Fitness of *Cassiopea polyps* Inoculated with Different Types of Symbionts

Mu'alimah Hudatwi[1]*, Diah Permata Wijayanti[2], Ambariyanto Ambariyanto[2], Michio Hidaka[3]

[1]Marine Science Department, Faculty of Agriculture Fisheries and Biology, Universitas Bangka Belitung
Kampus Terpadu Balunijuk, Merawang Bangka - Prov Kep. Bangka Belitung, Indonesia
[2]Faculty of Fisheries and Marine Science, Universitas Diponegoro
Jl. Prof. H. Soedharto, SH, Tembalang, Semarang, 50275 Indonesia
[3]Faculty of Science, University of the Ryukyus
〒903-0213 1, Sembaru, Nakagami Gun Nishihara Cho, Okinawa Ken, Japan
Email: alihudatwi@gmail.com

Abstract

The specificity of the relationship between cnidarian hosts and symbiotic dinoflagellates (zooxanthellae) differs among host species. Some cnidarian hosts can establish symbiotic relationship with various types of zooxanthellae, while others exhibit high fidelity to specific symbiont type. It is not known how compatibility or specificity of the relationship is determined. We hypothesized that some cnidarian hosts select symbiont type that leads to highest fitness when the host is flexible with symbiont type and more than one types of symbionts are available. As a first step to study this possibility, compatibility of clonal polyps of Cassiopea sp. with six strains of cultured zooxanthellae and the fitness of the host associated with different types of symbionts were studied. Polyp diameter was measured and the number of asexual buds were calculated as a measure of host fitness. The number of zooxanthellae in host and in asexual buds was also measured as a measure of symbiont fitness. Three strains KB8 (clade A), Y106 (clade A), and K100 (clade B) were compatible with the Cassiopea polyps, while other three strains, Y103 (clade C), K111 (clade D), and K102 (clade F) were incompatible. No clear difference in the fitness was found among the polyps inoculated with compatible and incompatible symbiont strains. In one experiment, a compatible strain Y106 seemed to decrease host fitness, but this should be checked by further studies. This study suggests that feeding regimes and long observation period might be important when fitness of hosts associated with different types of symbionts is investigated.

Keywords: *Fitness, Cassiopea, Symbiont, Symbiosis*

Introduction

Coral reef is very complex ecosystem which comprises of various associations among all levels of organisms within the ecosystem (Thampi *et al*., 2018). This ecosystem is a home for many organisms, such as soft and hard corals, algae, marine vertebrates (fish), jellyfish, mollusc, echinoderm, and other benthic organisms. It is also known that coral reef also provides human with physical, chemical, economical, and biological benefits (Hoegh-Guldberg, 1999). The main composer of this rich ecosystem is the cnidarians group including corals and sea anemones. One important association which supports the sustainability of coral reefs is between cnidarians (host) and zooxanthellae (symbionts) (Davy *et al*., 2012). This interaction is symbiotic interaction where the microalgae recycle the hosts inorganic metabolic waste products and provide the hosts with part of carbon and energy requirements through translocation of photosynthetically fixed carbon (Pitt *et al*., 2009). The interaction between jellyfish and dinoflagellate algae can be found in one coronate jellyfish and rhizostome genera Mastigias, Phyllorhiza and Cassiopea (Pitt *et al*., 2009).

Many cnidarian hosts form symbioses with one or more symbiont clade types, and this interaction is affected by exogenous factors (geographic location and environmental condition) (LaJeunesse *et al*., 2018). The interaction between host and symbiont is not always in positive form (Pearse and Muscatine, 1971; Davies, 1984; Ambariyanto, 1997), since several researchers reported that there is a chance of zooxanthellae to become parasitic to the host (Sachs and Wilcox, 2006; Stat *et al*., 2008; Baker *et al*. 2018). Stat *et al*. (2008) suggested that clade A *Symbiodinium* are functionally less beneficial to corals than the dominant clade C Symbiodinium and may represent parasitic rather than mutualistic symbionts.

The objective of this research was to study compatibility between *Cassiopea* polyps and six strains of cultured zooxanthellae and to investigate

*) Corresponding author

https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.27.2.151-158
Received : 10-11-2021
Accepted : 12-01-2022

whether symbiont type affects the host fitness. First, we studied compatibility of different types of symbionts *Cassiopea* host measuring their growth rate in the host polyps. Second, we investigated the fitness of *Cassiopea* polyps associated with different types of zooxantellae measuring the rate of bud production and growth of polyps.

Materials and Methods

The methods of this research consisted of four phases; first phase was preparation of clonal polyps of *Cassiopea* sp. Vegetative buds were collected from a single Schyphistomae polyp and induced to metamorphose into polyp stage. Second phase was preparation of aposymbiotic, clonal polyps of *Cassiopea* sp. The new buds and newly formed polyps were kept in darkness and checked for the absence of symbiont before use for the experiment. Third phase was inoculation of aposymbiotic polyps with *Symbiodinium* of different clades derived from different host species. Last phase was fitness measurement of symbiont and *Cassiopea* host, respectively.

