Improving the quality of tridacnid clam juveniles through crossbreeding broodstock from different zones across the Spermonde Archipelago

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Abstract. Tridacnid clam populations have declined, and they are considered as endangered. There is increasing concern over their status in the Spermonde archipelago, South Sulawesi, where some species can no longer be found, including larger species such as Tridacna gigas and T. dera sa as well as the shallow-water clam Hippopus hippopus. This research aimed to produce high-quality juvenile tridacnid clams by crossbreeding distantly related broodstock from different zones as a contribution to the effective conservation of endangered species. The research was conducted in the Spermonde archipelago and at the Hasanuddin University Marine Station Hatchery in Barrang Lompo Island. Broodstock of T. dera sa and T. squamosa were collected from zones III and IV of the Spermonde Archipelago. Juvenile clam production followed established methods for inducing spawning and larval rearing through trocophore, veliger, and pediveliger stages to produce juveniles that were reared in land-based and ocean nurseries. Parameters measured were the juvenile survival rate and the quality of zooxanthellae (density, chlorophyll content, and mitotic index). Data were analyzed descriptively. The mean density of zooxanthellae in juvenile clams was 3.17 x 10^6 cells/ind (range 3.15x10^6 - 5.27x10^6 cells/ind). The mean chlorophyll-a content of these zooxanthellae was 2.7 mg/m^3 (range 1.1 mg/m^3 - 4.1 mg/m^3), which is consistent with the mean phaeopigment concentration of 5.5 mg/m^3 (range 4.2 mg/m^3 - 6.2 mg/m^3). The mitotic index peaked at 8.5% between 09:00 and 12:00.

1. Introduction

The tridacnid clams are large tropical bivalve mollusks belonging to the Family Tridacnidae [1,2]. Often referred to collectively as giant clams, the real giant clam is Tridacna gigas [3]. An important food source since prehistoric times[4], increased levels of exploitation in recent decades, are widely considered unsustainable [1,5,6]. Tridacnid clam population declines are reported across much of their respective ranges, to the point where larger species, especially T. gigas (but also T. dera sa and Hippopus hippopus), are considered vulnerable to extinction or at least extirpation [1,6–8]. Of the eleven recognized tridacnid clam species worldwide [1,9], the World Conservation Union’s Red List of Threatened Species (http://www.iucnredlist. org) lists eight species as either “Lower Risk Conservation Dependent” or “Vulnerable,” with T. crocea classified as ‘Lower Risk Least Concern’; however, these assessments (in 1996) can be considered incomplete and out-of-date. The recently described species T. costata, [8], and the recently resurrected T. noae [10] have not been assessed. It is possible there may be further cryptic species [2].

Seven tridacnid clams are reported from Indonesia: the giant clam T. gigas; smooth giant clam T. dera sa; the fluted clam T. squamosa; the maxima clam T. maxima, the boring clam T. crocea, the
horse’s hoof clam \( H. \text{hippopus} \), and the china clam \( H. \text{porcelainus} \) [1,5]. Three species have restricted known ranges: the tevoroa clam \( T. \text{mbalavuana} \) is found in Fiji, \( T. \text{rosewateri} \) in the Indian Ocean, and \( T. \text{costata} \) in the Red Sea [1,5,8]. The distribution of \( T. \text{noae} \) is unclear [10], but could include the Indonesian Archipelago.

Although all seven reported species are still found at some sites in the Indonesian Archipelago, some species, especially \( T. \text{gigas} \), had been extirpated from several areas in Indonesia (and other countries) by the early 1990s [4]. Since then, many studies have found evidence of overexploitation and other threats to tridacnid clams in Indonesia. In particular, surveys in the Spermonde Archipelago in 2007 found strong indications of overexploitation, particularly of the larger species \( T. \text{gigas} \), \( T. \text{derasa} \), and \( H. \text{porcelainus} \) (Niartiningsih et al., unpublished data 2007). The giant clam \( T. \text{gigas} \) has not been found in more recent surveys in the Spermonde Archipelago. In 2010 [11], only four and tridacnid clam species were found (\( T. \text{squamosa} \), \( T. \text{maxima} \), \( T. \text{crocea} \), and \( H. \text{hippopus} \)), while surveys in 2013-3014 also found very few \( T. \text{derasa} \) [12].

