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RESEARCH

The interplay of temperature, light, and substrate type in driving growth and reproduction of an important tropical crustose coralline alga

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Abstract

Crustose coralline algae (CCA) from the genus *Titanoderma* are reported to induce high levels of coral larval settlement across a wide diversity of species. Consequently, *Titanoderma* is a promising taxon to cultivate in aquaculture facilities for application in coral reef restoration projects. However, knowledge on the optimum conditions to promote growth and reproduction in *Titanoderma* is limited. To investigate this, we cultured adult fragments of *Titanoderma* sp. at two temperatures (27.5 or 30 °C) and two light levels (mean maximum midday irradiance of 10 or 40 µmol photons m^{-2} s^{-1}) on three different tile materials ($CaCO_3$, concrete, or PVC). We found that the combination of 27.5 °C and 40 µmol photons m^{-2} s^{-1} were best for adult fragment growth. Greater number of conceptacles were formed under higher light intensities, while temperature did not have an influence. Sporeling settlement and subsequent growth into juveniles were only evident at 40 µmol photons m^{-2} s^{-1}, with substantially higher recruitment on substrates made of concrete. These results provide important insights for developing optimal conditions to cultivate *Titanoderma* sp. in aquaculture facilities to support reef restoration projects using sexually produced corals.

Keywords CCA · Macroalgae · Aquaculture · Recruitment · Coral restoration

Introduction

Crustose, non-geniculate coralline algae (CCA) are calcified red algae that encrust hard substratum in many marine environments, from tropical to polar regions and from intertidal to deep-sea floors (Adey and MacIntyre 1973; Steneck 1986; Littler et al. 1991). On tropical coral reefs, CCA play fundamental roles in consolidating and cementing the reef framework (Littler and Littler 1984; Adey 1998) and are key contributors to the coral reef carbonate budget (Cornwall et al. 2023). CCA also provide important chemical cues that induce metamorphosis and settlement of numerous benthic marine invertebrates (Morse 1992; Hadfield and Paul 2001), including coral larvae (Heyward and Negri 1999; Tebben et al. 2015; Abdul Wahab et al. 2023).

Similarly to fleshy macroalgae, CCA growth is strongly regulated by light and temperature (Adey 1970; Hurd et al. 2014; Rodríguez-Prieto 2016). Within the range of light and thermal environments that a CCA species are found, growth rates of CCA typically increase with light and temperature until they reach an optimum (Fortes and Lüning 1980; Brown et al. 2004; Hurd et al. 2014). Beyond this optimum, photoinhibition occurs and metabolic performance deteriorates rapidly, resulting in physiological stress and even mortality (Brown et al. 2004; Hurd et al. 2014). Previous studies have reported that the optimum light and temperature for tropical shallow subtidal macroalgae range between 10 and 250 µmol photons m^{-2} s^{-1} and 25 and 30 °C; these values vary depending on species and the environmental conditions

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to which the local populations are adapted to (Lüning 1981; Pakker et al. 1996; Bischoff-Bäsmann et al. 1997; Hurd et al. 2014).

Light and temperature also play critical roles in CCA reproduction (Hurd et al. 2014). They may provide cues for triggering the switch from vegetative growth to reproduction and induce the development and maturation of reproductive structures (i.e., conceptacles; Chamberlain 1987; Lüning 1990; Santelices 1990; Liu et al. 2017). Light and temperature may act as 'ultimate factors' by controlling reproduction directly or as 'proximate factors' by regulating a circadian clock (Lüning and tom Dieck 1989; Liu et al. 2017). Reproductive patterns in CCA are also largely species-specific and may vary among climate zones (e.g., Adey and Steneck 2001), with most species in the temperate regions exhibiting peak fertility in the summer, while other species are more fertile in the winter (Adey 1973; Chihara 1974; Edyvean and Ford 1986). Far less information is available on the conditions that promote the formation of reproductive structures or that affect early life history processes of CCA (Lewis et al. 2017; Page and Diaz-Pulido 2020).

When the spores contained inside the CCA conceptacles reach maturity, they are released into the surrounding water environment, where they settle on the substratum and germinate (Jones and Moorjani 1973; Chamberlain 1984). Several studies have shown that environmental factors including light and temperature affect the metabolic activity of spores, which may influence the speed of the germination process and subsequent growth into juveniles (Ichiki et al. 2000; Yoshioka et al. 2020). Additionally, the recruitment success of CCA sporelings is dependent on substrate types (Fletcher and Callow 1992; Kennedy et al. 2017). Substrate types can influence the physical and chemical conditions of the microenvironment that the non-motile CCA spores encounter as they sink and settle, which may affect the attachment process of the spores (Fletcher and Callow 1992). A previous study by Kennedy et al. (2017) compared the performance of settlement plates comprised of different material types (PVC, polycarbonate, terracotta, limestone, glass, and porcelain) in monitoring CCA recruitment and growth in a tropical coral reef. They demonstrated that total CCA cover was similar across material types, but the CCA community compositions were different, suggesting species-specific recruitment success on certain substrate types (Kennedy et al. 2017).

CCA from the genus *Titanoderma* are reported to induce high levels of coral larval settlement across a wide diversity of species (Harrington et al. 2004; Doropoulos and Diaz-Pulido 2013; Ritson-Williams et al. 2016; Gómez-Lemos et al. 2018; Jorissen et al. 2021). A recent study by Abdul Wahab et al. (2023) identified *Titanoderma* cf. *tessellatum* (Lemoine) Woelkerling, Chamberlain & Silva as the most effective inducer among the 15 calcifying algae (13 CCA, 1 geniculate coralline alga, 1 Peyssonneliales) species tested, inducing > 50% larval settlement in 14 coral species. Reliably controlling larval settlement in aquaculture is a critical component in the sexual production of corals (Randall et al. 2020), and the efficient propagation of *T.* cf. *tessellatum* to settle larvae could advance the successful upscaling of coral reef restoration projects. However, knowledge on the optimum conditions to promote growth and reproduction in *T.* cf. *tessellatum* is limited.

In this study, we examined the effects of temperature, light, and substrate type in influencing the growth and reproductive responses of *Titanoderma* sp. (hereafter *Titanoderma*). Specifically, we compared how (1) the growth of *Titanoderma*, (2) the formation and loss of their conceptacles, and (3) the recruitment of new sporelings varied among four different temperature and light regimes on three different substrate types in an indoor aquarium system. Understanding the environmental conditions that promote growth and reproduction in *Titanoderma* is essential for developing optimal cultivation methods of this important CCA.