Preparation of clonal jellyfish polyps

In order to obtain genetically same, aposymbiotic *Cassiopea* polyps, a single zooxanthellate polyp of *Cassiopea* sp. was put in a plastic chamber (186 ml) filled with filtered seawater (0.22 um) and maintained in a dark incubator at 22±1°C. The polyp was fed with Artemia for 6 h every fourth days and seawater was replaced with fresh filtered seawater after feeding. The polyp produced asexual buds, which detached from the polyp. The asexual buds were kept in the same chamber until they metamorphosed into polyps. When the number of asexual polyps increased more than 20, they were then put into different chambers. Some of them contained zooxanthellae but other polyps were aposymbiotic. Aposymbiotic polyps were selected under an epifluorescent microscope (Nikon AZ100) and were put in wells of 24-well plates (one polyp in each well).

Two repeated experiments were done under slightly different conditions and observation period. The method was slightly modified in the second experiment. First experiment was conducted for 13 days after inoculation and second experiment for 23 days. In the first experiment, polyps were fed every third days with unlimited food (ca 50 *Artemia* nauplii), but in the second experiment, polyps were fed with limited (7-11 *Artemia* nauplii) food every fourth days. Seawater was changed after feeding period (2 h) in both experiments. The observation in the first experiment was done every third days, while in the second experiment observation was made for first three days and then at the end of the experiment (on 23rd day). All polyps were kept in darkness to avoid possible zooxanthellae infection, except when feeding and observing.

Inoculation of zooxanthellae

Thirty-five aposymbiotic polyps were prepared for inoculation of zooxanthellae. Thirty of them were used for zooxanthellae inoculation and remaining 5 polyps were used for aposymbiotic control. All polyps were placed in two new 24-well plates (one polyp per well) and kept in an incubator (NK System with CCFLs light control) at 23°C (Exp. 1) or 25°C (Exp. 2) under 12:12 h light-dark cycle. Light intensity was 61-75 µmol $m^{-2}s^{-1}$. A temperature logger (HOBO Water Temp Pro V2) was placed in the incubator to record the temperature.

Six strains of cultured zooxanthellae were used to inoculate the aposymbiotic polyps. They were KB 8 (clade A), Y103 (clade A), K100 (clade B), K111 (clade C), K102 (D), and Y106 (clade F). They were kept in f/2 medium under 12:12 h light-dark cycle at 30-50 µmol $m^{-2}s^{-1}$. They were used for inoculation about 1 month after last transplantation. Information of original hosts of the cultured zooxanthellae and their taxonomic status (LaJeunesse *et al*., 2018) are given in Table 1. Although zooxanthellae of different clade belong to different genera in the family Symbiodiniaceae (LaJeunesse *et al*., 2018), here we use the terms 'zooxanthellae' or 'symbionts' to describe the present experiment.

Five polyps were inoculated with each strain of zooxanthellae. Each polyp was placed into a 96-well plate filled with 100 µL MFSW (0.22 µm). For inoculation, 100 µl zooxanthellae suspension (10^6 cells.ml^{-1}) was added to the wells and mixed by pipetting. The inoculation time was 3 h. Homogenized *Artemia* was added to enhance inoculation for the last 1.5 h (Exp. 1) or for whole 3 h (Exp. 2). After inoculation, the polyps were rinsed in MFSW in wells of 6-well plate, and then put into wells of two new 24-well plates. They were kept in the same incubator (NK System with CCFLs light control).

Compatibility and host fitness

The total number of zooxanthellae inside *Cassiopea* polyps was counted as a measure of symbiont compatibility. The counting was done by summing up the numbers of zooxanthellae in 2 images (upper half and lower half views) of *Casiopea* polyp under epifluorescence microscope (NIKON AZ 100) with 4x magnification lens. The number of zooxanthellae was counted on the fluorescence pictures using a hand counter when the number of zooxanthellae was relatively low, less than one

hundred. When more zooxanthellae were present in the polyps, automatic cell counting was done using imgaeJ cell counting of ITCN (Image-based Tool for Counting Nuclei) program. The ITCN cell counter detects the dark peak of fluorescence image and counts the number of cells. Specific growth rate of zooxanthellae was obtained from the linear regression of semi-logarithmic plot of the number of zooxanthellae vs time.

Fitness of host polyp was determined by counting the number of buds released by each polyp (Sachs and Wilcox, 2006). Growth rate of polyps was determined by measuring the oral diameter of *Cassiopea* polyps. The diameter was measured by using imageJ software (ver.1.48) as distance from edge to edge of the calyx. To obtain good and steady image of polyps, individual jellyfish polyp was placed in the hole of a hole slide glass covered with another normal flat glass slide and both glass slides were fixed using a rubber ring. The polyp was allowed to stand for ca 2 minutes before taking pictures.

Statistical analysis was performed using software SPSS 16.0 (statistical package for social science) for Windows. In the first experiment, symbiont fitness (number of zooxanthellae), polyp diameter, and total budding of first experiment were analysed by one-way analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) test. In the second experiment, the number of zooxanthellae in each polyp was compared among different treatment groups using ANOVA and Tukey multiple comparison test, while budding number and polyp diameter were done by Mann-Whitney test.

