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Optimizing *Scylla serrata* Karyotyping Methods

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Abstract: Sexes of mud crabs (*Scylla serrata*) are usually determined through their morphological characteristics, such as abdomen shape, carapace size, and gonopore presence. These are sometimes unreliable on crabs with a combination of the morphological characteristics of both sexes, commonly known as "gay" crabs. Karyotyping is a genetic alternative to determining the sex of by visualizing metaphase chromosomes, whose numbers indicate the sex of an organism. Published protocols on crab karyotyping use testes, making them inapplicable to female crabs. Two karyotyping methods were adapted to the somatic cells of male mud crabs in this paper to determine the better karyotyping method for possible use in female and gender indeterminate crabs. The results of the karyotyping methods using somatic tissue were compared to published male mud crab karyotypes to determine the accuracy of the methods. A set of male crabs were incubated in 1 g colchicine/L artificial seawater, and another set of crabs were injected 1 µg colchicine/g body weight and incubated in artificial seawater. The crabs were incubated for at least 5 hours, and karyotypes were prepared from the gills of the former and from the hepatopancreas of the latter through the air-dry method. The karyotypes were visualized and photographed in HPO. Metaphase chromosomes were not visualized from the samples incubated in the colchicine-seawater solution, but 43 to 98 metaphase chromosomes were visualized from the samples injected with colchicine. However, previously published data indicate that male *Scylla serrata* have 56 pairs of chromosomes. The results show that the colchicine injection method is better than incubation in colchicine-seawater solution in visualizing metaphase chromosomes using somatic cells, but still requires further optimization to improve accuracy for sex determination in female crabs and crabs of indeterminate sex.

Key Words: Mud crab; *Scylla serrata*; Karyotype; Sex difference; Sex determination

1. INTRODUCTION

Wild-caught or captive bred *Scylla* spp. are commercially important mud crabs. The Philippines has been one of the leaders in global mud crab production, but Philippine mud crab production has decreased in recent years (FAO, 2013). Male, female, and sex-indeterminate crabs fetch different prices because of their differences in size, taste, texture, and roe presence. Male *Scylla serrata* generally have better taste and texture, but female *Scylla* generally are bigger and may contain roe. However, some crabs have a mixture of male and female morphological characteristics. These crabs of indeterminate sex are locally called *bakla*, which translates to "gay". There are many conflicting theories on the actual sex of these crabs.

Crabs of indeterminate sex are thought to be hybrids of different *Scylla* species because crabs of different *Scylla* species are usually found in the same habitat (Imai and Takeda, 2005). The local Bureau of Fisheries and Aquatic Resources states that these crabs eventually develop into female crabs, even though crabs of indeterminate sex are as large as mature female crabs (PIA, 2013). Crab parasites also alter male crab behavior and morphology to make it resemble female crabs, which promotes the propagation of the parasite through mating between male crabs (Jithendran et al., 2010; Knuckey et al, 1995). The key to defining the actual sex of the crabs may be hetarogamety. Most crustaceans exhibit chromosome number difference between sexes or heterogamety (Niiyama, 1959), but the heterogamety that determines *S. serrata* sex has not been previously described.

Karyotyping is a useful tool that can be used to visualize the metaphase chromosomes of an organism, which can be easily counted. There are previous studies on karyotyping crab species(Gopikrishna & Shekhar, 2003; Swagatika & Kumar, 2014), but these use testes, which makes their methods inapplicable to female crabs. Karyotyping methods that apply to both sexes are required to determine heterogamety and to establish the sex of crabs of intermediate morphologies. This paper aims to compare the results of two karyotyping methods adapted to the somatic cells of male crabs with published karyotypes.

2. METHODOLOGY

2.1. Crab collection

S. serrata were identified through their frontal lobe spines and carpus spines and were sexed through their apron morphology (Keenan et al, 1998). Only male *S. serrata* with narrow aprons were chosen. Six crabs were bought from a seafood retailer on Pasay City. The crabs were rinsed with double distilled water upon reaching the laboratory.