Captive breeding has been developed in several countries as a tool to support tridacnid clam conservation, for example, Australia, Hawaii, the Marshall Islands, Solomon Islands, and Seychelles [5,13–15]. In Indonesia, the Hasanuddin University Marine Station Hatchery on Barrang Lompo Island has successfully cultured several tridacnid clam species [6,16]. Low population densities will likely reduce the chances of fertilization and make inbreeding more likely in the wild, a risk that needs to be taken into account to minimize the likelihood of collecting closely-related broodstock from the wild for captive breeding programs [6]. Inbreeding could result in lower quality offspring, while the limited genetic diversity could make restocked clams more vulnerable to environmental fluctuations and disturbances, especially in the context of global climate change, which may pose significant challenges for tridacnid clam recruitment [17].

Research on the genetic structure of giant clam populations found substantial genetic distances between adult tridacnid clams from two zones in the Spermonde Archipelago (Niartiningsih et al., unpublished data 2007). That finding opens up the possibility of improving offspring quality through crossing tridacnid clam broodstock from the two zones in order to avoid inbreeding. High-quality juvenile clams could be used to support rare species conservation programs in the area [16,18,19]. The aim of this research was to test that hypothesis through crossbreeding distantly-related tridacnid clams (broodstock) from these two zones and to evaluate the quality of the juvenile clams produced in terms of survival rate and their symbiotic zooxanthellae (\( \text{Symbiodinium sp.} \)).

2. Methodology

2.1. Study site and broodstock collection

The research was carried out at the Hasanuddin University Marine Station hatchery on Barrang Lompo Island in the Spermonde Archipelago, Makassar City, South Sulawesi Province, Indonesia. Tridacnid clam broodstock was collected from Zone 3 (outer, middle zone) and Zone 4 (outer zone) of the Spermonde Archipelago (Figure 1). The species propagated were determined by the availability of suitable adult clams in these two zones. Based on [20], broodstock was collected from eight islands in Zone 3 (Sarappo Lompo, Kodingareng Keke, Pala, Sarappo Keke, Cangke, Badi, Lumu-Lumu and Bone Tambung Islands) and two islands in Zone 4 (Langkai and Lanjukang Islands).

Tridacnid clam broodstock was collected by SCUBA diving, using a swept area or belt transect method to observe target organisms in a specified area. The rectangular transects were made by rolling out a 100 m tape, and recording tridacnid clams up to 2.5 m either side of the tape by species. Clams were collected at two depth zones. The shallower transect was laid along the reef crest, at the transition between the reef flat and reef slope. The deeper transect was laid at 5-10 m or 7-12 m depending on reef conditions. The location of each transect was recorded using a GPS unit.
2.2. Production of juvenile tridacnid clams

Juvenile tridacnid clams were produced following [21], [22], and [20]. Spawning was induced through a combination of temperature shock and injection with a serotonin solution. Larviculture (trocophore, veliger, and pediveliger stages) and juvenile husbandry followed [21] and [20].

Survival rate was recorded during larval stages (trocophore, veliger, and pediveliger) as well as during the juvenile phase in the land-based nursery. Additional parameters measured during the juvenile phase to evaluate juvenile quality were zooxanthellate density, chlorophyll-a concentration, and mitotic index.

Water quality data were also collected daily during the larval phase and weekly during the juvenile phase. The parameters measured were water temperature (Bulk Packaged H-6S mercury thermometer), pH (Digital pH meter 0:00-14:00), salinity (Atago Master-93H refractometer), dissolved oxygen (DO, Hanna Hi 9146 portable DO meter), as well as ammonia (NH3), nitrate (NO3) and phosphate (PO4) concentrations measured through titration in the Marine Chemistry Laboratory of the Marine Science Department, Hasanuddin University.