Materials and methods

Sample collection

Specimens of *Titanoderma* were collected from shallow reefs (< 5 m depth) around Davies Reef and Little Broadhurst Reef in the central Great Barrier Reef (GBR), Australia using SCUBA in July 2023 (GBRMPA Permit G21/45348.1). Specimens shared the morphologies and anatomies of *T.* cf. *tessellatum* based on the presence of overlapping swirls (i.e., applanate branching, Woelkerling and Campbell 1992), secondary pit connections and a basal layer of palisade cells (Lemoine 1929; Adey et al. 1982; Littler and Littler 2003). The taxonomic identity of fragments of *Titanoderma* was also validated based on molecular approach detailed further below. However, as the taxonomy of *Titanoderma tessellatum* is still unresolved and its type specimen has yet to be sequenced, we referred to our study species as *Titanoderma* sp.

Specimens varied in size, but most were ~ 4–8 cm^2 and were collected using hammer and chisel. We were not able to distinguish the life stages of the samples based on morphology as both the haploid gametophyte and the diploid tetrasporophyte stages of *Titanoderma* bear uniporate conceptacles, but from the examination of the anatomy of select samples through cross-sectioning, we exclusively observed bispores inside the conceptacle chambers, indicating the diploid stage.

Samples were transported in flow-through tanks on board the research vessel to the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS) in Townsville, Australia, and were cleaned of epiphytes and other

invertebrates using scalpels and bone cutters. Samples were then maintained in indoor semi-recirculating tanks (280 L) for one week at maximum midday irradiance of 15 µmol photons m^{-2} s^{-1} and 23 °C, matching the water temperature recorded in the field during the collection. Samples were cut into ~ 1.2 × 1.2 × 0.2 cm fragments (surface area: 1.51 ± 0.27 cm^2; mean area ± SD; Fig. 1a) using a wet diamond band saw (Gryphon Corporation, USA). *Titanoderma* fragments were attached, using a minimum amount of cyanoacrylate glue (Gorilla Super Glue), to the centre of 4 × 4 cm tiles that were made of either calcium carbonate ($CaCO_3$), concrete, or polyvinyl chloride (PVC) (more details in *Experimental design*). They were allowed to recover for one week in 15-L flow-through tanks at 27.5 °C with maximum midday irradiance of 15 µmol photons m^{-2} s^{-1}; no sign of stress was detected after the acclimation period in the aquarium conditions.

Experimental design

We examined the growth and reproductive responses of *Titanoderma* fragments cultured at two temperatures (27.5 vs. 30 °C) and two light levels (mean maximum midday irradiance of 10 µmol photons m^{-2} s^{-1} at daily light integral [DLI] of 0.3 mol m^{-2} day^{-1} vs. mean maximum midday irradiance of 40 µmol photons m^{-2} s^{-1} at DLI of 1.1 mol m^{-2} day^{-1}) on three different tile materials ($CaCO_3$, concrete, or PVC). The temperatures tested were within the seawater temperature range in the central GBR between spring and autumn (25–30 °C; Australian Institute of Marine Science 2023), the period during which peak growth rates have been reported in CCA (Lewis et al. 2017). The light intensities applied reflect the low to moderate irradiance of the cryptic habitats (e.g., inside cavities) and overhangs or vertical walls where *Titanoderma* is typically found on coral reefs (G. Diaz-Pulido, personal observation).

Titanoderma fragments were grown on three different materials of tiles (4 × 4 cm), namely $CaCO_3$, concrete, and PVC. These materials were selected because they are commonly used for monitoring the growth and recruitment of marine benthic communities (Burt et al. 2009; Kennedy et al. 2017). $CaCO_3$ tiles were sourced from Oplusi (USA) and were fabricated from casting $CaCO_3$ powder that was initially dispersed in water using polyvinyl alcohol (PVA) binder. Concrete tiles were made following the standard AS 2350.12–2006 Sect. 6 and 7 (Standards Australia 2016) using Portland cement, fine sand (particle size < 500 µm), and water. PVC tiles were purchased from local suppliers in Townsville, Australia. Each temperature and light combination was replicated using four 15-L tanks; each tank contained three tiles made of each material (16 tanks; 2 temperature × 2 light × 3 materials × 4 replicate tanks = 48

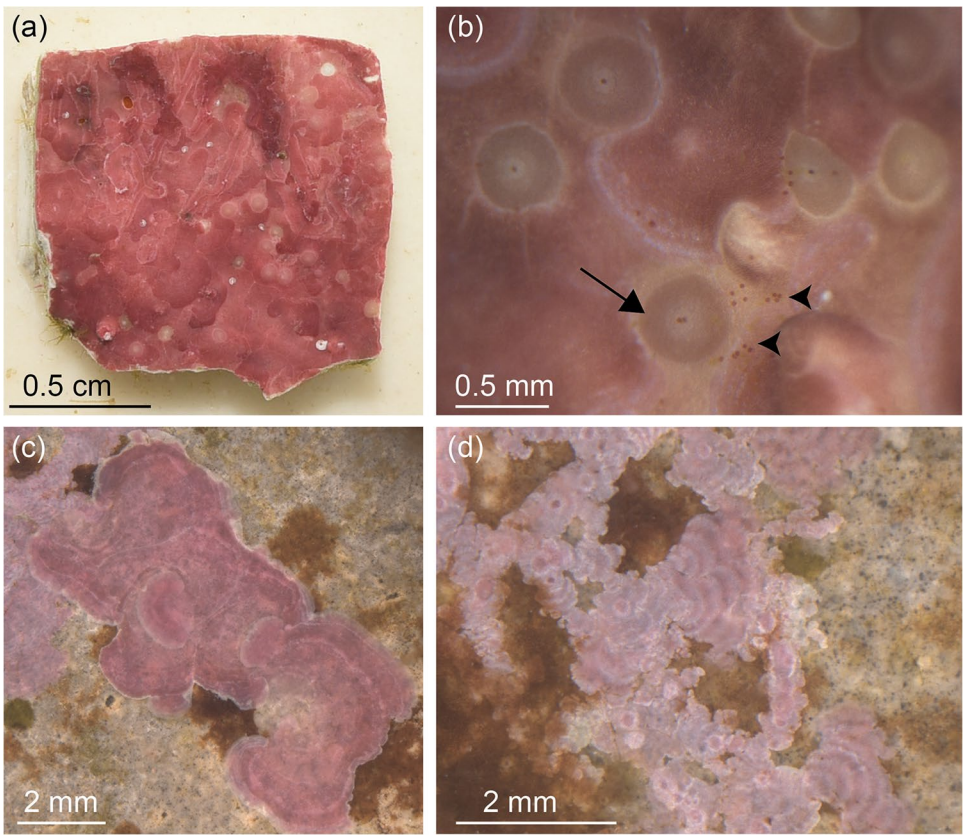

Fig. 1 **(a)** An example of a reproductively mature *Titanoderma* fragment used in this study, **(b)** uniporate conceptacles [arrow] with spores released [arrowheads], **(c)** *Titanoderma* juvenile with overlapping swirls at week 12, and **(d)** *Porolithon* juvenile with growth bands at week 12

fragments; Fig. 2). The experiment ran for 12 weeks (August–October 2023).

