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Comparative study on karyotypes of three *Mastacembelus* species (Synbranchiformes: Mastacembelidae) from Thailand

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Abstract. *Seree W, Supiwong W, Nuntaporn G, Ditcharoen S, Donbundit N, Seetapan K, Tongnunui S, Juntharat S, Tanomtong A. 2025. Comparative study on karyotypes of three* Mastacembelus *species (Synbranchiformes: Mastacembelidae) from Thailand. Biodiversitas 26: 246-254.* Karyotype study or chromosomal study is necessary for some cases of fish taxonomy. The chromosomes of three *Mastacembelus* species named Zig-zag eel or *Mastacembelus armatus*, Fire eel or *Mastacembelus erythrotaenia*, and Tire track eel or *Mastacembelus favus* using conventional Giemsa staining and Ag-Nucleolar Organizer Region (NOR) banding techniques were investigated. The results indicated that conserved diploid number (2*n*) of 48 and the fundamental number or chromosome arm number (NF) as 62 for all analyzed species of male and female specimens were revealed, although varying in their karyotype structures were observed. Karyotypes of three species are as follows: 12m+2sm+2a+32t, 10m+6sm+32t, and 8m+6sm+34t in *M. armatus*, *M. erythrotaenia*, and *M. favus*, respectively. In each species, positive Ag-NOR markers were differentially observed adjacent to telomeric and/or sub-telomeric regions (telomeric NORs) of metacentric or submetacentric chromosomes. In *M. armatus*, NOR locations are at the long arm of the first metacentric chromosome pair, while in *M. favus* and *M. erythrotaenia*, NOR locations are at the short arm of the first submetacentric chromosome and the first metacentric chromosome pairs, respectively. The findings show that *Mastacembelus* species, despite having a conserved diploid number, differ remarkably in the patterns of Nucleolar Organizer Regions (NORs) in their karyotypes. These species-specific patterns can be useful in further characterizing and identifying different species and investigating the evolutionary mechanisms driving the evolution of these fish karyotypes.

Keywords: Chromosome, cytogenetics, nucleolar organizer region, spiny eel fish

INTRODUCTION

Mastacembelidae is a family of fish in the order Synbranchiformes. It is found in freshwater and brackish habitats from Africa to Southeast Asia and Borneo (Nelson 2016). Up to date, 93 valid species are currently recognized in three genera (Fricke et al. 2024b). The Mastacembelidae family is important to humans in terms of being an economic fish for consumption. Moreover, several fishes in this family are also ornamental fish, especially the Zig-zag eel or *Mastacembelus armatus* Lacepède 1800 (Figures 1.A and 1.B), and Fire eel or *Mastacembelus erythrotaenia* Bleeker 1850 (Figure 1.D). They have a high value in the ornamental fish market (Vidthayanon and Daniels 2020). Thus, the main species in this family is the genus *Mastacembelus* which consists of three species recorded in Thailand including Tire track eel or *Mastacembelus favus* Hora 1924, *M. armatus*, and *M. erythrotaenia*. However, many species in this family are rather morphologically similar especially during the juvenile stage that may pose difficulties for their identification such as *M. armatus*, and *M. erythrotaenia*. It is difficult to identify the species due to the variation of color and reticulated patterns on the body of *M. armatus* (Figures 1.A and 1.B), that it is similar to *M. favus* (Figure 1.C). Therefore, not only morphology data is used to identify species, but other data such as behavior, molecular biology, genetics, and cytotaxonomy, also can be used as a tool for characterizing and identifying different species (Duong et al. 2020). The genus *Mastacembelus* is a poorly diagnosed group, and they are morphologically similar and diagnostic characteristics are usually complicated (Tran et al. 2013; Duong et al. 2020), leading to easy species misidentification.