2.3. Zooxanthelate density

The density of the symbiotic zooxanthellae in the mantles of juvenile clams was measured following [23] as modified by [24]. Each sampled juvenile tridacnid clam was homogenized to form a suspension in 100 ml of filtered seawater. The density of suspended zooxanthellae (Symbiodinium sp.) was measured using a hemocytometer under a microscope, with five replicates.

2.4. Chlorophyll-a and phaeopigment concentration

The chlorophyll-a concentration in juvenile tridacnid clams was measured using a trichromatic method [25] as modified by [24]. Each sampled juvenile tridacnid clam was homogenized to form a suspension in 100 ml of filtered seawater. The suspension was then filtered through Millipore paper (diameter 47 mm, pore size 0.45 µm) in a filter holder. To extract the pigments from within the zooxanthellae, which were retained on the filter paper, the paper was placed with 10 ml acetone 90 % in an electrical Eberback tissue grinder at 500 rpm for 2 minutes. The extract was then centrifuged at 2500 rpm for 30 minutes. The absorbance of the supernatant was read from a spectrophotometer (Mequeaturner Model 340 VIS) at wavelengths of 750, 664, 647, and 630 nm, and acetone 90 % was used as a blank (control). In order to ascertain whether the observed chlorophyll had become
degraded, the phaeopigment concentration was also measured using the same spectrophotometric method. After the chlorophyll–a was measured, a drop of hydrochloric acid (HCl) was added before repeating the spectrophotometric reading process.

2.5. Mitotic index of the zooxanthellae
The mitotic index of the zooxanthellae living in symbiosis within the body tissues of the juvenile tridacnid clams was determined following [26] as modified by [24]. Each sampled juvenile tridacnid clam was homogenized to form a suspension in filtered seawater. Stratified filtering was carried out to separate the zooxanthellae from the residue, using a series of 250, 175, and 50 µm sieves. The filtered product was placed in a measuring glass for analysis. The mitotic index was determined by counting the zooxanthellae in the process of karyokinesis or cytokinesis, which were visible as twinned cells under a microscope at 400 X magnification. Samples were observed over a 24 hour period at 03:00, 06:00, 09:00, 12:00, 15:00, 18:00, 21:00 and 24:00 with five replicates. The observations followed Becker (1986) in [27], distinguishing between two overlapping phases in cell replication. The first phase is the division of the nucleus (karyokinesis) and is followed by the physical process of cell division, which divides the cytoplasm of a parental cell into two daughter cells (cytokinesis). Data analysis. The data recorded for each parameter were analyzed descriptively and presented as tables and figures or graphs. The density of zooxanthellae was calculated using the following equation in [25]:

\[
\text{Zooxanthellae per mm}^2 = \frac{N \times A_e \times V_t}{A_c \times V_s \times A_s}
\]  
(1)

Where, \(N\) = number of counted organisms (cells/cm\(^2\)); \(A_e\) = cover glass area (mm\(^2\)); \(V_t\) = initial total sample volume (ml); \(A_c\) = scraped sample area (cm\(^2\)); \(V_s\) = volume of sample used (ml); \(A_s\) = micrometer area (mm\(^2\)).

The chlorophyll-a concentration of the zooxanthellae was calculated using the equation in [25], modified as follows:

\[
\text{Chlorophyll-a (mg/ml)} = \frac{((11.85 \times E_{664})-(1.54 \times E_{647})-(0.08 \times 630)) \times V_t}{V_s \times d}
\]  
(2)

Where, \(E_{664}\) = absorbance at 664 nm – absorbance at 750 nm; \(E_{647}\) = absorbance at 647 nm – absorbance at 750 nm; \(E_{630}\) = absorbance at 630 nm – absorbance at 750 nm; \(V_t\) = volume of the acetone extract (ml); \(V_s\) = volume of filtered water sample (ml); \(d\) = cuvette diameter (mm).