Each 15-L tank was independently supplied with aeration and filtered seawater (FSW, nominal 1 µm) at the respective temperature with an average 100% turnover h^{-1}. Tanks of the same temperature treatment were also held within water baths to maintain the temperature. Illumination was provided by custom LED panel lights (blue and white dominated) that followed a sinusoidal profile of 6 h ramping up from darkness to the maximum irradiance (either 10 or 40 µmol photons m^{-2} s^{-1}) and then 6 h of ramping down to darkness (12 h light: 12 h dark cycle; Fig. S1). Neutral-density filters were used to adjust the intensity of the LED lights to the desired levels. Both temperature and light were controlled by a programmable logic controller (PLC, Supervisory Control and Data Acquisition systems, Siemens PCS7) and were monitored continuously using PAR sensors (SKL26250 PAR Quantum Sensor, Skye Instruments Ltd, UK) and temperature probes (FEP RTD Pt100 Sensor, TC Direct, Australia). Weekly cleaning was performed to remove fouling diatoms, e.g., walls of the tanks were scrubbed with a sponge while tiles were gently brushed with a fine paint brush.

Growth, reproductive, and recruitment responses

We assessed the growth rates of *Titanoderma* fragments based on changes in their surface area; *Titanoderma* has thin crust habit and grows mainly by extending their margins laterally (Woelkerling et al. 1985). Fragments were photographed every two weeks using a Nikon D810 camera (Nikon AF-S 60 mm f/2.8G ED macro lens) and surface area was estimated using the freehand selection tool in the ImageJ software (v 1.53 k; Schneider et al. 2012). Growth rate was calculated as the percentage of change in the surface area of *Titanoderma* fragment $[(A_t - A_0) / A_0 \times 100]$, where A_t is the area of the fragment at the different time points and A_0 is the area of the fragment at the start of the experiment.

We assessed the reproductive responses of *Titanoderma* based on the changes in the numbers of conceptacles (Fig. 1b). Once *Titanoderma* spores reach maturity and are released from the conceptacles, the conceptacles will collapse and detach from the thallus surface or be overgrown by surrounding tissue (J. Fong, personal observation). From the photographs, we determined the number of newly produced conceptacles at each time point as well as the number of conceptacles that were lost. Fragments that suffered > 30% tissue loss were excluded from the analysis (Table S1). We then standardised the number of conceptacles gained and lost by the surface area of *Titanoderma* to obtain conceptacle density.

To investigate the effects of experimental treatments on the early life history stage process of CCA recruitment, we first counted the number of *Titanoderma* sporelings (< 1 mm in size) that successfully settled and germinated on each tile at week 6 to determine sporeling settlement density. As sporelings continued to grow and expand laterally to become juveniles (~ 1–5 mm in size), individuals coalesced. Therefore, at week 12, we quantified the total area of each tile covered by *Titanoderma* juveniles as a proxy for recruitment, which took into account spore settlement and post-settlement survivorship and growth. The area covered by the juveniles was measured using the *versatile wand* tool in ImageJ. Two different morphotypes of CCA juveniles were present, one having the morpho-anatomy characteristic of *Titanoderma* (i.e., presence of distinctive overlapping swirls; Fig. 1c), while the other lacked distinctive swirls, although growth bands may resemble poorly developed overlapping swirls (Fig. 1d). Their taxonomic identities were checked using the DNA barcoding method at the end of the experiment.

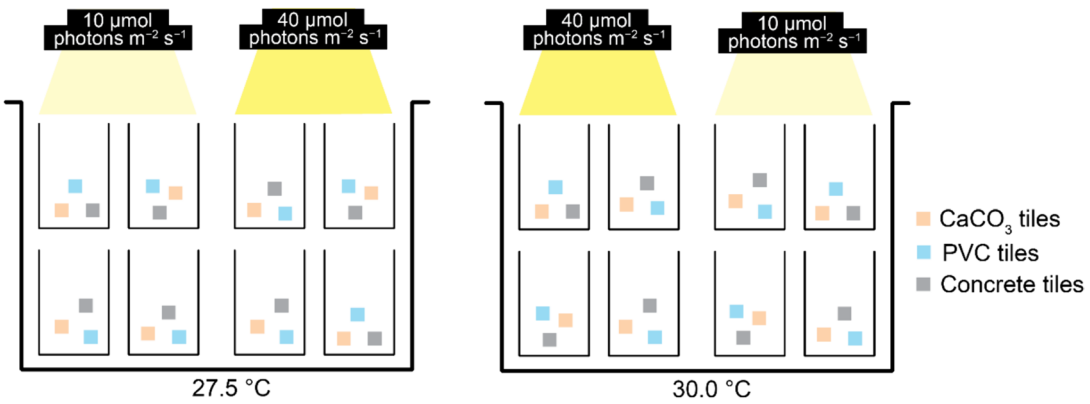

Fig. 2 Schematic diagram of the experimental set-up illustrating the four 15-L replicate tanks per light and temperature combination

Taxonomic confirmation using molecular approach

To validate the species identity of the *Titanoderma* fragments and the juveniles, representative individuals were sequenced at random. Tissue for DNA sequencing was gathered from the experimental tiles using a sterile single edge razor blade. The extraction of genomic DNA followed the protocol provided in Nucleospin Plant II Kit (Macherey–Nagel, Germany). PCR amplification tubes contained 40 µL of reaction mixture, consisting of 2–4 µL of genomic DNA, 1 µL each of 10 pM forward and 10 pM reverse primer, 14–16 µL of ultrapure distilled water and 20 µL of AccuPower Taq PCR PreMix (Bioneer Pacific, Australia). Two genes were amplified using either *psb*AF1/*psb*A600R (Yoon et al. 2002) or F993/R*rbc*Start (Freshwater and Rueness 1994). Successful amplicons were sent to Macrogen, Seoul, South Korea for sequencing and purification. Specimens used for molecular identification were deposited in the Herbarium in the Coral Reef Algae Laboratory, Griffith University with GenBank accession numbers provided in Table S2.