Chromosome studies, or karyotype analyzes, in fishes have provided basic information on the number, size, and morphology of chromosomes Tanomtong et al. 2019) which have made prominent contributions insights into evolution at the inter- and intra-specific levels, phylogenetics, systematics, taxonomy (Almeida et al. 2017; Hnátková et al. 2018; Gaaroglu et al. 2020; Supiwong et al. 2021;

Mingkwan et al. 2023), and genetic diversity (Cioffi et al. 2018; Ditcharoen et al. 2019, 2020; Chaiyasan et al. 2021a; Yeesin et al. 2021). Chromosomal analysis is important for fish breeding in terms of genetic control, the rapid production of inbred lines and the study of evolutionary processes (Maneechot et al. 2016; Khensuwan et al. 2023, 2024). Karyotypic studies are also important in aquaculture, involving the use of chromosome manipulation techniques such as induction of polyploidy, gynogenesis, androgenesis and inter- or intra-specific hybridization (Hou et al. 2014; Meng et al. 2016; Sreeputhorn et al. 2017). Chromosomal analyzes are also used in aquatic cytotoxicology (Promsid et al. 2015; Khamlerd et al. 2019; Phoonaploy et al. 2020; Thitiyan et al. 2022). The karyotypes of fishes belonging to the *Mastacembelus* genus have been studied in three reports (Arai 2011. Three species in Thailand and one species from India have been cytogenetically analyzed (Donsakul and Magtoon 1989; 1992; Arai 2011; Saowakoon and Saowakoon 2019). The diploid number is conserved as 2n = 48 and the NF = 62-64 have been presented (Arai 2011).

Consequently, there are a few reports of cytogenetics studies in this family of fishes, and most of them are only studied on conventional staining technique. Other staining techniques of the chromosome banding are very few. An important characteristic of Nucleolar Organizer Regions (NORs) in fish which detected by Ag-NOR staining is related to that it has inter- and intra-species polymorphism. NOR characteristics can be a cytogenetic marker for cytotaxonomic studies, and it also has been used for studying phylogenetic relationships among some fish groups (Supiwong et al. 2021; Yeesin et al. 2021; Mingkwan et al. 2023). Because of the scarcity of cytogenetic reports of this fish group, the present research aimed to study the comparative cytogenetics of fishes in the genus *Mastacembelus* by conventional staining and Ag-NOR banding techniques. Its obtained findings can be used to provide an important basis for genetics which will be utilized for improving fish breeding with good genetics characteristics according to market demand, even creating new ornamental fish species in the future, as well as further studies of genetic relationship in this family.

MATERIALS AND METHODS

Sampling and chromosome preparations

Ten male and ten female adult specimens of each analyzed species; *M. armatus*, *M. favus*, and *M. erythrotaenia* were collected from two river basins and fish market in Thailand, following *M. armatus* in the Mekong Basin, Nong Khai Province (17°56'16.9"N 102°48'59.2"E), *M. favus* in Kwai Basin, Kanchanaburi Province (14°06'47.8"N 99°08'41.7"E), and *M. erythrotaenia* in Fish and Aquarium Chatuchak Market, Bangkok Province, Thailand (13°48'09.6"N 100°32'55.6"E). The live specimens were carefully transferred to the laboratory aquaria and kept under standard conditions (in a well-aerated aquarium at 20-28℃) before the studies. Then, fish samples were identified for the correct species by using documents in the manuals of Tran et al. (2013) and Nelson et al. (2016). Both *M. armatus* and *M. favus* have reticulated forms on the body and similar ranges of countable characteristics. One distinguishing feature is the reticular pattern that covers the entire body of *M. favus*, which appears only on the upper two-thirds of the body of *M. armatus*. In *M. erythrotaenia*, the head and anterior part of the body have longitudinal red and black bands, while the rest of the body features red spots or elongated markings on a black background. The accepted scientific name is based on the Catalog of Fishes online database (Fricke et al. 2024a). Chromosomal preparation was performed directly in vivo. A 0.5% colchicine solution was injected intraperitoneally into all specimens at a dose of 1 mL/100 g of body weight, and all specimens were sacrificed after 1 hour using anesthetic tricaine mesylate (MS-222 euthanasia dose fish) by incorporating 25-30 mg/L of the anesthetic into the water in which the fish were immersed. The kidney tissues are the target for preparing metaphase chromosomes (Kumar et al. 2014; Khakhong et al. 2014; Kasiroek et al. 2017; Chaiyasan et al. 2018; Pissaparn et al. 2020). The procedures of ethical protocols were conducted under the approval of Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimental of the National Research Council of Thailand IACUC-KKU-65/64.