Mitotic index was calculated based on [26] and [24] using the following equation:

\[
\text{Mitotic index} = \frac{\text{Number of dividing zooxanthellae}}{50} \times 100\%
\]  
(3)

The survival rate (in %) of tridacnid clam larvae and juveniles was calculated at the end of the research period. Samples of 0.5 ml were collected from each 750-liter rearing tank with three replicates to obtain an average value representative of the entire water column. The number of living larvae and juveniles in each research unit was divided by the initial number of larvae and juveniles and multiplied by 100.

3. Results and discussion
3.1. Broodstock
The field survey only found adult specimens of two tridacnid clam species, \(T.\ squamosa\) and \(T.\ derasa\); therefore, the broodstock used in this research was limited to these two species (Table 1). The
T. derasa clams were relatively small for this species and were found in Zone 4 of the Spermonde Archipelago. This species would normally reach around 40-60 cm, but due to continued heavy exploitation, they no longer reach this size, and only two individuals could be found. The fishermen find T. derasa easy to find and collect because its byssus does not attach very firmly to the substrate. Individuals below 40 cm are not categorized as fully adult and are unlikely to be capable of producing eggs; however, they are generally capable of producing sperm and, therefore, could be used to fertilize other (larger) T. derasa individuals.

Table 1. Species and size (shell width) of tridacnid clams collected from Zones 3 and 4.

| No | Clam species | Size | Spermonde Zone |
|----|--------------|------|----------------|
| 1  | T. squamosa  | 29   | Zone 3         |
| 2  | T. squamosa  | 26   | Zone 3         |
| 3  | T. squamosa  | 40   | Zone 3         |
| 4  | T. squamosa  | 38   | Zone 3         |
| 5  | T. squamosa  | 27   | Zone 3         |
| 6  | T. squamosa  | 36   | Zone 3         |
| 7  | T. squamosa  | 39   | Zone 3         |
| 8  | T. squamosa  | 33   | Zone 4         |
| 9  | T. squamosa  | 35   | Zone 4         |
| 10 | T. squamosa  | 37   | Zone 4         |
| 12 | T. squamosa  | 35   | Zone 4         |
| 13 | T. squamosa  | 28   | Zone 4         |
| 14 | T. squamosa  | 29   | Zone 4         |
| 15 | T. squamosa  | 28   | Zone 4         |
| 16 | T. squamosa  | 30   | Zone 4         |
| 17 | T. squamosa  | 33   | Zone 4         |
| 18 | T. squamosa  | 31   | Zone 4         |
| 19 | T. derasa    | 30   | Zone 4         |
| 20 | T. derasa    | 28   | Zone 4         |

3.2. Spawning and fertilization
The response of the tridacnid clam broodstock to spawning induction by temperature shock (desiccation in full sun) was observed after around one hour. Three clams released sperm, but no clams released eggs with only temperature induction. The response of each clam to spawning induction (Table 2) shows that serotonin injection was more effective than the temperature in inducing spawning. Twelve of the twenty clams (60%) released sperm or eggs with serotonin. Of these 20 tridacnid clams, 40 % had spawned previously. The clams from Zones 3 and 4, which spawned all had a shell width greater than 28 cm.
Table 2. Spawning response of tridacnid clam broodstock to induction (temperature or serotonin).

| No | Clam species  | Shell Length (cm) | Temperature Induction | Serotonin Induction |
|----|---------------|-------------------|-----------------------|---------------------|
| 1  | *T. squamosa* | 29                | None                  | Sperm               |
| 2  | *T. squamosa* | 26                | None                  | None                |
| 3  | *T. squamosa* | 40                | None                  | Sperm               |
| 4  | *T. squamosa* | 38                | None                  | Sperm               |
| 5  | *T. squamosa* | 27                | Sperm                 | Sperm               |
| 6  | *T. squamosa* | 36                | None                  | Sperm               |
| 7  | *T. squamosa* | 39                | Sperm                 | Eggs                |
| 8  | *T. squamosa* | 33                | None                  | None                |

