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Cytogenetic of Brachyura (Decapoda): testing technical aspects for obtaining metaphase chromosomes in six mangrove crab species

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Abstract. Brachyura is one of the most specious infra-order belonging to Decapoda and it plays a central role from an ecological and economic point of view. Despite its importance, cytogenetic studies on Brachyura (Decapoda) are extremely limited due to the difficulties in obtaining chromosome preparations of good quality. Molecular cytogenetic have proven to provide basic knowledge on the genome organization of species and in methods for manipulating genomes. It is also very useful to support aquaculture. In this study we focused on six semi-terrestrial mangrove crab species to test several variations of technical steps to produce chromosome preparations in Brachyura. The best results were obtained using cells from early stage embryos incubated with 0.0005% nocodazole or 0.05% colchicine and hypotonized with 0.075 M KCl solution. The best method to analyze the chromosomes was the use of confocal microscope after DAPI staining. We recorded a high chromosome number for the six study species. Similar chromosome morphology was recorded for allied species likely due to phylogenetic relationship. Variable results with cytogenetic treatments in different species suggest that there may be a species-specific response to the techniques we tested. Chromosome number ranges reported in this study will be useful in future genome sequencing studies, i.e. to assess the quality of sequencing assemblies.

Keywords: aquaculture, chromosomes, confocal microscope, mangrove crabs, DAPI, metaphase.

INTRODUCTION

Decapoda represents the most species-rich crustacean order with more than 2,700 genera and 17,000 species inhabiting marine, intertidal, freshwater and terrestrial ecosystems (De Grave *et al.*, 2009). The infra-order Brachyura is particularly species-rich (about 6,800 species in 1270 genera)

and includes all the extant true crabs (Ng *et al.,* 2008; Ahyong *et al*., 2011; Tsang *et al*., 2014). The importance of this group is unquestionable both from an ecological and economic point of view. Many species of brachyuran crabs are edible, being extensively fished and eaten worldwide. According to recent data from the Food and Agriculture Organization (FAO 2019), the species of the genus *Portunus,* such as *P. trituberculatus* (Miers, 1876) and *P. pelagicus* (Linnaeus, 1975) represent the most fished crabs together with *Callinectes sapidus* Rathbun, 1896, *Cancer pagurus* Linnaeus, 1758 and species belonging to the genus *Scylla* De Haan, 1833.

Information about species karyotypes is a fundamental prerequisite for many advanced and applied studies. In genomics, for example, knowledge about species chromosome numbers is critical to assess the quality of assemblies and have an idea of the genome organization (e.g. Sharakhov *et al*., 2014). Moreover, comparing the sequence and structure of genes and their organization into chromosomes is now the best approach to understand genome evolution and consequently organism evolution (see Coghlan *et al*., 2005). Moreover, cytogenetic information is necessary in methods for modifying and manipulating genomes (see Abdelrahman *et al*., 2017). Aquaculture can also greatly benefit from improved cytogenetic analysis. It is fundamental in mapping loci involved in disease resistance and to improve commercial stocks by selecting cloned lines of aquacultured species (see Gui and Zhu 2012). It is necessary for controlling sex and inter-specific hybridization (see Colombo *et al*., 1998; Bartley *et al*., 2001; Shpak *et al*., 2017).

Despite their ecological and economic importance, little is known about the karyology of brachyuran crabs. Cytogenetics studies of brachyurans are relatively few, probably because they are technically more difficult than in other decapods as well as in mollusks and fishes (e.g. Sola *et al*., 1981; Galetti *et al*., 2000; Coluccia *et al*., 2004; Thiriot-Quievreux 2002, 2003; Scalici *et al*., 2010; Salvadori *et al*., 2012, 2014; Torrecilla *et al*., 2017; Guo *et al*., 2018). Brachyurans have a high number of chromosomes that are usually very small (e.g. Niiyama 1959; Lécher *et al*., 1995; Lee *et al*., 2004; Tan *et al*., 2004). In addition, despite cell culture might provide better and more abundant materials for karyological analyses, the few attempts to establish cell cultures in this taxon have not meet with great success and, thus, chromosome preparations are usually obtained directly from living tissues (e.g. in Toullec 1999; Sashikumar *et al*., 2008; Zeng *et al*., 2010; Hong *et al*., 2013).