Chromosome staining

Conventional and Ag-NOR staining techniques were performed using 20% Giemsa's solution (pH=6.8) and 50% silver nitrate solution, respectively, to stain the chromosomes, modified from Sangpakdee et al. (2015), Sangpakdee et al. (2017), Jantarat et al. (2017), Pinthong et al. (2017), Sreeputhorn et al. (2017), Supiwong et al. (2017b), Getlekha and Tanomtong (2020), and Pissaparn et al. (2020). Twenty metaphase spreads of each sample were recorded using a Cannon D-150 camera.

Data analysis

The chromosomal analysis was performed using Microsoft Excel 2016 software and Adobe Photoshop CS6. The parameters of chromosomal analysis including the length of short arm (Ls), the length of long arm (Ll), the length of total chromosome LT, relative length (RL, centromeric index (CI, were calculated (Pissaparn et al. 2020). The chromosomal classification was conducted using the CI (Ll/LT) values that there are between 0.500-0.599, 0.600-0.699, 0.700-0.899, and 0.900-1.000 described as metacentric (m), submetacentric (sm), acrocentric (a), and telocentric (t) chromosomes, respectively (Tanomtong et al. 2019; Pissaparn et al. 2020). The fundamental number or chromosome arm number (NF) was used the NF_1 values following Arai (2011) as the m and sm chromosomes have value as two while the a and t chromosomes have value as one. The methods for the classification of chromosomal sizes, karyotyping, and idiograming were performed according to the protocols of Tanomtong et al. (2019).

RESULTS AND DISCUSSION

Diploid chromosome number (2*n*), fundamental number (NF), and karyotype of the *Mastacembelus*

All *Mastacembelus* species had 2n = 48 and NF = 62 in both males and females. Heteromorphic sex chromosomes between male and female specimens could not be observed in the three species analyzed (Figure 1). The chromosomes of three *Mastacembelus* species were classified into large and medium sizes. There are differences in karyotypic structures among them as follows.

Mastacembelus armatus had 12 large metacentric, two large submetacentric, two large acrocentric, two large telocentric, and 30 medium telocentric chromosomes or 12 metacentric, two submetacentric, two acrocentric, and 32 telocentric chromosomes ($L^m_{12} + L^{sm}_2 + L^a_2 + L^t_2 + M^t_{30}$ or 12m + 2sm + 2a + 32t). *M. favus* had eight large metacentric, six large submetacentric, four large acrocentric, and 30 medium telocentric chromosomes or eight metacentric, six submetacentric, and 34 telocentric chromosomes ($L^m_8 + L^{sm}_6 + L^t_4 + M^t_{30}$ or 8m + 6sm + 34t) while *M. erythrotaenia* had ten large metacentric, six large submetacentric, two large acrocentric, and 30 medium telocentric chromosomes or ten metacentric, six submetacentric, and 32 telocentric chromosomes ($L^m_{10} + L^{sm}_6 + L^t_2 + M^t_{30}$ or 10m + 6sm + 32t) (Figure 2).

Idiogram represents a karyotype diagram of one haploid set of chromosomes (*n*) which include chromosome shapes and chromosome lengths by using the average of chromosome length data, chromosome shape and the centromere position from 20 metaphase chromosome plates. The standardized idiograms of *M. armatus*, *M. favus*, and *M. erythrotaenia* from conventional staining and Ag-NOR banding techniques are shown in Figure 3. All species herein had 18 large and 30 medium chromosomes. All bi-armed chromosomes and a few pairs of mono-armed chromosomes have large size, while most mono-armed chromosomes have medium size.

Figure 1. Pictures of analyzed *Mastacembelus* (Mastacembelidae). A-B. *Mastacembelus armatus*; C. *Mastacembelus favus*; D. *Mastacembelus erythrotaenia*