Results and Discussion

The aposymbiotic polyps successfully took up the zooxanthellae together with Artemia. In the first experiment, the inoculation during the first 1.5 h was done without homogenized Artemia, and most polyps did not take up the zooxanthellae cells during this period. Addition of homogenized Artemia to the zooxanthellae culture stimulated polyps to ingest the algae. Algal suspension of the same cell density was given to each polyp, but the number of algal cells ingested by polyps was different among zooxanthellae strains.

Symbionts resided in the upper gastric region when they were first taken up by the polyps. Compatible symbionts proliferated inside the host tissue and spread through all over the gastric region, and then to proximal part of the tentacles and stalk (Figure 1, strain KB8 clade A). Figure 1d shows that zooxanthellae transferred into a newly formed bud. Incompatible symbionts at first resided inside the upper part of gastric region of the polyps, but the number of zooxanthellae decreased and vanished almost completely from the host polyps (Figure 2, strain K102 clade F). Sometimes aggregates of zooxanthellae cells were attached to the pedal disc, but the polyps did not ingest them again. It is not clear whether the incompatible zooxanthellae were expelled or digested by the *Cassiopea* polyps.

Compatibility between Cassiopea polyps and six types of symbionts

Cassiopea polyps could take up all the 6 strains of zooxanthellae, but the number of zooxanthellae ingested was different among the strains of the symbionts. Figure 1a and C show the number of zooxanthellae present in *Cassiopea* polyp at the end of the observation period (13th day and 23rd day after inoculation in Exp 1 and Exp 2, respectively).

In the Exp 1, strains KB8 (clade A), Y106 (clade A) and K100 (clade B) were present at high density in the polyps. But clade A strains (KB8 and Y106) were found at higher density than K100 (clade B). Small number of Y103 (clade C) cells were present, but K111 (clade D) and K102 (clade F) were not found in the polyps (Figure 3a). Polyps inoculated with strains KB8, Y106, and K100 started with relatively high initial symbiont density, while those inoculated with strains Y103, K111, and K102 showed low number of ingested cells (Table 1). In Exp. 2, strains KB8 (clade A), Y106 (clade A) and K100 (clade B) were present at high density in the polyps. KB8 (Clade A) symbionts were present at significantly higher density than K100 (clade B). Other three strains (Y103, K111, and K102) were not found in the polyps on 23 days after inoculation, though small number of cells of these strains were found on the next day of inoculation (Table 1.).

The specific growth rates of strains KB8, Y106, and K100 were positive while those of strains Y103, K111, and K102 were negative in both Exp. 1 and Exp. 2 (Figure 3b, d). In Exp. 1, significant difference was found in the proliferation rate among three strains KB8, Y106, and K100) (ANOVA and Tukey multiple comparison test, $P<0.05$), while there was significant difference only between KB8 and K100 in Exp. 2.

The results of both experiments show that the three strains belonging to clade A or B are compatible with the *Cassiopea* polyps, while other three strains belonging to clade C, D, or F are incompatible strains. Lampert (2016) stated that *Cassiopea* polyps seem to be very flexible in their symbiont uptake and that,