A single concatenated supergene alignment using *rbc*L and *psb*A datasets were used to create the phylogenetic tree. GenBank sequences of type and voucher specimens were used to infer phylogenetic relationships of the newly generated specimens. For outgroups, we used the type sequences of closely related taxa of the order Hapalidiales, *Phymatolithopsis donghaensis* and *Phymatolithopsis prolixa*, to root the trees. Results were visualised and manually edited in MEGA 7 and MEGA 11 (Kumar et al. 2016; Tamura et al. 2021), and aligned using clustalW (Thompson et al. 1994). PartionFinder 2 (Lanfear et al. 2017) determined the best fit model for the partition scheme and model of evolution. The concatenated maximum likelihood (ML) analysis used the General Time reversible model (GTR) + G + I, with 1,000 bootstrap (BS) replications analysed within RaxML v2.0 (Stamatakis 2006; Stamatakis et al. 2008; Edler et al. 2021). Bayesian analysis was performed using MrBayes 3.2.7 following Jeong et al. (2023) using the schemes provided by PartionFinder 2.

Statistical analysis

To compare the growth responses of *Titanoderma* among treatments, we used generalised linear mixed-effects models (GLMMs; *glmmTMB* package; Brooks et al. 2017). We fitted the growth rates of *Titanoderma* against temperature, light, and their interactions as fixed factors, while tank identity and substrate type were included as random effects. Growth rates were modelled following Gaussian distribution, and only data from the final time point (i.e., week 12) were fitted into the models.

We used GLMMs with Poisson distribution to determine how the number of conceptacles gained and lost across the 12-week experiment might vary among the light and temperature treatments. Similar to the previous GLMMs for growth data, tank identity and substrate type were added as random effects. In addition, we included a temporal correlation with lag 1 (AR1) to account for repeated measurements of fragments across time. The surface area of *Titanoderma* was also added as an offset term in the models to standardise the number of conceptacles as densities.

As little to no recruitment was observed on tiles at 10 µmol photons m^{-2} s^{-1} treatments across the experimental period, we focused on comparing the settlement and recruitment rates of *Titanoderma* among substrate types and temperature levels at 40 µmol photons m^{-2} s^{-1} treatments. GLMMs with Poisson distribution were used to model the number of *Titanoderma* sporelings germinated at week 6, while the total area covered by *Titanoderma* juveniles at week 12 was fitted using Gamma distribution. To express the settlement of *Titanoderma* sporelings as density (i.e., number of sporelings per cm^2), we included tile area as an offset term. For both models, tank identity was added as a random effect. We also allowed the residual variance of the recruitment models to differ among temperature and substrate treatments to control for heteroscedasticity.

For all models, residual checks were conducted using the *DHARMa* package (Hartig 2022) to validate the model fit. Estimated marginal means (*emmeans* package; Lenth 2024) were computed to perform pairwise comparisons with Tukey adjustments to assess how the growth rates, number of conceptacles gained and lost, settlement and recruitment rates differed among treatments.

Results

Taxonomic identification

Algal tissues used for DNA sequences consisted of 5 adult *Titanoderma* fragments and 7 *Titanoderma* juveniles. For the adults, 4 *rbc*L and 3 *psb*A sequences were successfully obtained, while for the juveniles 6 *rbc*L and 3 *psb*A were obtained. The concatenated phylogenetic analyses showed that the sequences obtained from both *Titanoderma* adult fragments and recruits clustered together with the sequences of other *Titanoderma* specimens uploaded to GenBank (Fig. S2). The *Titanoderma* clade was well supported as a sister taxon from *Amphiroa*, and clustered separately from the type specimens of *Lithophyllum.* One sequence of the *Titanoderma* recruit (Accession No. PP763090) was nested in a clade along with other *Titanoderma* adult fragments (*Titanoderma* sp.1), while two additional genetically distinct *Titanoderma* were present among the other six recruits

(*Titanoderma* sp.2, *Titanoderma* sp.3; Fig. S2). The phylogenetic analyses therefore revealed that there were at least three species within the *Titanoderma* clade.

DNA sequencing of the CCA recruits confirmed the presence of two taxa, as intuited from the differences in the morphology of the juvenile crusts. Crusts with distinctive overlapping swirls belonged to the *Titanoderma* cluster, while sequences from the other morpho-taxon grouped together with *Porolithon maneveldtii*, separate from branches representing type specimens of other *Porolithon* species.

Growth rates

There were significant interaction effects of temperature and light on the growth rates of *Titanoderma* fragments (Table 1, Fig. 3). The greatest growth rate was recorded for fragments under the lowest temperature and highest light intensity. At 30 °C, fragments cultured at 10 µmol photons m^{-2} s^{-1} had 41.6% lower growth rates than those at 40 µmol photons m^{-2} s^{-1}, while fragments cultured at 27.5 °C had comparable growth rates at both light levels. While both positive growth and tissue loss were observed among the treatments (Fig. S3), on average, negative growth (represented by tissue loss on the surface of the CCA fragment) was recorded for each treatment (Fig. 3, Fig. S4).

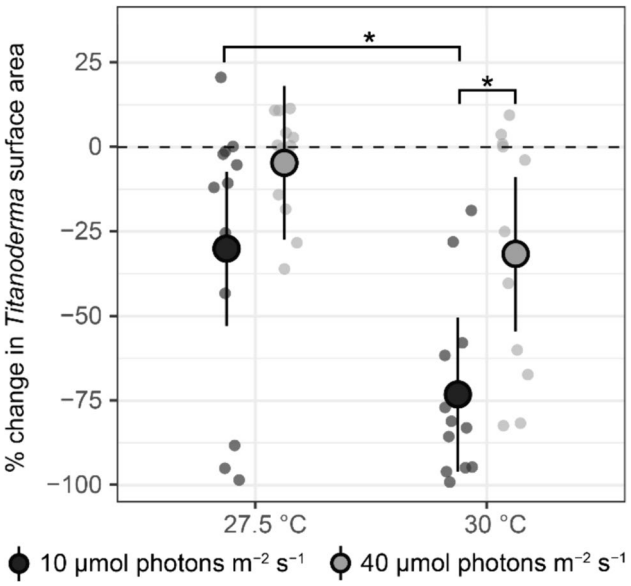

Fig. 3 The estimated marginal means and their 95% confidence intervals of the percentage change in the surface area of *Titanoderma* fragments at the end of the 12-week experiment ($n = 12$). Asterisks denote significant differences between treatments based on Tukey adjustments of *p*-values