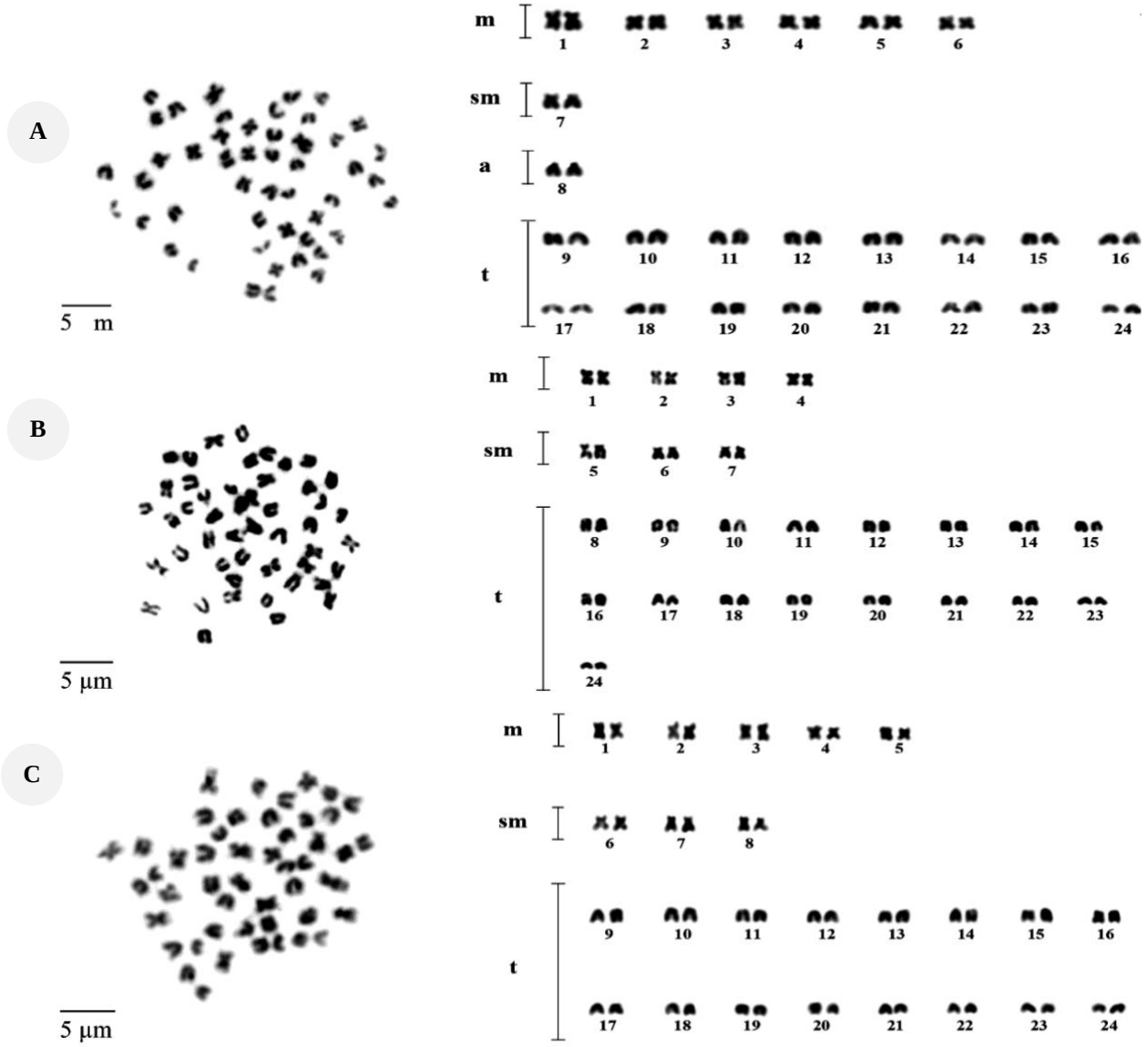

Figure 2. Metaphase chromosome plates (2*n* = 48) and karyotypes: A. *Mastacembelus armatus*, B. *Mastacembelus favus*, C. *Mastacembelus erythrotaenia* by conventional staining technique

Marker chromosomes of the *Mastacembelus*

The marker chromosomes or NOR-bearing chromosomes, are chromosomes that have a specific marker and can be detected in that organism and they also have a special character. All analyzed species had a single NOR-bearing chromosome pair and telomeric NOR positions (telomeric NORs) were also revealed. NOR sites are located at the regions adjacent to telomere of the long arm of the first submetacentric chromosome pair or the chromosome pair 1 in *M. armatus* (Figure 4.A), while those are the regions adjacent to telomere of the short arm of the first submetacentric chromosome pair or the chromosome pair 5 (Figure 4.B), and the first metacentric chromosome pair or the chromosome pair 1 (Figure 4.C) in *M. favus*, and *M. erythrotaenia*, respectively.