For these reasons, the preparation of good quality karyotyping and chromosome banding in Brachyura has been never obtained, the only works being restricted to descriptions of chromosome numbers (see Lécher 1995). Moreover, most of karyological studies on brachyurans are decades old (Niiyama 1942, 1959, 1966; Mittal and Dhall 1971; Vishnoi 1972; Trentini *et al*., 1989, 1992; Lécher 1995 and references therein), while recent works are scarce and mostly related to species of economic importance (Lee *et al*., 2004; Zhu *et al*., 2005; Swagatika and Kumar 2014; Cui *et al*., 2015). These recent papers reported that the mitten crabs *Eriocheir japonica* (De Haan, 1835) and *E. sinensis* (H. Milne-Edwards, 1853) have a diploid chromosome number of 2n = 146 (Lee *et al*., 2004; Cui *et al*., 2015), and the karyotype of *Portunus pelagicus* includes 51 pairs of chromosomes (Jazayeri *et al*., 2010), whereas the congeneric *P. trituberculatus* has 53 pairs (Zhu *et al*., 2005). Recently, Swagatika and Kumar (2014) recorded that the mud crab *Scylla serrata* (Forsskål, 1775) and the blue crab *P. pelagicus* have 2n = 106 and 2n = 98 chromosomes, respectively.

The present study aims to contribute a step forward in the crab cytogenetic methods by comparing different variables necessary to obtain chromosome preparations from live tissues. We selected six crab species, from four different brachyuran families, commonly found in the mangrove forest of the South China Sea, for which we systematically tested different technical variations in order to obtain metaphase chromosomes. The key elements for obtaining a high number of mitotic cells were scrutinized.

MATERIALS AND METHODS

Study species

About 5 adult males and 5 females, including 2 ovigerous, from six species of Hong Kong semi-terrestrial and mangrove crabs were collected at low tides, in October 2017. In particular, we collected *Parasesarma bidens* (De Haan, 1835) and *Metopograpsus frontalis* (Miers, 1880) at Tung Chung mangroves (Lantau Island); *Chiromantes haematocheir* (De Haan, 1833) and *Gelasimus borealis* (Crane, 1975) at Uk Tau (New Territories), and *Austruca lactea* (De Haan, 1835), *G. borealis* and *Metaplax tredecim* Tweedie, 1950 at Starfish Bay (New Territories). These species, belonging to four different brachyuran families, are common inhabitants of lowland forests, mangrove forests and adjacent mudflats. They are all active during low tide, despite occupying different supratidal and intertidal habitats. All are also sold in pet trade for aquariophily (e.g. Mong Kok market, Hong Kong). Taxonomical and ecological information concerning the studied species are summarized in Table 1.

Table 1. Biological and ecological information on the six mangrove crab species.

Species	Family	Habitat	Max CW (in mm)	Aquariophily
Gelasimus borealis	Ocypodidae	Mud flat, sublittoral fringe	28.1	Yes
Austruca lactea	Ocypodidae	Sand flat, eulittoral	16.4	Yes
Metopograpsus frontalis	Grapsidae	Mud flat, sublittoral fringe	26.0	No
Metaplax tredecim	Varunidae	Mud flat, sublittoral fringe	19.0	No
Chiromantes haematocheir	Sesarmidae	Lowland forests, Supralittoral	38.0	Yes
Parasesarma bidens	Sesarmidae	Mangrove forests, eulittoral	28.5	Yes

Data shown are: family; mangrove habitat occupied by adult populations (personal data); Max CW, maximum male carapace weight (Aiyun and Siliang 1991); presence in the pet trade for aquariophily.

Within few hours from collection, crabs were transported to laboratories of the School of Biological Sciences, The University of Hong Kong, divided according to their species and accommodated in terraria containing mangrove mud and sea water. In case of herbivore species (i.e. *P. bidens* and *C. haematocheir*) fresh *Kandelia obovatae* (Sheue, Liu and Yong, 2003) leaves (i.e. the dominant tree in their original habitats) were provided as food items. Each terrarium was also provided with stones and pieces of mangrove wood and bark as hiding places. Animals were kept at room temperature (around 22° C) and at natural light conditions.