Table 1 Summary of simple main effects based on the estimated marginal means and 95% confidence intervals (CI) of growth rate (% change), conceptacle gain and loss (conceptacles cm^{-2}), settlement density and recruitment rate of *Titanoderma*. Estimates of conceptacle gain and loss, settlement density, and recruitment rate are on log scales. Units of light levels (µmol photons m^{-2} s^{-1}) are abbreviated as PAR. Bold values indicate statistical significance at *p*-value < 0.05

Variables	Estimate (95% CI)	*p*-value	Variables	Estimate (95% CI)	*p*-value
1. Growth rate					
27.5 °C, 10 vs 40 PAR	–25.4 (-53.8, 3.0)	0.079			
30 °C, 10 vs 40 PAR	–41.5 (–69.9, –13.1)	**0.005**			
10 PAR, 27.5 vs 30 °C	43.1 (14.6, 71.5)	**0.004**			
40 PAR, 27.5 vs 30 °C	27.0 (–1.5, 55.4)	0.063			
2. Conceptacle gain			**3. Conceptacle loss**		
27.5 °C, 10 vs 40 PAR	–2.2 (–3.8, –0.7)	**0.005**	27.5 °C, 10 vs 40 PAR	–0.2 (–1.0, 0.6)	0.606
30 °C, 10 vs 40 PAR	–2.9 (–4.7, –1.1)	**0.002**	30 °C, 10 vs 40 PAR	–0.5 (–0.9, 0.9)	0.918
10 PAR, 27.5 vs 30 °C	0.8 (–1.2, 2.7)	0.431	10 PAR, 27.5 vs 30 °C	–0.6 (–1.5, 0.2)	0.158
40 PAR, 27.5 vs 30 °C	0.1 (–1.3, 1.5)	0.883	40 PAR, 27.5 vs 30 °C	–0.5 (–1.3, 0.4)	0.269
4. Sporeling settlement at week 6, 40 PAR			**5. Juvenile recruitment at week 12, 40 PAR**		
27.5 °C, $CaCO_3$ vs PVC	0.7 (–2.2, 3.6)	0.832	27.5 °C, $CaCO_3$ vs PVC	4.3 (1.3, 7.2)	**0.002**
27.5 °C, $CaCO_3$ vs Concrete	–3.6 (–5.3, –1.9)	**< 0.001**	27.5 °C, $CaCO_3$ vs Concrete	–3.3 (–5.5, –1.1)	**0.001**
27.5 °C, PVC vs Concrete	–4.3 (–6.6, –1.9)	**< 0.001**	27.5 °C, PVC vs Concrete	–7.6 (–9.6, –5.6)	**< 0.001**
30 °C, $CaCO_3$ vs PVC	–1.2 (–2.4, 0.0)	0.043	30 °C, $CaCO_3$ vs PVC	–2.4 (–5.3, 0.6)	0.144
30 °C, $CaCO_3$ vs Concrete	–2.7 (–3.8, –1.7)	**< 0.001**	30 °C, $CaCO_3$ vs Concrete	–4.3 (–6.5, –2.1)	**< 0.001**
30 °C, PVC vs Concrete	–1.5 (–2.1, –0.9)	**< 0.001**	30 °C, PVC vs Concrete	–2.0 (–3.9, 0.0)	0.054
$CaCO_3$, 27.5 vs 30 °C	–1.0 (–2.8, 0.8)	0.292	$CaCO_3$, 27.5 vs 30 °C	0.6 (–2.2, 3.4)	0.665
PVC, 27.5 vs 30 °C	–2.9 (–5.0, –0.7)	**0.009**	PVC, 27.5 vs 30 °C	–6.0 (–8.6, –3.5)	**< 0.001**
Concrete, 27.5 vs 30 °C	–0.1 (–0.9, 0.7)	0.774	Concrete, 27.5 vs 30 °C	–0.4 (–1.4, 0.7)	0.477

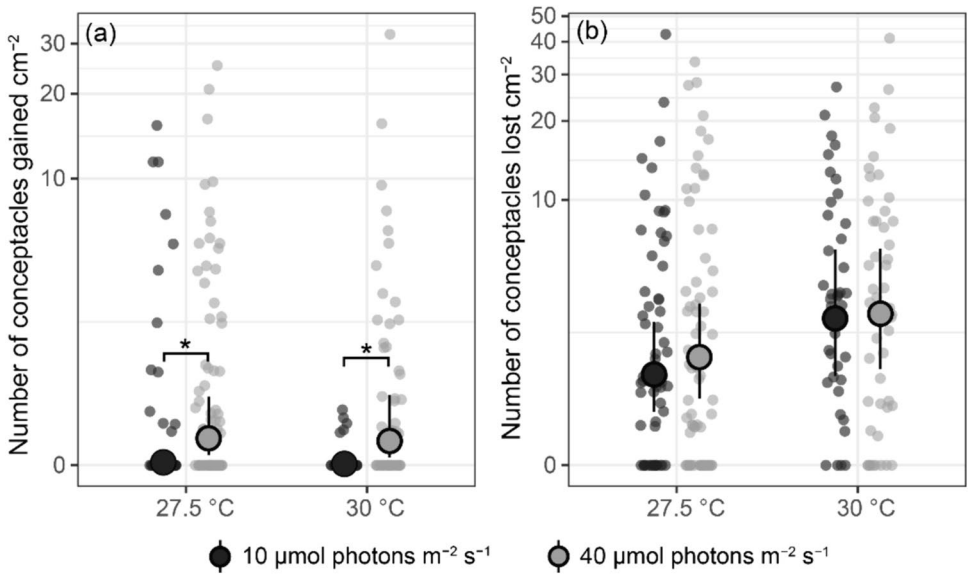

Fig. 4 The estimated marginal means and their 95% confidence intervals of the numbers of conceptacles **(a)** gained and **(b)** lost per cm^2 of *Titanoderma* fragments ($n = 12$, with six time-points; see Table S1 for more details). Y-axes are in logarithmic scales. Asterisks denote significant differences between treatments based on Tukey adjustments of *p*-values

Conceptacle formation and loss

The number of newly formed conceptacles differed significantly between the two light levels, but not the temperature treatments (Table 1, Fig. 4a). Notably, at both 27.5 and 30 °C, *Titanoderma* fragments that were cultured at 40 µmol photons m^{-2} s^{-1} had 9.4–18.3 × higher number of conceptacles being produced cm^{-2} compared to the fragments cultured at 10 µmol photons m^{-2} s^{-1} (Fig. 4, Fig. S5). In contrast, the number of conceptacles that were lost did not vary among temperature and light treatments (Fig. 4b). There were more observations with conceptacle loss ($n = 171$) than gain ($n = 83$). The greatest loss in the number of conceptacles in the two weeks' period was 55 conceptacles on two fragments cultured at 27.5 °C, 10 µmol photons m^{-2} s^{-1} and at 30 °C, 40 µmol photons m^{-2} s^{-1}, while the greatest gain was 44 on a fragment cultured at 30 °C, 40 µmol photons m^{-2} s^{-1}.