Discussion

*Diploid chromosome number (*2n*), fundamental number (NF), and karyotype of the* Mastacembelus

All *Mastacembelus* species demonstrated the same 2n = 48. They are also the same 2n as previous reports studied in *Macrognathus aculeatus* Bloch 1786, *Macrognathus circumcinctus* Hora 1924, *Macrognathus pancalus* Hamilton 1822, *Macrognathus siamensis* Günther 1861, M. *armatus*, *M. erythrotaenia*, and *M. favus* (Donsakul and Magtoon 1989; 1992; Arai 2011; Saowakoon and Saowakoon 2019). However, they differ from *M. siamensis*, as reported by Saowakoon and Saowakoon (2019), which is 2n = 50. Although the same diploid chromosome number is found in all *Mastacembelus* investigated, *M. erythrotaenia* differed in the fundamental number (NF = 64) compared to the previous investigation, which showed that NF = 62 (Donsakul and Magtoon 1989). Only seven of 93 species in this family

have been cytogenetically analyzed. The previous and present findings showed that the NF is more diverse than the 2n. The NF ranged between NF = 54 and NF = 78, while the 2n ranged between 48 and 50 chromosomes (Arai 2011). Mingkwan et al. (2023) suggested that a higher NF was referred to be the apomorphic or advanced character for fish species, whereas the karyotype with 2*n* = 48 is considered as the plesiomorphic or primitive karyotype. In addition, six of seven species of the Mastacembelidae family have 2*n* = 48. It indicates that this family has a conserved diploid chromosome number (2*n*) among fish families.

In the present study, the karyotypes of these fishes are presented in both types and sizes of chromosomes for the first time. There are differences in karyotype structures among the three *Mastacembelus* analyzed. Moreover, the karyotypes of each species were found to be different between the results of the present and previous studies. The karyotype formula for *M. armatus* is 12m + 2sm + 2a + 32t. It is inconsistent with reports of Donsakul and Magtoon (1992), and Arai (2011), who found 12m + 2sm + 30a + 4 subtelocentric (st), and 10m + 4sm + 2st + 32t, respectively. One for *M. favus* is 8m + 6sm + 34t. It differs from the report of Donsakul and Magtoon (1989), who found 10m + 4sm + 4st + 30t. For *M. erythrotaenia*, the karyotype formula is 10m + 6sm + 32t. It differs from the report of Donsakul and Magtoon (1989) who revealed 12m + 2sm + 4st + 34t. The hypothesis of karyotype differentiation has been referred to the intra-specific variation among populations, incorrect identification of one species as another because of species complexity, and/or chromosomal rearrangements such as deletions, pericentric and paracentric inversions, Robertsonian rearrangement, and chromosomal translocation (Esmaeili et al. 2015; Getlekha et al. 2016; Maneechot et al. 2016; Ditcharoen et al. 2019; Ditcharoen et al. 2020; Chaiyasan et al. 2021b; Supiwong et al. 2021; Yeesin et al. 2021; Mingkwan et al. 2023; Khensuwan et al. 2023, 2024; Buasriyot et al. 2024). Supiwong (2021) also explained that when chromosomal evolution occurs in populations divided by a geographic barrier, centric fusion and pericentric inversion may reorganize chromosomes, leading to this variance. Considering the NF and karyotypic structure, the high NF and more bi-armed chromosomes seem to be more advanced characters than the low NF and more mono-armed chromosomes (Yeesin et al. 2021). Accordingly, *M. erythrotaenia* is considered to have more advanced karyotypic characters than in *M. armatus* and *M. favus*. Moreover, there is no evidence of heteromorphic sex chromosomes in these species, according to all species of this family (Donsakul and Magtoon 1989; Donsakul and Magtoon 1992; Arai 2011; Saowakoon and Saowakoon 2019). Like several fishes in Thailand, sex chromosomes may be in the early stage of differentiation as heteromorphic pattern. Only one species, *Belontia hasselti* Cuvier 1831 has the ZZ/ZW sex chromosome system (Chaiyasan et al. 2021a).