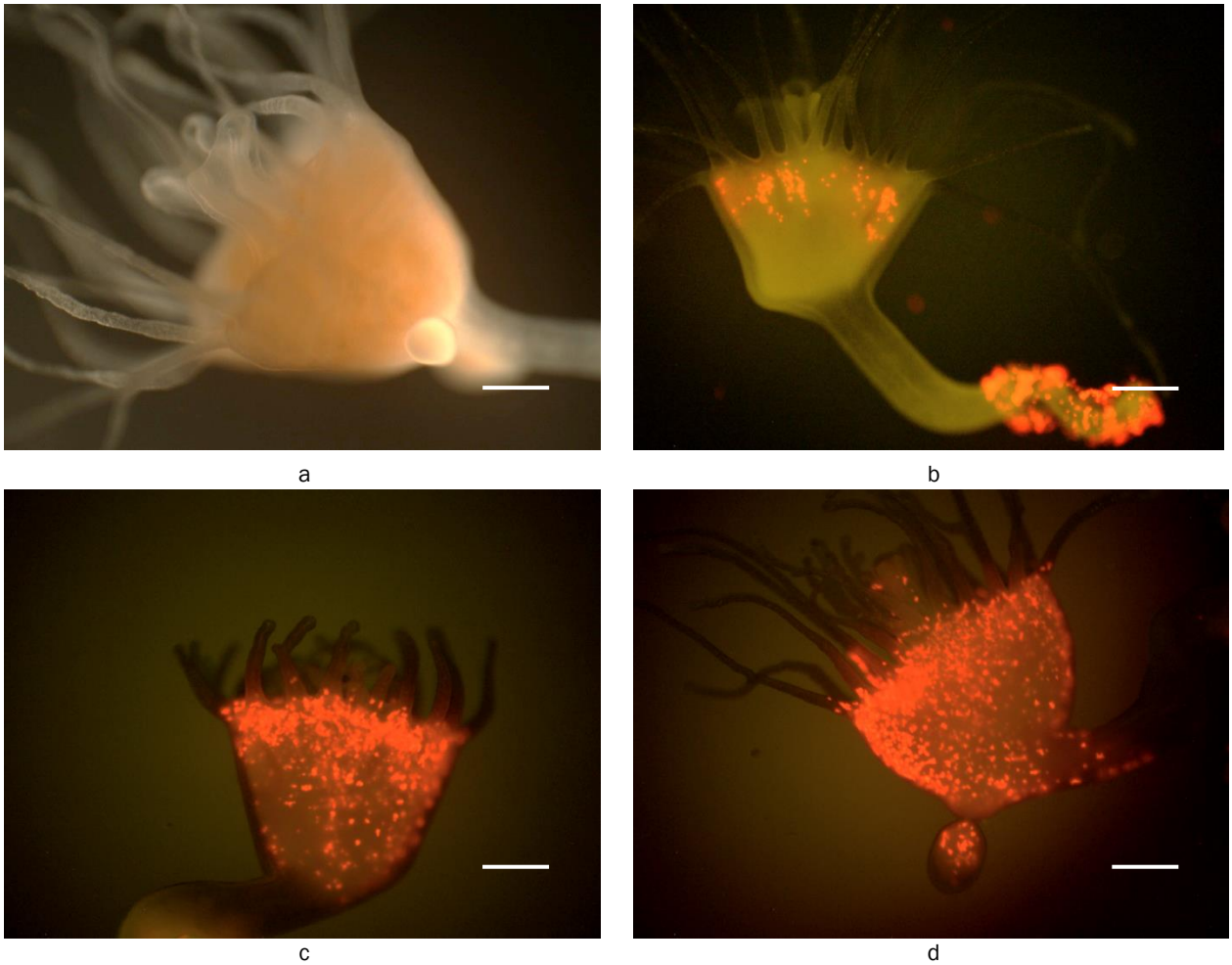

Figure 1. The spreading pattern of a compatible symbiont KB8 strain (clade A) in a *Cassiopea* polyp in Exp.1. a = before inoculation. b-d = after inoculation. b = Symbiont first appeared at the upper gastric region; c = spread to whole gastric region; d = finally spread to the proximal parts of tentacles and stalk. Scale bars: 0.3 mm in a, 0.5 mm in b-d.

Table 1. Symbiont density in the *Cassiopea* polyps on the next day of inoculation and at the end of observation period. (Mean ± SE, n=5).

Exp. 1		Symbiont density (cells.polyp^{-1})	
Symbiont strain*	Symbiont taxon based on Ref1**	1 day after inoculation	13 day after inoculation
KB8 (clade A)	*Symbiodimnium* sp.	64 ± 17	1516 ± 270
Y106 (clade A)	*Symbiodimnium* sp.	107 ± 24	565 ± 97
K100 (clade B)	*Breviolum* sp.	103 ± 35	149 ± 40
Y103 (clade C)	*Cladocopium* sp.	9.2 ± 2.2	3.8 ± 1.7
K111 (clade D)	*Drusdinium* sp.	0.8 ± 0.4	0.4 ± 0.4
K102 (clade F)	*Fugacium* sp.	4.8 ± 1.7	0

Exp. 2		Symbiont density (cells.polyp^{-1})	
Symbiont strain	Symbiont taxon based on Ref1**	1 day after inoculation	23 day after inoculation
KB8 (clade A)	*Symbiodimnium* sp.	118 ± 48	5827 ± 1473
Y106 (clade A)	*Symbiodimnium* sp.	72 ± 15	2488 ± 707
K100 (clade B)	*Breviolum* sp.	117 ± 26	676 ± 329
Y103 (clade C)	*Cladocopium* sp.	16.2 ± 2.3	0
K111 (clade D)	*Drusdinium* sp.	3.6 ± 1.0	0
K102 (clade F)	*Fugacium* sp.	8.6 ± 4.5	0

*Original host of symbionts: KB8 (*Cassiopea* sp.), Y106 (*Tridacna crocea*), K100 (*Aiptasia pulchella*), Y103 (*Fragum* sp.), K111 (*Sarcophyton glaucum*), K102 (*Montipora verrucosa*); ** Ref1: LaJeunesse *et al*. (2018))