ZONE 4

| 9  | *T. squamosa* | 35                | None                  | Sperm               |
| 10 | *T. squamosa* | 37                | Sperm                 | Eggs                |
| 12 | *T. squamosa* | 35                | Sperm                 | Sperm               |
| 13 | *T. squamosa* | 28                | None                  | None                |
| 14 | *T. squamosa* | 29                | Sperm                 | Eggs                |
| 15 | *T. squamosa* | 28                | Sperm                 | Sperm               |
| 16 | *T. squamosa* | 30                | None                  | Sperm               |
| 17 | *T. squamosa* | 33                | None                  | None                |
| 18 | *T. squamosa* | 31                | None                  | None                |
| 19 | *T. derasa*   | 30                | None                  | None                |
| 20 | *T. derasa*   | 28                | None                  | None                |

Tridacnid eggs were sampled from the broodstock holding tanks at a rate of 0.5 ml from each 87 Litre stock tank (Table 3). The sampled eggs came from a clam with 39 cm shell length from Zone 3, 37 cm and 29 cm Shell length (SL) from Zone 4. Of these three spawned tridacnid clams, the total number of eggs produced by the single 39 cm wide clam from Zone 3 was 29,172,840 eggs, while 51,812,800 eggs were produced by the two tridacnid clams (29 and 37 cm shell width) from Zone 4 (Table 3).

Table 3. The number of eggs sampled from three giant clams from Zone 3 and Zone 4.

| Sample number | Zone 3 (n=1) | Zone 4 (n=2) |
|---------------|--------------|--------------|
| 1             | 167          | 311          |
| 2             | 171          | 321          |
| 3             | 181          | 269          |
| 4             | 201          | 376          |
| 5             | 120          | 323          |
| 6             | 166          | 343          |
| Mean          | 167.66       | 323.83       |
| Total         | 29,172,840   | 51,812,800   |

The eggs were fertilized by mixing the eggs and sperm in hygienic tanks. The results of the fertilization were observed two hours after mixing the eggs and sperm (Table 4). The fertilization data show a similar percentage of fertilized eggs for the three clams, with around 91.8% of eggs fertilized in the first 3 hours. This fertilization rate can be considered high because the rate is above 90%. It
takes a certain length of time for eggs and sperm to come together. This process seems somewhat slower in *T. squamosa* than in corals, where fertilization can reach 95% during the second hour [28].

**Table 4.** Data on fertilization of eggs from clam broodstock collected in Zone 3 and Zone 4.

| Sample number | Zone 3 Clam | Zone 4 Clam |
|---------------|-------------|-------------|
|               | Fertilized  | Not yet fertilized | Fertilized | Not yet fertilized |
| 1             | 37          | 4            | 33         | 4               |
| 2             | 87          | 11           | 52         | 1               |
| 3             | 16          | 2            | 31         | 1               |
| 4             | 62          | 8            | 24         | 2               |
| 5             | 35          | 0            | 25         | 2               |
| 6             | 68          | 8            | 54         | 4               |
| 7             | 106         | 10           | 53         | 10              |
| 8             | 120         | 10           | 76         | 7               |
| 9             | 60          | 0            | 41         | 3               |
| 10            | 81          | 7            | 29         | 3               |
| Rata-rata     | 672         | 60           | 418        | 37              |
| Total         | 91.80%      | 8.19%        | 91.86%     | 8.13%           |

### 3.3. Environmental conditions and survival rate and density

The ranges of the environmental variables measured in the *T. squamosa* nursery environment were: water temperature 28 – 34 °C; salinity 32-35 ppt; pH 7.0–8.5; DO 3.32 – 7.68 ppm; PO4 0.038 – 0.078 ppm; NH3 0.0015-0.0021 ppm; and NO3 0.26 - 2.43 ppm. These results indicate that the culture medium parameters remained within appropriate ranges for the husbandry of tridacnid clam juveniles, except for the water temperature, which on some days, reached a peak of 34 °C.

The number of larvae observed was 19,625,000, with a survival rate to the juvenile stage of 4.49%. This relatively low survival rate may have been due to the high temperatures (up to 34 °C) experienced during part of the study period.