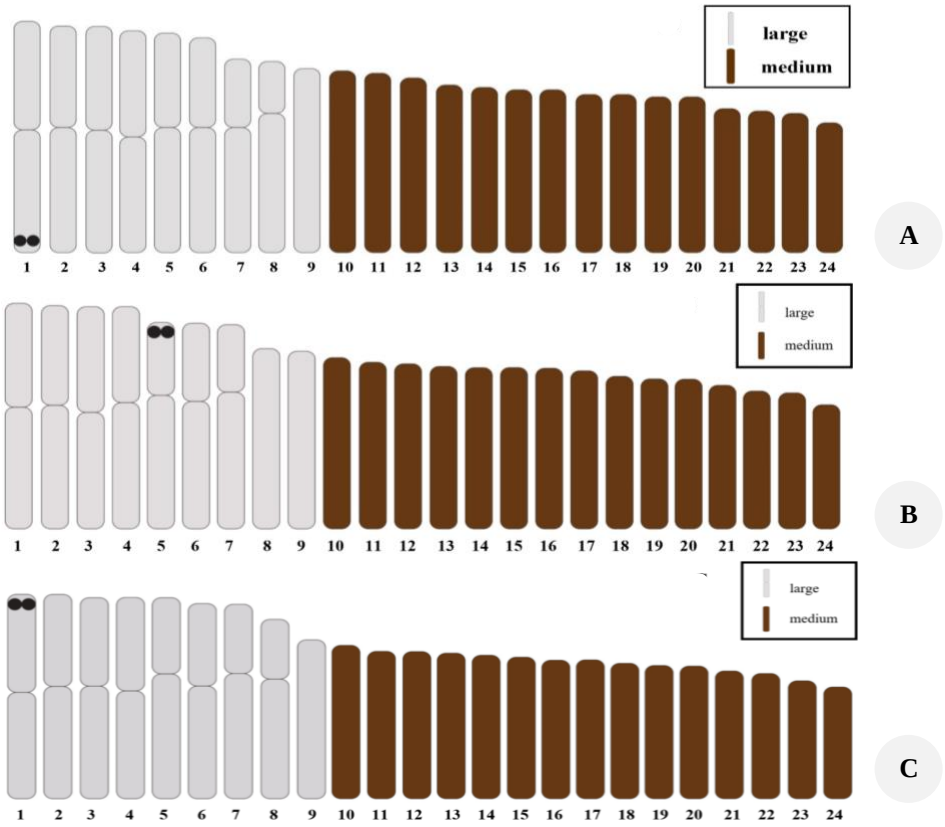

Figure 3. Standardized idiograms of: A. *Mastacembelus armatus*, B. *Mastacembelus favus*, C. *Mastacembelus erythrotaenia* (*n* = 24) by conventional staining and Ag-NOR banding techniques. Chromosomes of three species are divided into two sizes, large and medium. Black dots indicate Nucleolar Organizer Regions (NORs)