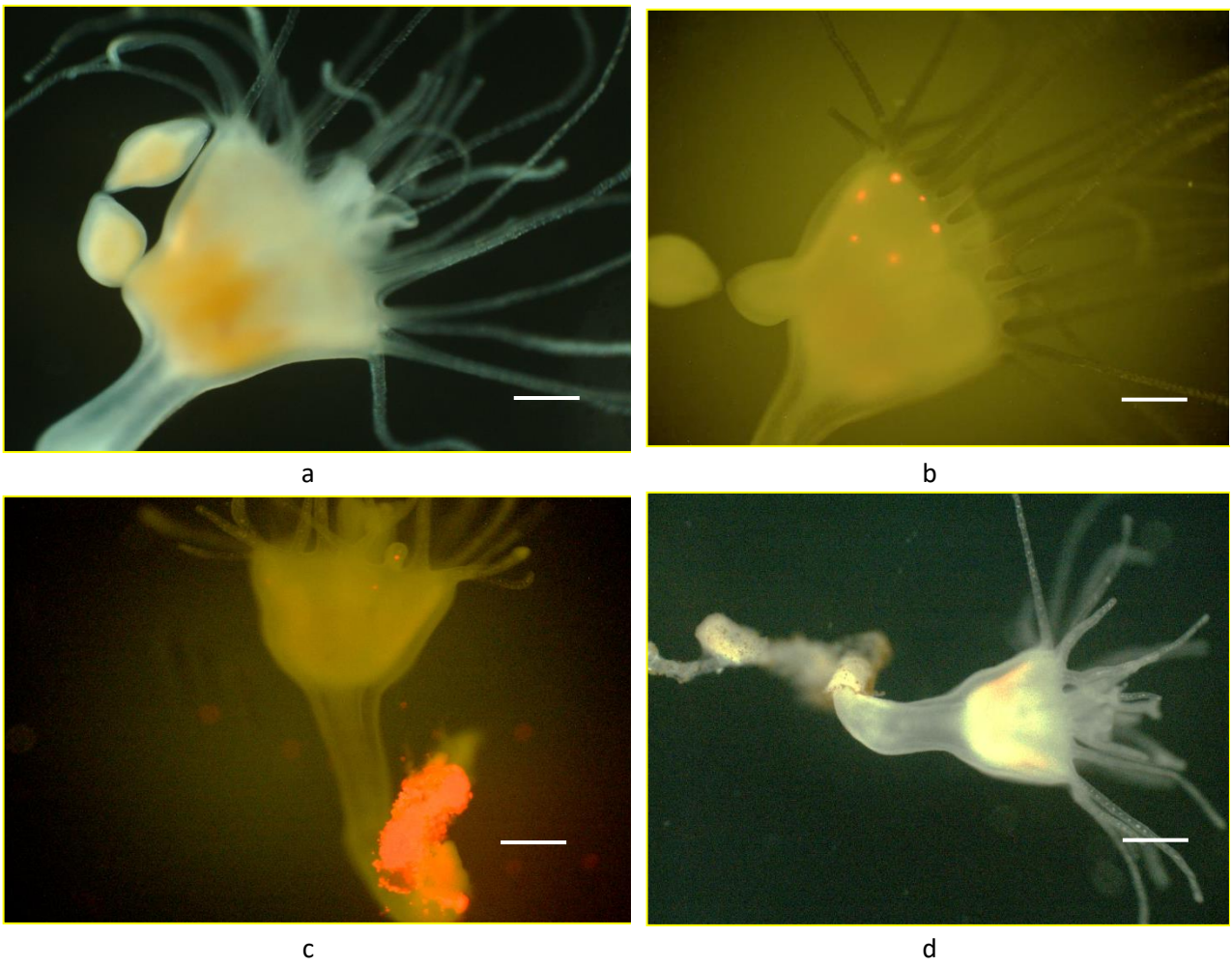

Figure 2. The incompatible symbiont K102 strain (clade F) failed to grow in the *Cassiopea* polyp during Exp.1. (a) before inoculation. b-d, after inoculation. Although the polyp ingested a small number of symbionts on the first day of inoculation (b), zooxanthellae decreased in number (c) and finally lost on tenth day after inoculation (d). Scale bars: 0.5 mm.

if more than one clade was present in the water, all available clades would be found in the polyps. Our results also show that *Cassiopea* polyps can take up all the symbiont strains initially. But three strains (clade A and B) proliferated in the polyps, while other three strains (clade C, D, and F) disappeared from the polyps in 13-23 days after inoculation. The polyps we used were asexually produced via budding and it is an interesting question whether sexually produced polyps, which might be younger than asexually produced polyps, exhibit more flexibility with symbiont types.

Fitness of host polyps inoculated with different strains of symbionts

The number of asexual buds produced by each polyp during the observation period (13 days in Exp. 1 and 23 days in Exp. 2) was used as a measure of host fitness. In the Exp. 1, no remarkable difference in the number of buds was found among polyps inoculated with different algal strains or the control polyps except in one case: polyps inoculated with K102 (clade F) produced significantly less buds than the control polyps (Figure 4a). In the Exp. 2, the number of buds produced during the 23-day observation period appeared to vary among polyps inoculated with different types of symbionts. However, significant difference was found only between polyps inoculated with Y106 and the control polyps: polyps with Y106 produced smaller numbers of buds compared with the control polyps (Mann-Whitney test, $P<0.05$).