DNA Barcoding

Identification of species was made based on morphological traits and verified by DNA barcoding analysis performed on an individual per species. DNAs were extracted from muscle tissue, removed from one pereiopod, using the Puregene Kit (Gentra System), then resuspended in distilled water and stored at -20°C. A fragment of the cytochrome oxidase subunit I (COxI), corresponding to the barcoding region and consisting of 656 base pairs (bp), was amplified using polymerase chain reaction (PCR) with the following primers: COL6b 5'-acaaatcataaagatatygg-3' (Schubart and Huber 2006) and HCO2198 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer *et al*., 1994). The amplifications were performed in a Perkin Elmer 9600 thermal cycler with the following PCR conditions: 40 cycles of denaturation for 45 s at 94°C, annealing for 1 min at 48°C, extension for 1 min at 72°C, preceded by an initial denaturation for 10 min at 94°C followed by a final extension for 10 min at 72°C. Subsequently, PCR products were visualized on an agarose gel, purified by precipitation with Sure Clean (Bioline) and then resuspended in water. The sequence reactions were performed with the Big Dye terminator mix (Big Dye Terminator1V 1.3 Cycle Sequencing kit; Applied Biosystems) followed by electrophoresis in an ABI Prism automated sequencer (ABI Prism™ 310 Genetic Analyzer; Applied Biosystems). The sequences were corrected manually with the program CHROMAS v. 1.55 (Technelysium Pty Ltd, Queensville, Australia). We then used the software BLAST (available on the website of the National Center for Biotechnology Information NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare our sequences to sequence databases and calculate the statistical significance of matches. We also compared the obtained sequences to our own reference sequences.

Cytogenetic analysis

The general workflow used to obtain chromosome preparation is as follows: metaphase blocking, tissue preparation, hypotonization and fixation, slides preparation and staining. For all these steps several tests were performed. A schematic representation of the experimental plan is provided in Figure 1.

Tissue preparation

Adult tissues: after injection or incubation with the metaphase blocking agent (colchicine or nocodazole, see below), animals were anesthetized for 10 min at -20°C, and then sacrificed. Gonads, gills and hepatopancreas were dissected and placed in a small Petri dish with 1-2 ml of hypotonic solution. Tissues were either kept intact, or mashed by rubbing against a stainless-steel grid with curved forceps, and then transferred to a 15 ml tube containing the pre-warmed (28°C, i.e. the average environmental temperature during this season) hypotonic solution. Hemolymph samples were also collected by extraction of 0.5-1.0 ml of liquid with a 6 mm insulin syringes in proximity of the coxa of the fourth pair of legs, and directly placed in pre-warmed hypotonic solution.

Fertilized eggs: clusters of fertilized eggs were removed from ovigerous females by cutting the proximal part of pleopods. For *G. borealis*, eggs at two different embryonic stages were used, stage I and V (Simoni *et al*., 2011). For all other species, we utilized embryos at stage V of development. Eggs were incubated in metaphase blocking agent (see below), hypotonized, and then either fixed directly on slides or minced. Mincing of eggs was performed with needles in a small Petri dish in 1-2 ml of fixative.

Metaphase blocking

Two different metaphase blocking agents were tested to arrest the mitotic spindle and visualize chromosomes: nocodazole (15 mg/ml in dimethyl sulfoxide, DMSO) and colchicine (powder). Metaphase blocking agents were diluted/dissolved in sterile sea water to obtain different final concentrations and applied to tissues and eggs via injection and incubation respectively.

Injection: animals were injected in proximity of the coxa of the fourth pair of legs. Both agents were tested for the amount of 0.2 and 2 μg/g crab weight. Animals were kept in terraria at room temperature for 8, 16 or 24 hours and then dissected as described above.

Incubation: eggs were transferred to 15 ml tubes and incubated in 10 ml of 0.0005, 0.005 or 0.05% of colchicine or nocodazole solution for 2, 4, 8, 16 or 24 hours at 28°C, and then transferred to pre-warmed hypotonic solutions. To guarantee eggs an appropriate level of oxygenation, tubes were kept without lids and gently stirred during incubation process.

Hypotonization

Tissues (intact or mashed) and eggs were incubated in hypotonic solution to achieve a good cell swelling and metaphase spreading. To facilitate the access of the hypotonic solution to the embryos, part of the eggs was punctured before incubation. Two different solutions were tested, 0.1% sodium citrate and 0.075 M potassium chloride, with incubation times of 15, 30 or 45 min. After centrifuge at 150 rcf for 10 min, hypotonic solution was removed from mashed tissues, while it was removed from intact tissues and eggs by gently pipetting out the liquid.

Fixation

After hypotonic removal, tissues and eggs were fixed by applying 3–5 ml of cold, freshly prepared fixative (3 parts methanol or absolute methanol: 1 part glacial acetic acid). Eggs were then minced as described above and transferred to a 15 ml tube. Tubes containing cell suspensions from eggs and mashed tissues were centrifuged at 200 rcf for 10 min. The supernatant was removed, and fresh fixative was added. This step was repeated three times. Intact tissues were left in 15 ml tubes containing fixative solution for 20 min, then fixative was changed, and tissues stored at -20°C, until slides preparation.