### 3.4. Zooxanthellate density

The mean density of zooxanthellae in juvenile clams was $3.17 \times 10^6$ cells/ind, with a range of $3.15 \times 10^6$ to $5.27 \times 10^6$ cells/ind (Figure 2).

![Figure 2. Zooxanthellate density in juvenile fluted clams (*T. squamosa*).](image)

The zooxanthellate densities measured are similar to those reported by [23] for *T. squamosa* ($3.14 \times 10^6$ cells/cm²) but lower than those reported by [24]) ($1.13 \times 10^6$ cells/cm²) for *T. squamosa* with 25 cm shell width. However, it should be noted that the units of measurement used differ between our research and previous research, because our research focused on small juvenile clams so that a unit area (cm²) was not appropriate, unlike in adult tridacnid clams.
3.5. Mitotic index
During observations of mitosis under the microscope, the zooxanthellae appeared brownish-yellow in color. The time taken to count 500 dividing cells was, on average 10 minutes, so that with five replicates, the process took around 50 minutes for each observation period, as in [22]. The observations were made every 3 hours (9 observation periods) over a 24 hour period (Figure 3), beginning at 12:00, with the final observation at 09:00 on the following day. The mitotic index was highest at 09:00 and 12:00 with a peak value of 8.5 %. The mean water temperature over the period was 30.1ºC, but at the peak of mitotic activity, the water temperature was 32ºC. Mitosis was slower at 15:00 and declined to a minimum of 1.2% at 00:00 and 03:00. The peak mitotic index in this study (8.5%) was similar to that reported by [24] for *T. squamosa* (8.28 %).

![Figure 3. Mitotic index of zooxanthellae from juvenile fluted clams (*T. squamosa*).](image)

3.6. Chlorophyll and phaeopigment concentration
The mean chlorophyll concentration was 2.7 mg/m³ with a range of 1.1 to 4.1 mg/m³ (Figure 4). Mean phaeopigment concentration was 5.5 mg/m³, with a range of 4.2 to 6.2 mg/m³ (Figure 5). It is interesting to note that phaeopigment concentrations were higher than chlorophyll concentrations.

![Figure 4. Chlorophyll-a concentration in zooxanthellae from *T. squamosa* juveniles.](image)

![Figure 5. Phaeopigment concentration in zooxanthellae from *T. squamosa* juveniles.](image)
Several studies report phaeopigment concentrations lower than chlorophyll-a concentrations. For example, [24] found that chlorophyll concentrations increased in line with decreases in phaeopigment concentration. The presence of phaeopigments indicates that some chlorophyll cells have died [24], indicating that in this research, the number of dead chlorophyll cells exceeds that of live chlorophyll cells. The high proportion of dead chlorophyll cells could be due to environmental factors, in particular, the high peak temperature (34°C) experienced during the research. High temperatures can cause the surface layer of the mantle to become saturated with light [24]. Furthermore, the shells of small tridacnid clam juveniles were still transparent, resulting in higher exposure of the mantle to solar irradiation than in older specimens, and the chlorophyll-a measurements may change as the clams grow until they reach the adult stage.

4. Conclusion
From this study, we draw five main conclusions.

1. The survival rate of fluted clam Tridacna squamosa juveniles was 4.49%.
2. The mean density of zooxanthellae in individual juvenile clams was 3.17 x 10^6 cells/ind.
3. The mean chlorophyll-a and phaeopigment concentrations in zooxanthellae from juvenile clams were 2.7 mg/m^3 and 5.5 mg/m^3, respectively.
4. The mitotic index of zooxanthellae from the juvenile clams peaked from 09:00 to 12:00 at 8.5%.
5. Water quality remained conducive to fluted clam larviculture and juvenile husbandry except for elevated peak temperatures of 34°C during part of the study period.

We recommend periodic measurement of several parameters for the symbiotic zooxanthellae in these fluted clams as they grow to adulthood. In particular, zooxanthellate density, chlorophyll-a concentrations, and mitotic index.

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