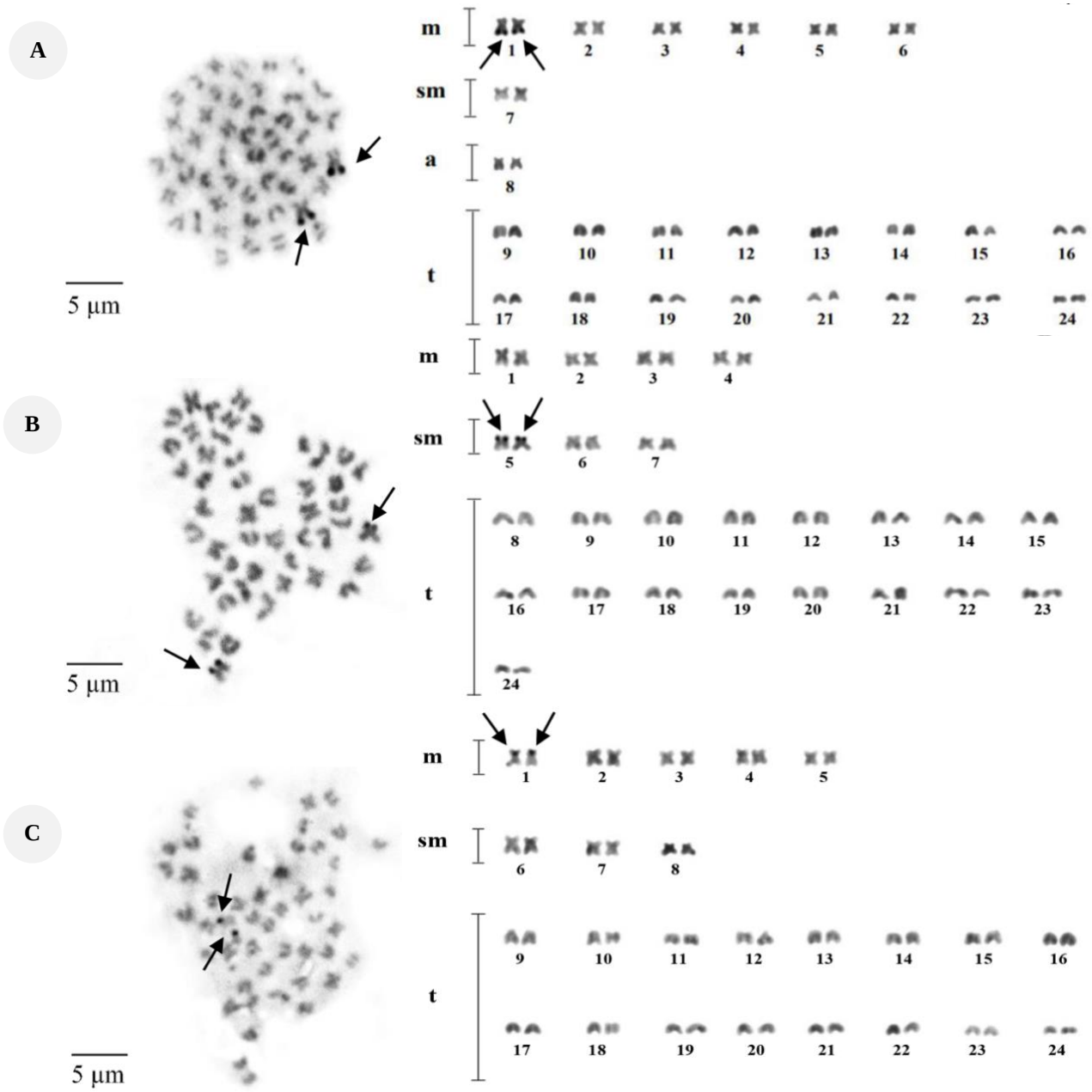

Figure 4. Metaphase chromosome plates (2*n* = 48) and karyotypes: A. *Mastacembelus* armatus, B. *Mastacembelus favus*, C. *Mastacembelus erythrotaenia* by Ag-NOR banding technique. Arrows indicate Nucleolar Organizer Regions (NORs)

The comparison of the karyotypic structures among three *Mastacembelus* exhibited that there are differences in each species as four chromosome types (m, sm, a, t) observed in *M. armatus* and three chromosome types (m, sm, t) observed in *M. erythrotaenia* and *M. favus*. In addition, the number of mono- and bi-armed chromosomes in *M. erythrotaenia* as 16 bi- and 32 mono-armed chromosomes, differs from *M. armatus* and *M. favus* as 14 bi- and 34 mono-armed chromosomes. Considering chromosome sizes, all species had 18 large and 30 medium chromosomes. However, there are differences of chromosome types in the large sized group such as all bi-armed chromosomes with two, two, and one pair of mono- armed found in *M. armatus*, *M. favus*, and *M. erythrotaenia*, respectively. From the obtained finding, the number of chromosome types in karyotype can be classified between *M. armatus* and *M. favus*.

Marker chromosomes of the Mastacembelus

The present research is also the first report on NOR-bearing chromosomes detected by Ag-NOR staining method. This technique is used to identify the active ribosomal RNA gene loci on chromosomes. These locations are considered as species-specific markers for several fishes (Supiwong et al. 2014; Almeida et al. 2017; Ditcharoen et al. 2019; Supiwong et al. 2021; Aiumsumang et al. 2021;

