tropical Eastern Pacific region. Our observations differ from these previous studies in that *Symbiodinium* KLAp1 and KLAp2 originate from a single host population/habitat. Since opportunities for niche-specialisation in these two strains are comparatively limited, we are reluctant to speculate as to the specific drivers of their physiological divergence. It might stem from the variability and spatial complexity of their natural habitat among mangrove roots at the site of collection, but equally, we should not be surprised to see physiological variation between different individuals within a species (see Parkinson *et al*., 2016). Further work is needed to quantify the distribution of these *Symbiodinium* within natural host populations, as one strain may only represent a background constituent in the majority of animals sampled (Santos *et al*., 2001). Overall, our data provide additional evidence for the existence of substantive physiological diversity within *Symbiodinium* species (Parkinson and Baums, 2014; Suggett *et al*., 2015; Parkinson *et al*., 2016).

Effects of temperature on the infectiousness of Symbiodinium

Although KLAp1 and KLAp2 were phylogenetically similar and isolated from the same host population, we observed differences in their ability to re-infect host animals and the degree to which this was affected by heating (Table 2, Fig. 3). Host-infection at 26°C was faster for KLAp2 than for KLAp1 (Fig. 3; effect of "Strain" at Days 4, 7: $P < 0.001$, Day 14: $P < 0.05$), a pattern that persisted until Day 21 ($P < 0.001$). This was reflected in final-day host protein-normalised *Symbiodinium* densities (two-way ANOVA, "Temperature" × "Strain", $F_{(1,11)} = 11.02$, $P = 0.007$; Tukey's *post-hoc*, $P < 0.001$), and supported by the fact that KLAp1 and KLAp2 *Symbiodinium* had similar *in hospite* fluorescence intensities per cell (Supporting Information Fig. S3). The comparable magnitudes of the genotypic differences on Day 7 – after which anemones were no longer exposed to *Symbiodinium* cells – and Day 21 indicate that *in hospite* proliferation rates for the two strains were similar at 26°C. This is in contrast to their differing *in vitro* growth rates, suggesting that KLAp1 might receive greater benefit from symbiosis than does KLAp2 or, conversely, that *in vitro* conditions such as N:P or trace metal concentrations may have been sub-optimal for KLAp1. The genotypic differences noted at 26°C were not detected when anemones were exposed to the two *Symbiodinium* strains at 30.5°C (Fig. 3; Chl *a* fluorescence, effect of "Strain" at Days 4, 7, 14, 21: $P > 0.05$; *Symbiodinium* density, Tukey's *post-hoc*, Day 21: $P > 0.1$). This was driven by increases in the extent to which KLAp1 infected and proliferated within anemone tissues and by a reduction in the initial uptake of KLAp2 into anemones (Days 0-7; Fig. 3). As for 26°C, temporal changes in tentacle chl *a* fluorescence after Day 7 at 30.5°C were similar for both sets of anemones (Fig. 3).

Concretely, the effect of algal strain on the establishment of *Symbiodinium* A4-*E. pallida* symbiosis appears to be determined more by differences in initial infectiousness than by variability in algal proliferation rate. The interactive effect of heating complicates matters, as it slightly increased the infectiousness and capacity for *in hospite* proliferation of KLAp1, but reduced the initial infectiousness of KLAp2. Together, these findings imply that host-symbiont communication at the onset of symbiosis might be sensitive to intraspecific variability among *Symbiodinium* and can be modified by temperature to affect the uptake of closely related and host-compatible *Symbiodinium* strains. The generally positive effect of heating observed here—contrary to previous investigations (Abrego *et al*., 2012; Schnitzler *et al*., 2012)—probably reflects the relatively high thermal tolerance of the *E. pallida*-