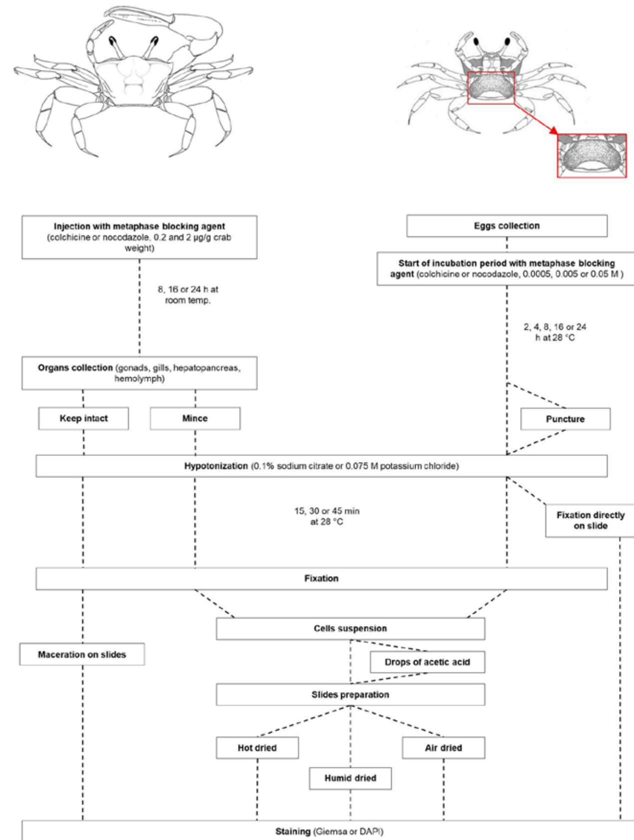

Figure 1. Schematic representation of the experimental plan. Crabs representations modified from www.fiddlercrab.info.

Part of hypotonized eggs were also transferred directly to a clean slide and macerated using a thin needle. After the maceration, we applied a fixation solution of 3:3:4 ethanol:acetic acid:distilled water three times, followed by a fixation solution of 1:1 acetic acid:ethanol and finally a few drops of glacial acetic acid. Between each application of the fixation solutions, the excess was removed with the aid of a filter paper. The slides were left to dry at room temperature.

Slides preparation

Intact tissues were macerated with the help of two needles directly on slides. Few drops of glacial acetic

acid were used to help maceration. Slides were then air dried. Cell suspensions obtained from eggs and mashed tissues were gently shacked and left decanted to separate cells from egg chorion and largest pieces of tissues. The upper part of decanted preparation was transferred to a 2 ml tube and centrifuged at 1,200 rcf for 10 min. Fixative was removed and resuspended cell pellet was used to drop slides. Few drops of acetic acid were added to part of the cell suspensions to improve chromosome spreading. For cell suspensions, three different protocols for slides preparation were tested:

Air dried: cell suspension was dropped with a siliconized Pasteur pipette from a height of about 10 cm onto a pre-cleaned microscope slide and dried at room temperature before staining.

Hot dried: the above procedure was applied, but slide was pre-heated and dried at 50°C.

Humid dried: the cell suspension was dropped from a height of about 10 cm onto a pre-cleaned microscope slide chilled to -20°C. After a short drying period at room temperature in which the fixative was partially evaporated, the slides were held two to three times briefly into water steam. The slides were then dried on a metal block which was half submerged in a 75°C water bath.

After drying, the slides were stained with Giemsa 10% solution for 20 min or 4',6-diamidino-2-phenylindole (DAPI) and mounted.

Image capture and chromosomes counting

Metaphases were observed under optical, fluorescence and confocal microscopes. Leitz Dialux 20 optical microscope was mounted with Moticam Pro 205B. Zeiss Axio Imager.D2 fluorescence microscope was mounted with Zeiss Axiocam 503 mono. Zeiss LSM 710 NLO confocal microscope was mounted with Airyscan Module for super resolution. Images were edited with Adobe® Photoshop® CS5 extended (Adobe Systems Inc., San Jose, California, USA). The mode of diploid chromosome numbers was calculated, using Excel, after counting 42, 18, 13, 21, 19 metaphases of *G. borealis*, *A. lactea*, *M. frontalis*, *M. tredecim* and *C. haematocheir* respectively.