Settlement and recruitment

At week 6, most tiles at 10 µmol photons m^{-2} s^{-1} treatments had zero ($n = 18$ tiles) or less than three sporelings settled ($n = 5$ tiles), except for one concrete tile at 27.5

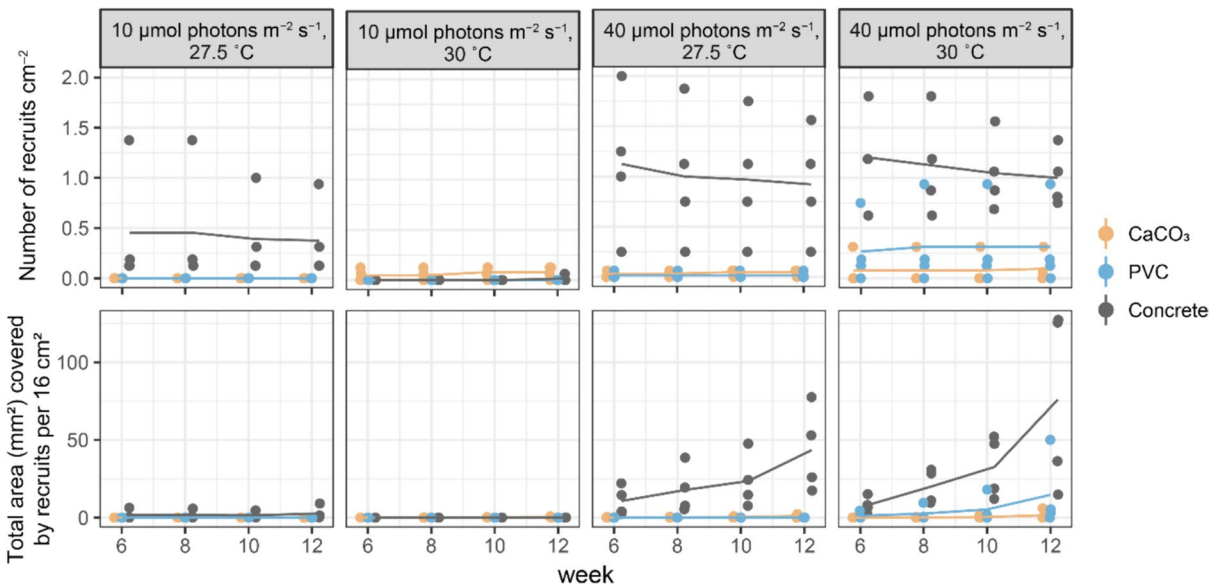

Fig. 5 Changes in the density of *Titanoderma* recruits (sporelings + juveniles) (top panel) and the total area covered by *Titanoderma* recruits on each 16 cm^2 tile (bottom panel) across the 12-week experimental period ($n = 4$). Circles represent raw data points, while lines represent the mean

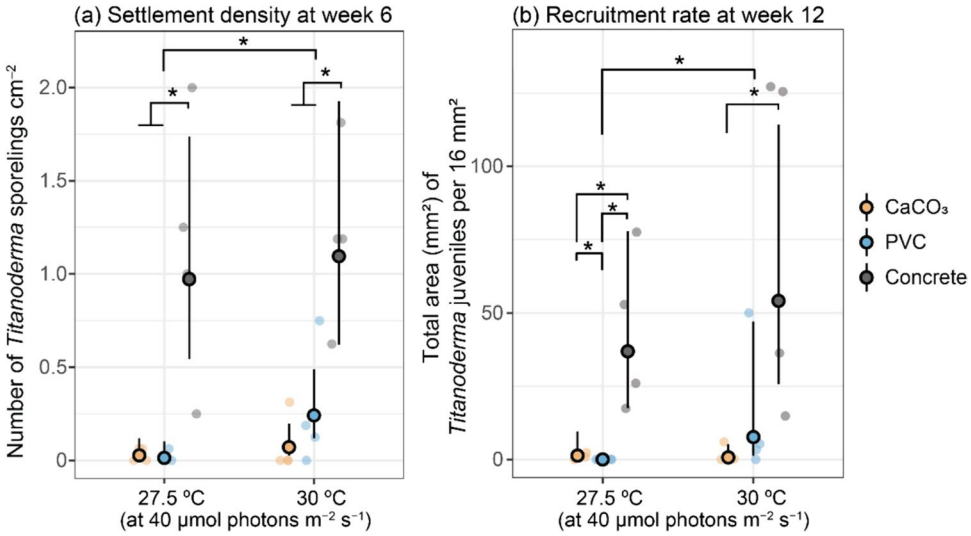

Fig. 6 Settlement density and recruitment rate of *Titanoderma* at 40 µmol photons m^{-2} s^{-1}. The estimated marginal means and their 95% confidence intervals of **(a)** the density of sporelings on each tile at week 6 and **(b)** the total area occupied by juveniles at week 12 ($n = 4$). Asterisks denote significant differences between treatments with Tukey adjustments of p-values

°C that had 22 sporelings (Fig. 5). As such, at week 12, little to no recruitment of *Titanoderma* juveniles were observed on tiles at 10 µmol photons m^{-2} s^{-1} (Fig. 5). In contrast, successful settlement and recruitment on tiles was observed from week 6 at 40 µmol photons m^{-2} s^{-1} (Fig. 5).