Diameters of polyps were also measured in an attempt to use them as a measure of host growth rate. However, the changes in the polyp diameter appeared almost the same among the polyps inoculated with different symbiont strains or without inoculation (Figure 4c and Figure 4d.). No significant

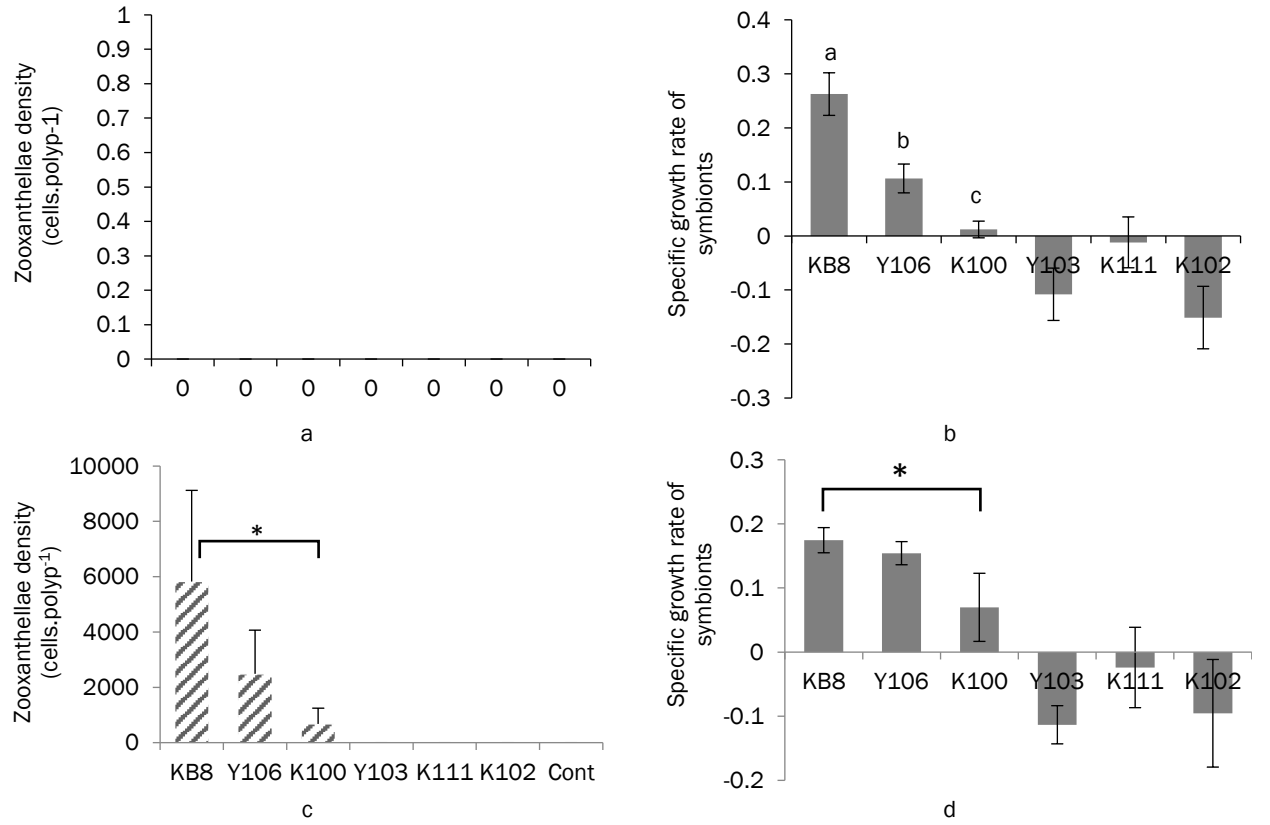

Figure 3. Density and specific growth rate of different types of symbionts in *Cassiopea* polyps. A and C, The number of symbionts in the polyps at the end of the observation period (13th day in Exp 1 (A) and 23rd day in Exp 2 (C). B and D, specific growth rate of symbionts during the observation period in Exp 1 (B) and Exp 2 (D). Different letters in A & B and the asterisk in C & D indicate significance differences (Tukey HSD test, $p<0.05$).

difference was found in the polyp diameter among polyps inoculated with different types of symbionts and the control polyps both on the last day of the experiments (day 13 in Exp. 1, and day 23 in Exp. 2). The increase in polyp diameter was small, in the range of 0.2–0.5 mm after 23 days maintenance in Exp. 2.

In Exp. 1, polyps inoculated with incompatible symbiont strains (Y103, K111, and K102) and the control polyps without inoculation produced almost the same number of buds as those inoculated with compatible symbionts (KB8, Y106, and K100). There was no significant difference in the polyp diameter at the end of the experiment. These suggests that the presence of compatible symbionts did not increase the number of buds produced in this experimental condition (ca 50 Artemia nauplii were applied to each polyp every third day).

In Exp. 2, we tried to limit food by applying 7-11 Artemia nauplii to each polyp every fourth day. Contrary to our expectation, we could not find tendency that polyps inoculated with compatible symbionts produced more buds than those inoculated with incompatible symbionts or the control polyps without inoculation. No significant difference in the polyp diameter was found among polyps inoculated with different symbiont strains at the end of the experiment. It is difficult to explain why polyps inoculated with Y106 (clade A) produced less buds than the control polyps. This may suggest that Y106 can proliferate in the polyp but give harmful effect on the host polyp. However, in the Exp. 1, polyps inoculated with Y106 produced almost the same number of buds as those inoculated with other symbiont strains. So, further study is necessary to conclude whether Y106 is parasitic or not.