RESULTS

Barcoding analysis

The PCR successfully amplified the mtDNA COxI gene in the six species, resulting in sequences about 600 bp long, excluding the primer. All the sequences have an A-T rich nucleotide composition as expected for the mitochondrial DNA of arthropods (Simon *et al*., 1994). The DNA barcoding confirmed the morphological identification of the six species. The sequences have been deposited in GenBank ((access numbers: MT265074-79).

Tissue preparation

Injection of the metaphase blocking agents did not cause any visible damage to the animals, and individuals of all species survived the treatments. No metaphases were observed in cytogenetic preparations obtained from mashed and intact tissues, regardless of the concentrations and exposure times to metaphase blocking agents. Slides obtained from intact tissues presented well preserved nuclei at different cell cycle stages. Preparations obtained from cell suspensions of mashed tissue showed well separated cells, indicating that manipulation and maceration procedures were correctly performed.

Metaphases were observed in the cell suspensions obtained from eggs of all the six species (Fig. 2). The highest number of metaphases was observed in preparations obtained from embryonic stage I eggs (Fig. 2A). Both nocodazole and colchicine were effective in metaphase arrest of embryonic cells. Optimal results were obtained with colchicine and nocodazole at 0.05% and 0.0005%, respectively. No visible differences were observed between colchicine and nocodazole treatments (i.e. chromosome condensation or metaphase spreading). Preparations obtained with higher concentrations of nocodazole did not show any metaphases. On the other hand, few metaphases were also detected in preparations obtained with lower concentrations of colchicine.

Same hypotonization treatments gave different level of spreading in the six species, as described below. No relevant differences were registered between the two hypotonic solutions, and the three incubation times. However, results obtained with 0.075 M potassium chloride solution, with an incubation time of 30 min produced better spreading metaphases in *M. tredecim* as described below (Figs. 2J, K, L). No differences were registered between punctured and unpunctured eggs. An increase of incubation time with hypotonic solution up to 3 hours did not affect or improve metaphase quality.

Chromosome preparation obtained from mashed eggs suspensions showed well separated cells and very few residues of chorion, indicating that the manipulation and fixation procedures were adequate, and the decantation step was useful. No metaphases were observed in preparations obtained from eggs directly fixated on slides. Moreover, cells were sparse and clustered, preventing an accurate observation of the preparation.