At this higher light intensity, significantly greater densities of *Titanoderma* sporelings successfully settled and germinated on concrete tiles at week 6 compared to PVC and $CaCO_3$ tiles at both temperature levels (Table 1, Fig. 6a). There were no differences in the sporeling settlement between PVC and $CaCO_3$ tiles. On average, each concrete tile had 1.16 ± 0.20 sporelings cm^{-2} (mean ± SE) while there were 0.14 ± 0.09 and 0.05 ± 0.04 sporelings cm^{-2} on each PVC and $CaCO_3$ tile, respectively. Comparable settlement rates were observed between the two temperature levels, except PVC tiles having higher number of sporelings at 30 °C compared to 27.5 °C. Similar patterns were observed for the recruitment of *Titanoderma* juveniles grown at 40 µmol photons m^{-2} s^{-1} at week 12 (Table 1, Fig. 6b). Compared to PVC and $CaCO_3$ tiles, concrete tiles had significantly greater (3.3–7.6×) total area covered by *Titanoderma* juveniles at both temperature levels, with the exception of PVC tiles having comparable recruitment rates to concrete tiles at 30 °C (Fig. 6b). The average area occupied by *Titanoderma* juveniles on concrete tiles was 59.7 ± 16.2 mm^2, while it was 1.3 ± 0.7 mm^2 on $CaCO_3$ tiles and 7.4 ± 6.1 mm^2 on PVC tiles. Similar to sporeling settlement, only PVC tiles had higher recruitment rates at 30 °C compared to 27.5 °C, while the recruitment on concrete and $CaCO_3$ tiles did not vary between the temperature treatments.

Discussion

This study aimed at identifying the environmental conditions of light, temperature, and substrate type that favoured the growth, reproduction, sporeling settlement, and juvenile recruitment of *Titanoderma*—an important crustose coralline alga that is a key inducer of coral larval settlement. We found that the combination of moderate temperature (27.5 °C) and moderate light (40 µmol photons m^{-2} s^{-1}) were best for adult fragment growth. Adult fragments formed greater number of conceptacles under moderate light treatment compared to low light treatment, while temperature did not have an influence. Sporeling settlement and subsequent recruitment were only evident in moderate light treatment, with substantially higher rates on substrates made of concrete. Our findings provide important insights for developing optimal cultivation methods to promote growth and reproduction of *Titanoderma*, a promising taxon for application in coral aquaculture and reef restoration.

Growth responses

Within the range of environmental variables tested, adult fragments exhibited negative growth rate (tissue loss > growth) under most conditions. Nevertheless, fragments cultured at 27.5 °C, 40 µmol photons m^{-2} s^{-1} had the least tissue loss (-4.68 ± 4.58%). In contrast, fragments cultured at 30 °C, 10 µmol photons m^{-2} s^{-1} had the lowest growth rate (-73.2 ± 7.7%). Some CCA are notably difficult to cultivate ex situ and multiple experimental studies have reported negative growth rates in CCA maintained

in conditions matching local environmental parameters (Rodríguez-Prieto 2016; Cornwall et al. 2019). Other environmental parameters not investigated in this study such as water motion, nutrient levels, interactions with herbivores might play key roles in maintaining growth in *Titanoderma*. In addition, the tissue loss in *Titanoderma* fragments may have been a result of physiological stress due to recent fragmentation, followed by a relatively short acclimation duration to the experimental temperatures (i.e., one-week acclimation from 23 to 27.5 °C, and subsequent exposure to either 27.5 or 30 °C for 12 weeks). Longer acclimation time with slow ramping of temperature (1 °C per week; Cornwall et al. 2019) might allow us to draw better inferences on the optimum temperature and light conditions for adult *Titanoderma* fragments. Nevertheless, at moderate light treatment (40 µmol photons m^{-2} s^{-1}), *Titanoderma* fragments successfully produced first-generation of sporelings, which grew into juveniles (1–5 mm in size) in 6 weeks, highlighting the potential to propagate *Titanoderma* from first generation fragments in aquaculture facilities.

Similar to the patterns observed in the adult fragments, growth rate of sporelings cultured at 40 µmol photons m^{-2} s^{-1} was higher than at 10 µmol photons m^{-2} s^{-1}. Among the few sporelings that successfully settled at 10 µmol photons m^{-2} s^{-1}, minimal growth was detected over the course of the experiment (Fig. 5). In contrast, the total area covered by *Titanoderma* juveniles grown at 40 µmol photons m^{-2} s^{-1} increased progressively while the densities of sporelings on each tile did not fluctuate, indicating that the increase in the recruitment of *Titanoderma* juveniles was driven by the growth of the sporelings (Fig. 5). At 40 µmol photons m^{-2} s^{-1}, comparable sporeling density and recruitment was observed between 27.5 or 30 °C, suggesting that growth rates of sporelings did not differ between the temperature treatments. Taken together, these results indicate that 10 µmol photons m^{-2} s^{-1} was less than optimal for *Titanoderma* growth across life stages. Although *Titanoderma* can be found in low light habitats such as crevices and inside cavities, most specimens are typically found at shallow to intermediate depths (2–6 m depth) under moderate light environment. *Titanoderma* is also less abundant on inshore, turbid reefs compared to on mid- and outer-shelf reefs of the Great Barrier Reef, where water clarity is much higher (Fabricius and De′ath 2001; Dean et al. 2015). The inability of adult *Titanoderma* fragments and *Titanoderma* juveniles to maintain tissue growth and survive at 10 µmol photons m^{-2} s^{-1} suggests that this light level might be below the compensation point to obtain enough energy from photosynthesis to balance respiration. Future studies should not only investigate a wider range of higher irradiances to determine the saturation level for *Titanoderma*, but also the requirements for water flow and inorganic nutrient supply. However, it should be noted that if live *Titanoderma* is used as a settlement cue for coral larvae in reef restoration projects, higher light regime is likely to encourage more rapid algal growth, increasing competition and overgrowth of the newly settled coral spat (Ramsby et al. 2024).