Lampert (2016) stated that, if only one single clade of algae was present, polyps survived best with clades A or B and polyps with clade D died rather quickly. It is likely that there is difference in fitness among polyps with different strains of symbionts. It is possible that we could not detect difference in fitness among polyps with different symbiont strains because polyps were able to acquire enough nutrition from feeding on Artemia nauplii even in Exp. 2 where we intended to limit food supply. Hoffman *et al*. (1978) mentioned that food allocation and environmental condition affect the number of bud production in *Cassiopea andromeda polyps. Fitt and Costley (1998) mentioned that scyphistomae stage*

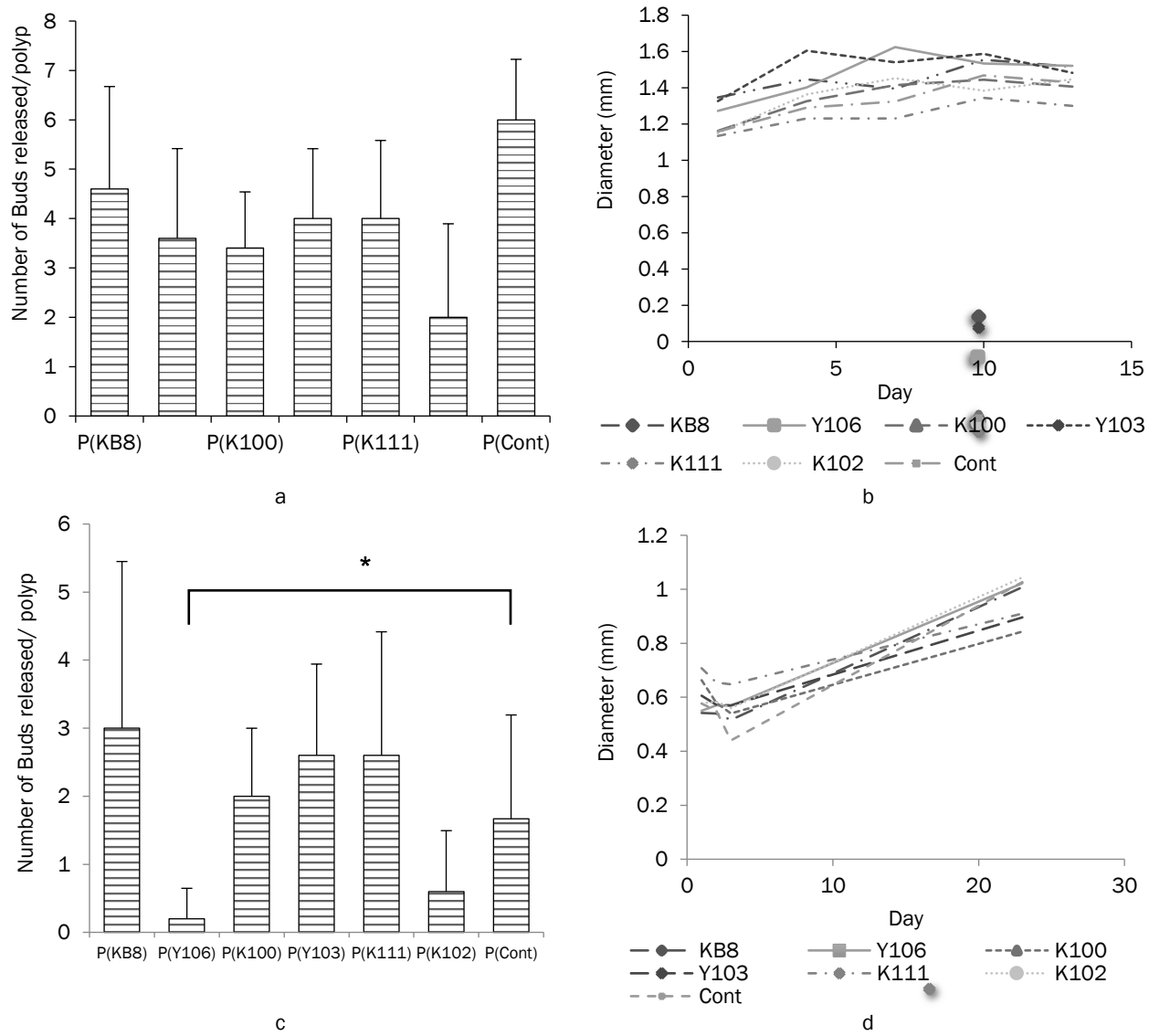

Figure 4. Fitness of *Cassiopea* polyps associated with different symbiont types in Exp. 1 (A and B) and Exp. 2 (C and D). A and C, the number of buds released from the polyps on 13th day with unlimited *Artemia* feeding (A) and on 23rd day with limited *Artemia* feeding (C). B and D, Changes in the oral diameter of polyps in Exp. 1 (B) and Exp. 2 (D). P(KB8) indicates the polyps inoculated with KB8 strain and so on. Asterisks indicate significant difference from the control (Mann-Whitney test, $p<0.05$).

of Cassiopea rely on food supply from outside, because they cannot fulfill all needed nutrition for growing and reproduction from photosynthetic products translocated by algal parter.