2.2. Crab incubation

2.2.1. Incubation in colchicine

A method for preparing metaphase chromosomes from freshwater crayfish gill tissue(Indy et al, 2010) was adapted for mud crabs. Artificial seawater was created using instant saltwater mix and sterile distilled water. The solution was adjusted to 30 ppm, which is within the range of ideal salinity for mud crab culture(Shelley and Lovatelli, 2011). The artificial seawater solution was aerated using a 2500L/h pump for at least 12 hours before incubating the crabs. Just before submerging the crabs in the artificial seawater, 1 g of colchicine was dissolved in the artificial seawater per liter of solution.

Three crabs were submerged in the colchicine-artificial seawater solution for at least 5 h to allow for cell division. The pump was continually used to aerate the artificial seawater. Gill tissues were dissected from the crabs after incubation. The gill tissues were rinsed with sterile artificial seawater after dissection.

2.2.2. Colchicine injection

A method for preparing chromosome spreads from insects(Tüzün & Yüksel, 2010) was adapted for mud crabs. Artificial seawater was created like in the previous section, but without adding colchicine. The artificial seawater was also aerated for 24 h using a 2500 L/h pump. The crabs were intramuscularly injected 1 µg of colchicine/g crab bodyweight diluted in 1 mL of sterile artificial seawater solution. The crabs were incubated in the aerated artificial seawater for at least 5 hours to allow for cell division. The pump was continually operated during incubation. The hepatopancreas of the crabs were dissected after incubation. The hepatopancreas tissues were rinsed with sterile artificial seawater after dissection.

2.3. Slide preparation

The air-dry method for tissue swelling, fixing and staining(Cokendolpher & Brown, 1985) was used, with some modifications. The dissected tissues were separately submerged in 0.06 M KCl for 15 min to swell the cells. The tissues were homogenized using a mortar and pestle during the swelling. The tissues were removed from the hypotonic solution and were submerged in 4 mL of cold 4:1 methanol:glacial acetic acid for 1 h, replacing the solution every 15 min. Each tissue preparation was finally transferred to tubes containing 1 mL of 4:1 methanol:glacial acetic acid and refrigerated for 24 h. The tissues were suspended in the methanol:glacial acetic acid mixture by gently inverting the tubes. A drop of each tissue-methanol:glacial acetic acid suspension was aspirated from each tube and dropped on clean

glass slides from at least 30 cm height to rupture the cells. The slides were allowed to air-dry until the liquid on the slide has fully evaporated.

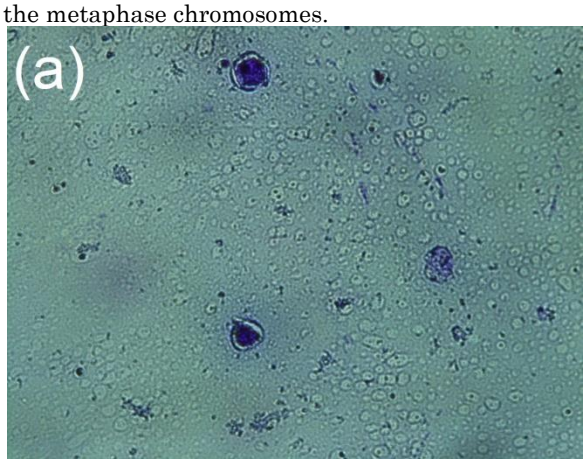
The dried slides were immersed in 0.05 g trypsin/100 mL PBS for 30 s, rinsed in cold phosphate buffered saline (0.15 M NaCl-0.05 M NaHPO4, pH 7.4) for 5 min, and finally immersed in 5% Giemsa in pH 6.8 Sorenson buffer(4.54 g KH_2PO_4 and 4.75 g Na_2HPO_4 in 1 L water) for 12 min. The slides were finally rinsed with tap water and air-dried until the liquid on the slide has fully evaporated.