Conceptacle generation and loss

Our findings revealed that conceptacle formation in *Titanoderma* was strongly regulated by light. The number of conceptacles produced was 9.4–18.3 × greater on fragments cultured at 40 µmol photons m^{-2} s^{-1} than 10 µmol photons m^{-2} s^{-1} at both 27.5 and 30 °C. In contrast, there were no differences in the rate of conceptacle loss across light and temperature treatments. While the influence of environmental variables in controlling CCA reproduction has been documented in several temperate species (Adey 1973; Edyvean and Ford 1986; Chamberlain 1987), only limited knowledge is available for tropical CCA species. By examining skeletal bands in *Porolithon onkodes*, Lewis et al. (2017) deduced that *P. onkodes* in the southern reefs of the GBR produced conceptacles annually during the summer, although it was uncertain which environmental factors drove the seasonal variability in the CCA reproduction. Our results were similar to the patterns reported by Jones and Woelkerling (1983), who found that conceptacles of *Fosliella cruciata* (now known as *Hydrolithon cruciatum*) from the temperate region of southern Australia developed faster under higher light intensities. At 12–25 µmol photons m^{-2} s^{-1}, *H. cruciatum* generated new conceptacles within 26–33 days, while it took 65–72 days for conceptacles to be produced under 3–6.5 µmol photons m^{-2} s^{-1} (Jones and Woelkerling 1983). Furthermore, Jones and Woelkerling (1983) noted that conceptacles of *H. cruciatum* were formed only at higher temperatures of 15 and 22 °C, but not at 10 °C. We did not find any differences in the conceptacle production by *Titanoderma* between 27.5 and 30 °C; however, we did not test the lower limit of the annual temperature range (i.e., 23 °C), where production may have been lower. It remains unclear why conceptacle formation was promoted at higher irradiance in our experiment, but it might be related to greater availability of resources that could be allocated to growth and development of reproductive tissues. Other studies in fleshy macroalgae have identified increased reproduction in response to light via alleviation of resource limitation (Ngan and Price 1980; Santelices 1990).

Sporeling settlement and recruitment of juveniles

Rates of sporeling settlement and juvenile recruitment of *Titanoderma* responded dramatically to variations in light and substrate type, while temperature had a minimal effect. The majority of tile replicates at 10 µmol photons m^{-2} s^{-1} treatment had little to no settlement of

sporelings and recruitment of *Titanoderma* juveniles at week 6 and 12, respectively. This was likely driven by the low success rate in spore germination compounded by slow growth of sporelings due to limited resources as a result of low light conditions, supporting the critical role of light in algal ecophysiology (Hurd et al. 2014; McCoy and Kamenos 2015). Ordoñez et al. (2017) reported that *Porolithon* cf. *onkodes* spores had higher occurrences of abnormalities and lower germling growth rates at 40–60 µmol photons m^{-2} s^{-1} compared to 140–160 µmol photons m^{-2} s^{-1}. Furthermore, *Titanoderma* fragments cultured at 10 µmol photons m^{-2} s^{-1} had lower conceptacle densities (Fig. 4); consequently, fewer spores were present, and this may have also led to reduced settlement and recruitment under these conditions. Other environmental factors not considered in our study such as slower microbial colonisation might also limit the settlement success of early life history stages under low light conditions (Qian et al. 2007; Dang and Lovell 2016).

Among the three substrate types tested, concrete tiles were the best in promoting settlement of *Titanoderma* sporelings and recruitment of *Titanoderma* juveniles. While there were < 0.2 sporelings cm^{-2} present on the PVC and $CaCO_3$ tiles, concrete tiles had an average of 1.16 ± 0.20 sporelings cm^{-2}. As the sporelings grew into juveniles, the differences among substrates became even more evident: the total area covered by *Titanoderma* juveniles was > 20 × greater on concrete tiles than PVC and $CaCO_3$ tiles. These results were consistent with a recent study that examined macroalgal establishment rates across three material types (concrete, $CaCO_3$, and alumina-based ceramic) and five roughness levels (Fong et al. 2024), which also found higher abundance of the crustose coralline algae *Crustaphytum* sp. and *Lithophyllum* sp. on the concrete tiles. Physicochemical properties of concrete including high porosity and low surface hydrophobicity (Hayek et al. 2021) likely play important roles in achieving higher spore attachment and germination success (Fletcher and Callow 1992). These properties might also help in promoting rapid colonisation of concrete tiles by microorganisms, providing suitable biofilm for settlement and growth of CCA sporelings (Hayek et al. 2021). Nevertheless, it should be noted that $CaCO_3$ tiles used in the study were fabricated from compacted $CaCO_3$ powder, so the results might not be comparable with natural $CaCO_3$ substrates. Regardless of the mechanisms involved, results from our experiment clearly showed among the three material types tested, concrete achieved by far the highest recruitment of *Titanoderma* juveniles under typical aquaculture conditions.

Conclusion

Our study identified irradiance and temperature levels that favoured the growth and reproduction of adult and juvenile *Titanoderma* crusts, with the greatest growth observed under lower temperature (27.5 °C) and higher light intensity (40 µmol photons m^{-2} s^{-1}). We also demonstrated that concrete promoted higher sporeling settlement and juvenile recruitment over $CaCO_3$ and alumina-based ceramic surfaces. However, we also highlight the potential importance of longer acclimation durations before experimental manipulation to minimise tissue loss in adult fragments. The DNA sequencing confirmed the presence of several (cryptic) species of *Titanoderma* juveniles, which shared similar morpho-anatomical characters and could not be distinguished with the naked eye, emphasising the importance of applying molecular tools to validate the taxonomic identification of CCA species. Clearly, the taxonomy of *Titanoderma* requires further attention so this species can be clearly delineated as a fundamental step towards the development of successful aquaculture of these taxa. Finally, as the genus *Titanoderma* (including *T.* cf. *tessellatum*) is one of the most effective inducers of coral larval settlement in both the Pacific (Harrington et al. 2004; Gómez-Lemos et al. 2018; Jorissen et al. 2021; Abdul Wahab et al. 2023) and Caribbean (Ritson-Williams et al. 2016) corals, further studies are required to optimise its growth and propagation, particularly regarding inorganic nutrient and water flow requirements. Developing axenic cultures of *Titanoderma* (e.g., excluding other macroalgae and CCA) should also be a priority of future research, as this would help in maximising tissue growth. The present study and further research will contribute to develop the cultivation of this taxa to support reef restoration projects using sexually produced corals.

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Author contributions **Jenny Fong:** conceptualization, investigation, formal analysis, writing – original draft preparation, writing – review & editing. **Timothy Jackson**: molecular work, writing – original draft preparation, writing – review & editing. **Florita Flores**: conceptualization, writing – review & editing. **Elsa Antunes**: resources, writing – review & editing. **Muhammad Azmi Abdul Wahab**: conceptualization, writing – review & editing, funding acquisition. **Andrew P. Negri**: conceptualization, writing – review & editing, funding acquisition. **Guillermo Diaz-Pulido**: conceptualization, writing – original draft, writing – review & editing, funding acquisition.

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Data availability The data generated from this study are openly available in AIMS Data Repository at https://doi.org/10.25845/gh39-nf23. The sequences were deposited in GenBank (Accession numbers in Supplementary Table 2).

Declarations

Competing interests The authors declare no competing interests.

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