The present study shows that the number of buds released from the polyps as well as the oral diameter of each polyp may be a good index to measure host fitness (Sachs and Wilcox, 2006), but that careful control of feeding regimes and light conditions as well as longer time of observation period would be necessary to study the effect of different symbiont strains on fitness of *Cassiopea* polyps. *Cassiopea* polyps would serve as a good model to investigate symbiotic relationship between cnidarian host and symbiotic algae, especially effects of different symbiont types on the fitness and stress tolerance of the holobiont (Sachs and Wilcox, 2006; Lampert, 2016; Ohdera *et al*., 2018).

Conclusion

This study shows that the interaction between *Cassiopea* jellyfish polyps and algal symbionts depending on the type of zooxanthellae. The six strains of zooxanthellae showed different compatibility with the *Cassiopea* polyps. KB8 (clade A), Y106 (clade A), and K100 (clade B) proliferated in

the polyps while other 3 strains were incompatible and did not proliferate in the polyps. No significant difference in host fitness was found between polyps inoculated with compatible symbionts and those inoculated with incompatible symbionts. This suggests that further limitation of food and longer observation period would be necessary to detect possible differences in host fitness.

Acknowledgement

Thanks the Double Degree Program between Diponegoro University and University of the Ryukyus, which enabled first author to do the present research.

References

Ambariyanto, A., 1997. The role of symbiotic zooxanthellae on giant clam nutrition. *J. Coast. Dev.,* 1(1): 43-48.

Baker, D.M., Freeman, C.J., Wong, J.C.Y., Fogel, M.L. & Knowlton, N. 2018. Climate change promotes parasitism in a coral symbiosis. *The ISME Journal,* 12: 921–930. https://doi.org/10.1038/s41396-018-0046-8.

Davies, P.S. 1984. The role of zooxanthellae in the nutritional energy requirements of *Pocillopora eydouxi*. *Coral Reefs*, 2(4): 181-186.

Davy, S.K., Allemand, D. & Weis, V.M. 2012. Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol. Mol. Biol. Rev.,* 76: 229-261.

Fitt, W.K. & Costley, K. 1998. The role of temperature in survival of the polyp stage of the tropical rhizostome jellyfish *Cassiopea xamachana*. *J. Exp. Mar. Biol. Ecol*. 222: 79-91.

Hoffman, D.K., Neumann, R. & Henne, K. 1978. Strobilation, budding and initiation of scyphistoma morphogenesis in the rhizostome *Cassiopea andromeda*. *Mar. Biol.* 47: 161-176.

LaJeunesse, T.C., Parkinson, J.E., Gabrielson, P.W., Jeong, H.J., Reimer, J.D., Voolstra, C.R. & Santos, S.R. 2018. Systematic revision of Symbiodiniaceae highlights the antiquity and diversity of coral endosymbionts. *Current Biol.,* 28: 2570-2580.

Lampert, K.P. 2016. *Cassiopea* and its zooxanthellae. Pp 415-424 In: Goffredo, S., & Dubinsky, Z. (ed.) 'The Cnidarita, Past, Present and Future' Springer International Publishing Switzerland, https://doi.org/10.1007/978-3-319-31305-4_26.

Ohdera, A.H., Abrams, M.J., Ames, C.L., Baker, D.M., Suescún-Bolívar, L.P., Collins, A.G., Freeman, C.J., Gamero-Mora, E., Goulet, T.L., Hofmann, D.K. & Jaimes-Becerra, A., 2018. Upside-down but headed in the right direction: review of the highly versatile *Cassiopea xamachana* system. *Front. Ecol. Evol*., 6: p35.

Pearse, V.B. & Muscatine, L. 1971. Role of symbiotic algae (zooxanthellae) in coral calcification. *The Biol. Bull.,* 141(2): 350-363.

Pitt, K.A., Welsh, D.T. & Condon, R.H. 2009. Influence of jellyfish blooms on carbon, nitrogen and phosphorus cycling and plankton production. *Hydrobiologia,* 616: 133–149.

Stat, M., Morris, E. & Gates, R.D. 2008. Functional diversity in coral-dinoflagellate symbiosis. *Proc. Nat. Acad. Sci. USA,* 105(27): 9256-9261.

Sachs, J. L. & Wilcox, T.P. 2006. A shift to parasitism in the jellyfish symbiont *Symbiodinium microadtriaticum*. *Proc. Royal Soc. London, B.* 273: 425-429.

Thampi, V. A., Anand, M. & Bauch, C. T. 2018. Socio-ecological dynamics of Caribbean coral reef ecosystems and conservation opinion propagation. *Sci. Rep.*, 8: 2597. http://doi.org/10.1038/s41598-018-20341-0