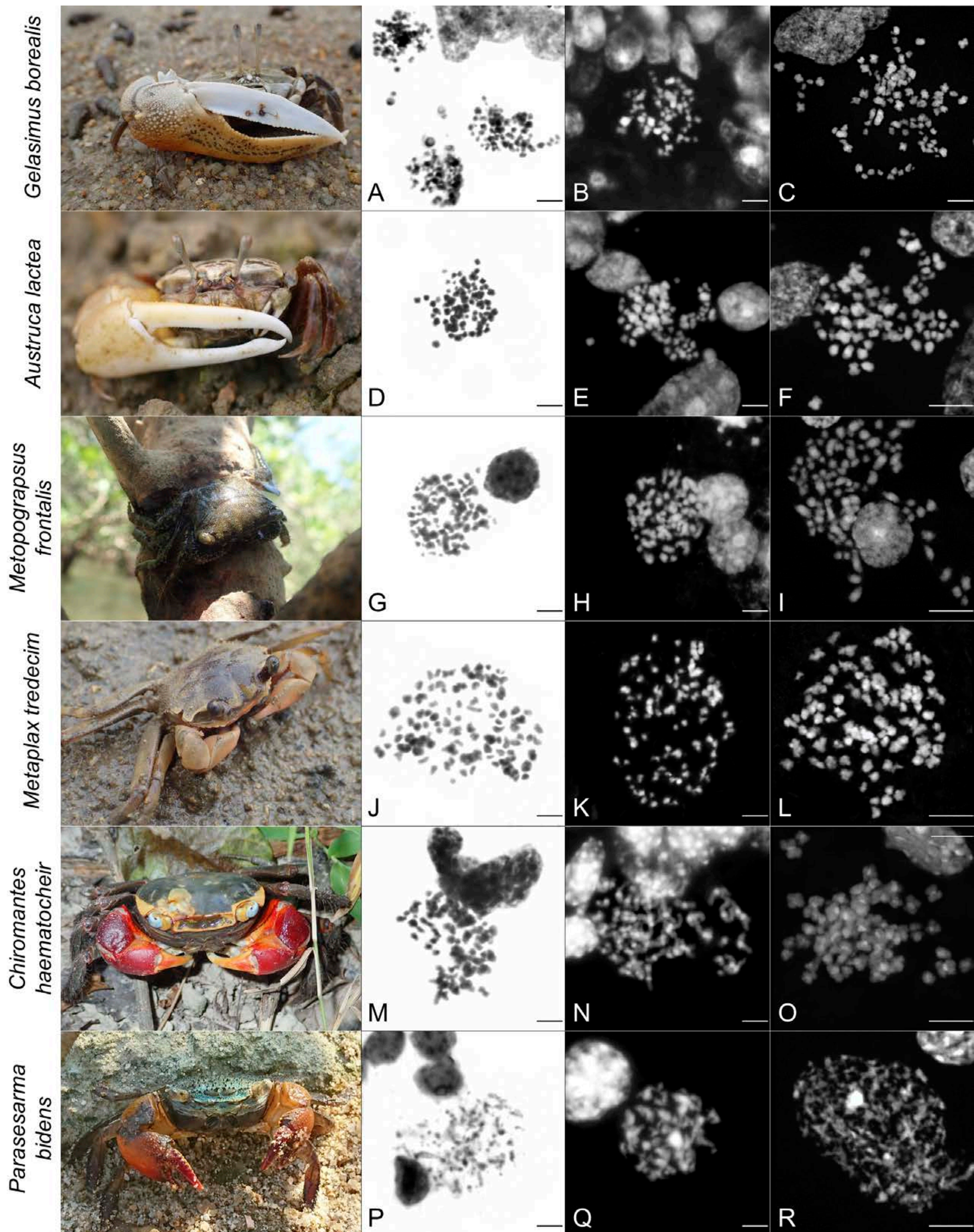

Figure 2. Metaphase spreads, obtained from eggs incubated with 0.05% colchicine and hypotonized with 0.075 M KCl of *Gelasimus borealis* (**A**, **B**, **C**), *Austruca lactea* (**D**, **E**, **F**), *Metopograpsus frontalis* (**G**, **H**, **I**), *Metaplax tredecim* (**J**, **K**, **L**), *Chiromantes haematocheir* (**M, N, O**), *Parasesarma bidens* (**P**, **Q**, **R**). Slides were either stained with Giemsa and observed under an optical microscope (**A**, **D**, **G**, **J**, **M**, **P**) or stained with DAPI and observed under a fluorescent (**B**, **E**, **H**, **K**, **N**, **Q**) and confocal microscope (**C**, **F**, **I**, **L**, **O**, **R**). Photos by Stefano Cannicci.

Slides dropping methods were all successful, and no differences were registered among the three methods. The addition of a few drops of acetic acid to cell suspensions visibly increased the spreading of chromosomes. Metaphases were better detected when DAPI staining was applied as, contrastingly to Giemsa, it did not stain organic and inorganic residual material, making it easier to detect metaphases. Confocal microscopy gave better resolution of chromosome morphology and heterochromatic regions were visible.

Chromosome number and morphology

We obtained metaphase chromosomes for all the studied species but *P. bidens*, for which only broken metaphases were observed with all treatments (Figs. 2P-R). We analyzed an average of 50 metaphases for each species.

In *G. borealis* the number of chromosomes per cell ranged between 30 and 60 with a mode at 48. Chromosomes morphology was hardly distinguishable due to their small size. Metaphase spreading was limited (Figs. 2A-C), however a high number of metaphases per slides was observed when preparations were made with eggs at embryonic stage I (Fig. 2A).

Chromosome morphology and metaphase spreads in *A. lactea* were very similar to preparations obtained from the eggs of the allied species *G. borealis* (Figs. 2D-F). In this ocypodid the number of chromosomes per cell ranged between 56 and 74 with a mode at 60.

We obtained a very low number of metaphases for *M. frontalis*, and the hypotonic treatment was less successful in this species than in the other ones (Figs. 2G-I). The number of chromosomes per cell ranged between 38 and 62 with a mode at 55.

The hypotonization treatment gave the best results for the varunid *M. tredecim*, despite its chromosomes appeared very small (Fig. 2J-L). The number of chromosomes per cell ranged between 78 and 108 with a mode at 80.

For the two sesarmid species concerned, we obtained reliable results for *C. haematocheir* only. Chromosomes of *C. haematocheir* were larger than those of the other species, and their morphology could be better observed (Figs. 2M-O). Confocal images showed that most chromosomes were biarmed, and DAPI staining revealed AT-rich pericentromeric regions (Fig. 2N, O). The number of chromosomes per cell ranged between 58 and 74 with a mode at 66.