3. RESULTS AND DISCUSSION

3.1. Incubation in colchicine

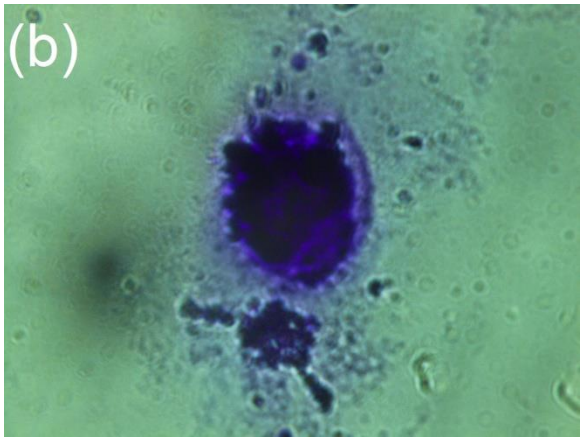
Representative photos of the slides are shown in Fig. 1. Giemsa stain stains chromosomes with a purple color. The slides do not show spread groups of chromosomes. Chromosomes are visible, but are only found close together within intact cells. Debris can also be found throughout the slides.

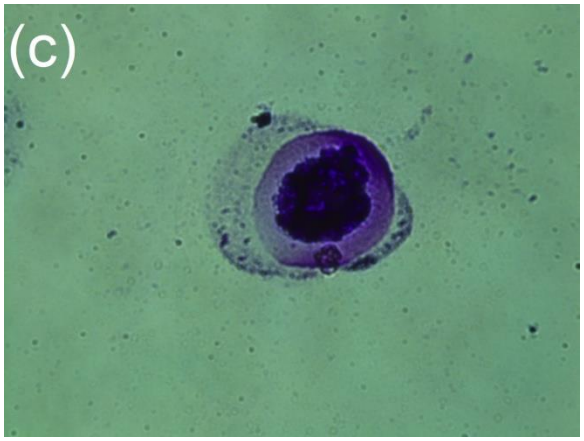
The formation of blue bodies shows that incubation in colchicine-seawater solution effectively arrested the gill cell chromosomes in the metaphase stage. However, the chromosome spreads cannot be used for karyotyping because the chromosomes overlap when viewed, which prevents chromosome counting. The intact cells denote that the cells require further swelling by lowering the concentration of the hypotonic solution or by increasing the dropping height to properly spread the metaphase chromosomes.



2.4. Slide observation

Slides were observed using a light microscope. Photographs were taken using a digital camera apparatus directly attached to the microscope.





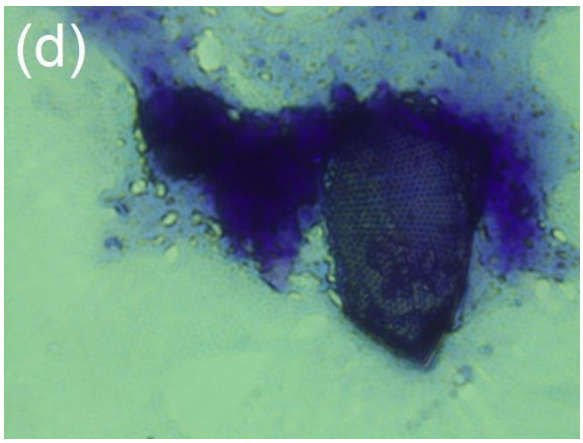


Figure 1. Representative photos of crab chromosome spreads from colchicine incubation. (a) intact cells at 100x magnification. (b & c) intact cells at 400x magnification. Blue bodies denote metaphase chromosomes. (d) Gill remnant at 100x magnification.