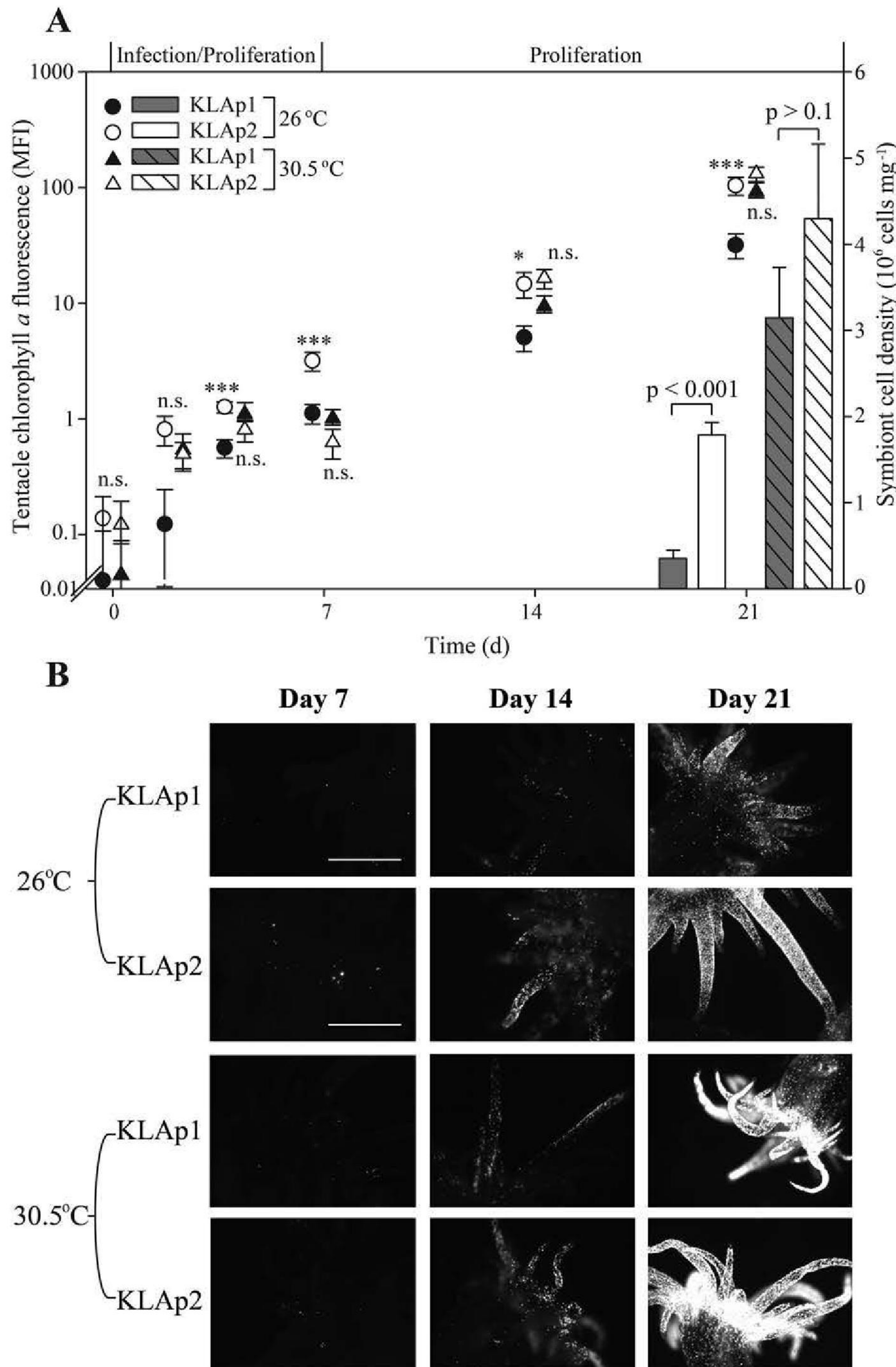

Fig. 3. Panel A) Rates of infection of aposymbiotic *Exaiptasia pallida* by *Symbiodinium* KLAp1 and KLAp2 at either 26°C or 30.5°C. Note logarithmic scale on the y-axis. Tentacle chlorophyll *a* fluorescence values (MFI: mean fluorescence intensity) were analyzed using a three-way non-parametric repeated-measures analysis ("nparLD" statistical package, see text for details). Significant differences between anemones exposed to KLAp1 or KLAp2 are indicated at each level of "Temperature" and "Day" by asterisks ($n = 12$ anemones per *Symbiodinium* strain; ****P* < 0.001, **P* < 0.05, n.s. not significant) and are the results of permutation analysis of the effect of "*Symbiodinium* strain" ($n = 1000$ replications, see text for details). End-point *Symbiodinium* KLAp1 and KLAp2 densities were compared using two-way ANOVA ($n > 3$ with pooled individuals). (B) Visualization of tissue chlorophyll *a* fluorescence ($k_{excitation} = 628 \pm 20$ nm, $k_{emission} = 692 \pm 20$ nm) in anemones separately exposed to *Symbiodinium* KLAp1 and KLAp2 at two temperatures (26°C and 30.5°C).

Symbiodinium A4 association (Goulet *et al.*, 2005) and the natural variability of its habitat (Florida Keys; mean annual temperature range ~ 17–31°C; https://www.nodc.noaa.gov/dsdt/cwtg/all_meanT.html). Certainly, the temperature found to inhibit coral-*Symbiodinium* symbiosis establishment in previous experiments (31°C; Abrego *et al.*, 2012; Schnitzler *et al.*, 2012) is close to the upper thermal limits of the respective study species (Berkelmans and Willis, 1999; Bellantuono *et al.*, 2012). It remains to be seen whether the establishment of cnidarian-dinoflagellate symbioses is inhibited by temperatures below those that are stressful for established holobionts.