DISCUSSION

This study, reporting the results of several cytogenetic technical trials on six semi-terrestrial and mangrove crab species selected as representatives of the infraorder Brachyura, provides insights on the technical aspects necessary to obtain chromosome preparations in brachyuran crabs.

The best results were obtained with pre-hatching embryos incubated with colchicine or nocodazole at 0.05% and 0.0005%, respectively, hypotonized in 0.075 M KCl solution for 30 min and fixed in freshly prepared fixative. The best method to analyze the chromosomes resulted to be the use of confocal microscope after DAPI staining.

Embryos and post-hatched larvae of decapods contain rapidly growing tissues with a high mitotic activity (Anger 2001). Thus, these life stages represent an optimal material to obtain metaphases for karyological studies. Optimal results for chromosome preparations using fertilized eggs have already been obtained by Campos-Ramos (1997) for another suborder of the Decapoda, the Dendrobranchiata. In agreement with the present study, this author tested colchicine concentrations from 0.006% to 0.1% in *Penaeus vannamei* (Boone, 1931) and *P. californiensis* (Holmes, 1900) eggs and obtained optimal results using 0.05% colchicine, with no differences in chromosomes condensation at variable colchicine concentrations. Recently Martin *et al.*, (2016) also obtained optimal chromosome preparations from *Procambarus virginalis* (Lyko, 2017) (Decapoda: Pleocyemata: Astacidea) eggs using 0.05% colchicine. Larvae at early zoeal stages were used by Cui *et al.* (2015) who obtained good chromosome preparations for the Chinese mitten crab *Eriocheir sinensis*. In the present study, clear differences were obtained using embryos of *G. borealis* at different stages, with early stage embryos presenting the highest number of metaphases with respect to the late stage ones. This suggests that the highly dividing tissues of embryos at initial stage are even more suitable for chromosome preparation, despite the abundance of yolk, which reduces the cleanliness of the preparations. Absence of metaphases in preparations obtained with higher concentrations of nocodazole is likely due to the toxicity of DMSO present in nocodazole solution, which caused an arrest of cell cycle in embryos (Moralli *et al.*, 2011).

The hypotonic treatment was the most critical phase as several metaphases did not spread sufficiently and overlapping of the chromosomes made it difficult to make reliable chromosome counts. The best results were obtained with 0.075 M potassium chloride as hypotonic

solution, which is the most commonly used in decapods (e.g. in Salvadori *et al.*, 2012; Cui *et al.*, 2015; Martin *et al.*, 2016; Torrecilla *et al.*, 2017).

However, it was also evident that the same hypotonization treatments yielded different level of chromosome spreading in each of the six analyzed species. Such differences may be due to differences in the characteristics of the chorion, or in the osmotic concentration and physiological characteristics of the embryos of different species. Our target species, in fact, occupy different habitats in Hong Kong, from the lowland forests, in the case of *C. hematocheir*, to the true mangrove forests, such as *P. bidens* and *M. frontalis*, to the lower intertidal sand and mud flats, such as *A. lactea*, *G. borealis* and *M. tredecim*. Indeed, the permeability and osmotic characteristics of their chorions, as well as the osmotic and physiological traits of their embryos are adapted to different conditions in terms of salinity, temperature, submersion and water availability. It is known that eggs of semi-terrestrial and intertidal families are permeable to air-borne gasses and can intake oxygen from air, while the embryos of marine species can only rely on water (Cannicci *et al.*, 2011; Simoni *et al.*, 2011). Moreover, the osmolarity of tissues of brachyuran crabs is strictly related to their microhabitat, since they are osmoconformers (Charmantier 1998). It is plausible that the differences we obtained for the six species using the same treatments may be related to differences in permeability to solutes of their chorion and in osmoregulation mechanisms of their embryos, which may have influenced the response of cells to hypotonization. The best spreading results were obtained for *M. tredecim* which is the only species colonizing the lower intertidal belt.

We failed to obtain metaphases from adult tissues. In decapods, there are a few cytogenetic studies using gills and hepatopancreas as tissue of choice for chromosome preparation (e.g. Indy *et al.*, 2010; Salvadori *et al.*, 2012, 2014; González-Tizón *et al.*, 2013). A few other studies concluded that testes were a suitable tissue for chromosome preparations (e.g. Lee *et al.*, 2004; Tan *et al.*, 2004; Awodiran *et al.*, 2016; Milnarec *et al.*, 2016). However, the inactivity of testes in species with seasonality of reproductive activity (commonly described in male crustaceans, especially in representatives from colder regions: Adiyodi 1988) may lead to a scarcity of dividing cells in this organ, and thus to the lack of metaphases. This could be the case in our samples, whose sperm ducts appeared reduced in size as expected during the "resting" reproductive phase. This result is plausible since our sampling was performed at the very end of the reproductive period for Hong Kong crab species, when only very few females were still ovigerous.