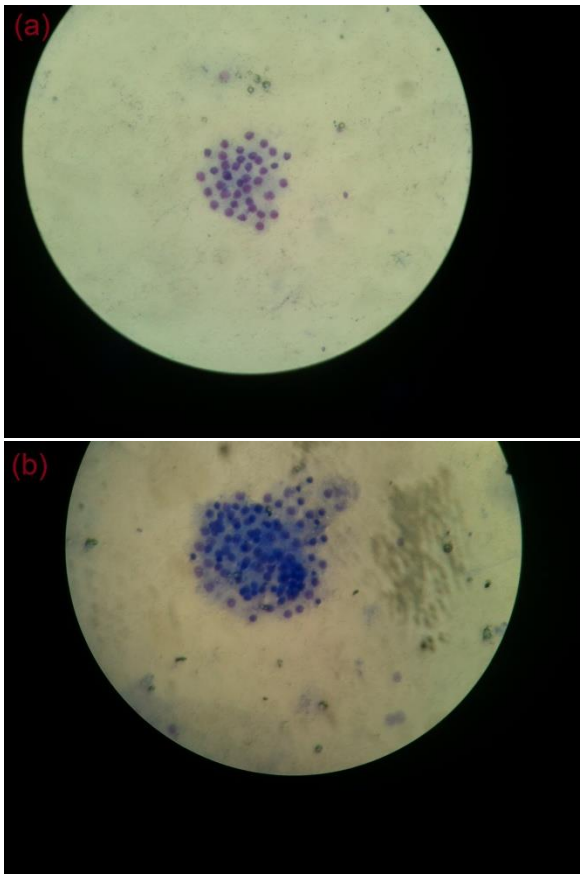
Another possibility is that crab gills are too sturdy for use in karyotyping. The gills of living crabs are exposed to the external environment. Thus, it may have mechanisms for adaptation to hypotonic solutions, which prevents swelling. Another reason is that gills are supported by gill arches, which increases the integrity of the cells. This is supported by Fig. 1d, which shows an intact remnant of the gill, and the presence of debris in the other slides. Despite being manually ground using a mortar and a pestle, a large part of the gill was still intact.

3.2. Colchicine injection

Representative photos of the slides are shown in Fig. 2. Chromosome spreads are visible, but the numbers of chromosomes per spread range from 43 to 98. Chromosome spreads are not bound by a cell membrane and are mostly distinct. Some debris are still visible in the slides. A total of three chromosome spreads were successfully identified.

The formation of blue bodies indicates that colchicine injection also effectively arrested hepatopancreas cells in the metaphase stage, like in the first method. Cell swelling and rupturing by dropping were also successful because the cell membranes of the spreads are not intact. This is an advantage of using hepatopancreas cells over the first method. This is possibly because hepatopancreas cells are exposed to an almost constant environment inside the body of crabs, unlike the gills. Also, hepatopancreas cells do not have as much supporting connective tissue as the gills.

Despite the proper spreading, the numbers of chromosomes in the spreads differ from the number established in the previously mentioned studies on *Scylla serrata* karyotyping. All identified spreads have fewer chromosomes than what is in the previously mentioned karyotyping studies. This shows that the method still requires further optimization. Applying this method as it has been described on female and gender-indeterminate crabs may yield false answers.



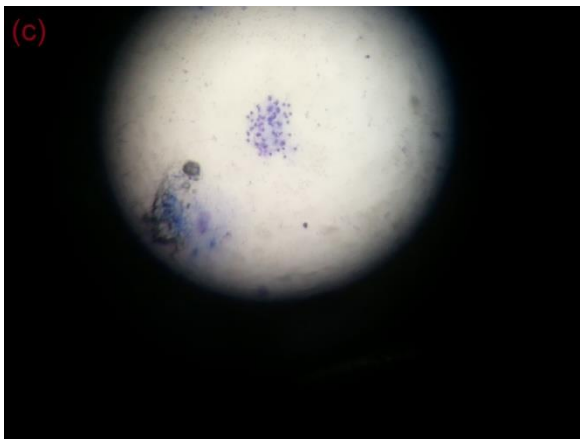


Figure 2. Representative photos of crab chromosome spreads from colchicine injection. (a) Spread of 43 chromosomes at 400x magnification. (b) Spread of 98 chromosomes at 400x magnification. (c) Spread of 47 chromosomes at 100x magnification.

4. CONCLUSIONS

Incubation in colchicine does not yield metaphase chromosomes that can be used for karyotyping studies from gill cells. Colchicine injection yields metaphase chromosomes from hepatopancreas cells. However, the number of visualized chromosomes did not match that which is previously published. Thus, the colchicine injection method must be improved to be applied for use in determining heterogamety in *Scylla serrata*.

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