As discussed above, the emergence of a specific holobiont is a product of interactions between the abiotic environment, host and symbiont ecophysiologies, and communication between symbiotic partners (Schoenberg and Trench, 1980; Knowlton and Rohwer, 2003; Finney *et al.*,

2010; LaJeunesse *et al.*, 2010a; Thompson *et al.*, 2014; Skelton *et al.*, 2016). From the perspective of the *Symbiodinium*, any beneficial effects of mildly elevated temperature on photosynthetic performance might enhance productivity and enable the cells to better infect- and proliferate within a host organism. Equally, heating may influence the host's ability to recognise and respond to the presence of symbionts. Indeed, a number of heat-sensitive cellular processes such as apoptosis (Dunn *et al.*, 2007), sphingolipid- and nitric oxide signalling (Detournay and Weis, 2011; Hawkins *et al.*, 2013) and lectin-glycan recognition (Vidal-Dupiol *et al.*, 2009; Pinzón *et al.*, 2015), appear to be active at the onset of symbiosis (Wood-Charlson *et al.*, 2006; Dunn and Weis, 2009; Bay *et al.*, 2011; Detournay and Weis, 2011). Yet, the cellular and molecular interactions between cnidarians and symbiotic dinoflagellates are yet to be fully revealed (Davy *et al.*, 2012).

Current climate projections indicate that tropical coral-*Symbiodinium* associations will experience near-annual thermal stresses within the next 50 years (van Hooidonk *et al.*, 2014). While "shuffling" of the *in hospite Symbiodinium* population may enable some corals to withstand these conditions (Silverstein *et al.*, 2014; Cunning *et al.*, 2015), the events necessary to bring about such responses (e.g., coral "bleaching") have significant costs (Jones, 2008; Grottoli *et al.*, 2014). However, in a warmer environment, faster and more successful host infection by comparatively heat-resistant *Symbiodinium* strains could promote the emergence of thermally tolerant holobionts without the need for stressful bleaching. In this context, it would be instructive to know how the mechanisms of symbiosis establishment are thermally labile. Given that a number of symbiotic microbes infect their hosts faster in a warmer environment (Shah *et al.*, 2002; Mouton *et al.*, 2006; Anbutsu *et al.*, 2008), a closer examination of the effects of current global warming on the outcome of initial coral-*Symbiodinium* interactions is warranted.

In summary, it appears that variability among conspecific *Symbiodinium* strains, a phenomenon with implications for the functioning of established holobionts (Howells *et al.*, 2012; Parkinson and Baums, 2014; Leal *et al.*, 2015), also plays a role in determining the outcome of initial host-symbiont interactions. The ecological significance of this remains unclear, but variability in the degree to which *Symbiodinium* infectiousness is sensitive to environmental conditions could potentially provide additional material for the natural selection of different holobionts (LaJeunesse *et al.*, 2010b; Parkinson *et al.*, 2015).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Figure S1. Agarose gel electrophoresis (1% [w/v] agarose, 4 V cm^{-1}, 90 min) of (Panels A and B) PCR-amplified and (C) TOPO-cloned psbAncr DNA fragments from two Symbiodinium cultures (“KLApl” and “KLAp2”) established from cells isolated from symbiotic *Exaiptasiapallida* anemones. DNA ladder is “Generuler 1kb-plus” (Thermo Fisher Scientific, Waltham, MA, USA).

Figure S2. *Symbiodinium* Clade A phylogeny based on chloroplast 23S ribosomal sequences obtained from Dr. Todd C. LaJeunesse (Penn State University, USA). Bold type refers to the ITS2-type of the respective culture/isolate. Scientific names in parentheses indicate the putative host

species. The A4 genotypes described in the present study are indicated by an asterisk.

Figure S3. Panel A) Baseline (26°C) chlorophyll a content per cell, measured spectrophotometrically on methanol-extracts prepared according to the methods of Rogers and Marcovich (2007); (B) Baseline dark respiration rate; (C) Forward-scatter (a cell size proxy) determined using flow cytometry of glutaraldehyde-fixed samples. Each replicate measurement represents *n* = 10,000 cells, selected above background material on the basis of their chlorophyll fluorescence (k [ex] = 488 nm, k [em] >650 nm); (D) net photosynthetic rate determined as oxygen flux in cell suspensions maintained in sealed 20-mL glass chambers at 26°C and exposed to darkness or 100 mmol photons m^{-2} s^{-1}, respectively; (E) Chlorophyll fluorescence (k [ex] = 618-638 nm, k [em] > 682-702 nm) of algal cells within anemone tissues. Boxes are means +/- 1 s.e.m, and vertical lines indicate +/- 1 SD from the mean. Statistics represent the outputs of Student's T-tests (*n* = 6 flasks per genotype for panels A-D; 50 cells per genotype for panel E). Panels F and G) Visual appearance of *Symbiodinium* cultures KLAp1 and KLAp2, respectively (see text for culture growth conditions).