We recorded a high chromosome number for our species, as known for other brachyuran crabs and Decapoda in general (Niiyama 1959; Lécher *et al.*, 1995; Lee *et al.*, 2004; Cui *et al.*, 2015). The highest chromosome numbers were recorded for the varunid *M. tredecim*, with a mode value of 80. This is indeed lower than what reported for other two varunid species, the mitten crabs *Eriocheir japonica* and *E. sinensis* whose diploid chromosome number is 2n = 146.

While we recorded different chromosome numbers for *G. borealis* and *A. lactea* (numbers ranging between 30-60 and 56-74, respectively), their chromosomes are more similar to each other than to the rest of the study species. This similarity is likely due to their close phylogenetic relationship: the two species being part of the same ocypodid subfamily Gelasiminae (Shih *et al.*, 2016).

The wide range of chromosome number registered in our study species can be attributed to a poor metaphase spreading. This was mainly due to an ineffective hypotonic treatment, a step that proved to be one of the most crucial ones, as previously stated. A further evidence of this comes from the fact that the numerical counts of chromosomes registered for metaphases analyzed under the confocal microscope are greater than the mode (all these values fall into the right tail of the frequency distributions). Indeed, the higher resolution of confocal microscope allowed a better visualization of the smallest chromosomes when the metaphase was poorly spread, resulting in a higher chromosome number (e.g. Fig 2E, F). The issue of poor spreading of metaphases is known to affect the counts of chromosomes in crustaceans, and some authors suggested to use the scanning electron microscope (SEM) to gain more resolution for a better analysis of chromosomes (Lee *et al.*, 2004, 2008). However, this technology is very laborious and thus not very effective.

The wide variability in distribution of chromosome numbers as well as the small size of chromosomes prevented us from proposing a reference karyotype for the species. Such problems were also registered for the other species described so far, for which authors did not provide a karyotype. To our knowledge, the only karyotypes available for Brachyura are those of *Scylla serrata* and *Portunus pelagicus* (Swagatika *et al.*, 2014). Analyses under a confocal microscope gave the best resolution, allowing discernment of chromosome morphology and revealing the presence of AT-rich pericentromeric regions in *C. haematocheir*. This is the first observation of this kind for crab chromosomes, albeit being a common feature of eutherian species (Sumner 2008).

Nonetheless the lack of a reference karyotype for any of the study species, our results on species' chro-

mosome numbers will be extremely valuable in future genomic studies, i.e. for assessing genome assembly quality. It is known, in fact, that the comparison between the number of final scaffolds in assemblies and the chromosome number range of a given species may provide a clear indication of the level of fragmentation of the assembly. In particular, when the number of final scaffolds is much higher than the chromosome number mode, the assembly needs more refinement; while if the number of final scaffolds is much lower than the mode, the assembly presumably includes chimeric scaffolds and thus needs to be revised (Ma *et al*., 2012; Burton *et al*., 2013; Sharakhov *et al*., 2014).

CONCLUSIONS

Brachyura are undoubtedly an ecologically and economically important taxon, however, so far, very few studies have targeted their karyology, with information generally limited to the description of chromosomes numbers. Many authors report difficulties in obtaining cytogenetic information in this taxon due to high number and small size of chromosomes (Lécher *et al*., 1995; Lee *et al*., 2004; Tan *et al*., 2004). Our results corroborate the presence of such methodological issues and stress the fact that several improvements are still needed to reach the quality standard needed for molecular cytogenetic researches. This study also underlines that ecological and physiological adaptations of a species can affect its responses to the sequential steps of karyotyping analysis. This outcome makes the design of a standard protocol for cytogenetic analyses in brachyurans even more difficult. However, our comparative approach highlighted the critical steps that must be improved to obtain high quality material in true crabs. We believe therefore that this study provides a step forward in the cytogenetic of brachyurans and represents an important basis for further cytogenetic methods in this taxon.

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The authors declare that all the experiments were performed in the respect of ethical rules. They also declare that there is no conflict of interest in publishing the present paper. This study was funded to SC by the HKU Seed Fund for Basic Research no. 201605159004 and the Faculty of Sciences RAE Improvement Fund no. 000250449.088562.26000.100.01.

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