Mingkwan et al. 2023). All *Mastacembelus* investigated had a single pair of NOR-bearing chromosomes. It is consistent with *M. circumcinctus*, but it differs from *M. siamensis*, which had two pairs of NOR-bearing chromosomes (Saowakoon and Saowakoon 2019). Single NOR pair are also widespread in numerous fishes (Arai 2011; Kaewsri et al. 2014a, 2014b; Khakhong et al. 2014; Pinthong et al. 2014; Jumrusthanasan et al. 2015; Maneechot et al. 2015; Phimphan et al. 2015; Pinthong et al. 2015; Getlekha et al. 2017; Jantarat et al. 2017; Kasiroek et al. 2017; Phimphan et al. 2017; Pinthong et al. 2017; Supiwong et al. 2017a, 2017b, 2017c; Sochorová et al. 2018; Phimphan et al. 2020; Pissaparn et al. 2020; Aiumsumang et al. 2021; Chaiyasan et al. 2021a, 2021b; Ditcharoen et al. 2021; Supiwong et al. 2021; Yeesin et al. 2021). Supiwong et al. (2014) mentioned that the occurrence of multiple NORs in fishes was considered to be apomorphic or advanced condition, whereas single pair of NORs was considered to be plesiomorphic or a primitive condition. Thus, the *Mastacembelus* species seem to be conserved of NOR number in karyotype.

Considering NOR positions, they are located at regions adjacent to telomere of the short arm revealed in *M. favus* and *M. erythrotaenia*, while those are regions adjacent to telomere of the long arm observed in *M. armatus*. This character is considered as telomeric NOR. It differs from *M. circumcinctus* and *M. siamensis* that NOR sites are at pericentromeric regions or centromeric NOR (Saowakoon and Saowakoon 2019). However, three *Mastacembelus* herein have different NOR-bearing chromosome pairs such as pairs 1 (long arm), 5 (short arm), and 1 (short arm) in *M. armatus*, *M. favus*, and *M. erythrotaenia*, respectively. Interestingly, NOR character is suitable for species identification in the case of *M. armatus* and *M. favus*. These two species are sibling species that are more similar in morphology (Duong et al. 2020). The difference of NOR locations in karyotype between *M. armatus* and *M. favus* may be caused by the pericentric inversions during chromosomal evolution. This process can change NOR positions and NOR-bearing chromosomes i.e., the long arm of metacentric chromosome in *M. armatus* or the short arm of submetacentric chromosome in *M. favus*. In fishes, the location of NORs in a terminal position, and close to the centromere (centromeric NOR), is also considered to be a primitive feature (Supiwong et al. 2021).

Accordingly, the *Mastacembelus* seems to be more advanced trait than that in the *Macrognathus* due to their NOR locations. Telomeric NORs in three *Mastacembelus* are the same as found in several fish families such as Bagridae, Clariidae, Chanidae, Cyprinidae, Epinephelinae, Labridae, Scaridae, Siganidae and Sirulidae (Kaewsri et al. 2014a, 2014b; Khakhong et al. 2014; Pinthong et al. 2014; Supiwong et al. 2014; Jumrusthanasan et al. 2015; Phimphan et al. 2015; Pinthong et al. 2015; Maneechot et al. 2016; Getlekha et al. 2017; Pinthong et al. 2017; Supiwong et al. 2017c; Chaiyasan et al. 2018; Ditcharoen et al. 2019; Phimphan et al. 2020; Ditcharoen et al. 2021; Yeesin et al. 2021; Mingkwan et al. 2023; Khensuwan et al. 2023, 2024; Buasriyot et al. 2024). However, there are difference from some fish families such as Chaetodontidae, Heamulidae, Lutjanidae, Notopteridae, and Osphronemidae (Maneechot et al. 2015; Jantarat et al. 2017; Phimphan et al. 2017; Supiwong et al. 2017a, 2017b; Chaiyasan et al. 2021a, 2021b; Supiwong et al. 2021; Mingkwan et al. 2023) that these fishes had centromeric NORs. The chromosomal evolution processes in this family have been related to the pericentric inversions in the genus *Mastacembelus*, while in the genus *Macrognathus*, both pericentric inversions and chromosomal fissions may be the processes during chromosomal evolution from the ancestor. Moreover, in the genus *Mastacembelus*, the chromosomal evolution has occurred through pericentric inversion process in NOR-bearing chromosomes. This may be the reason for the description of NOR loci difference between the three *Mastacembelus* species analyzed. Thus, the NOR characteristics can be used as taxonomic markers in the *Mastacembelus* species in the present study. They are also used as taxonomic markers in many fish groups (Khakhong et al. 2014; Supiwong et al. 2014; Sochorová et al. 2018; Supiwong et al. 2021; Yeesin et al. 2021; Mingkwan et al. 2023).

Duong et al. (2020) stated that *M. armatus* may be confused with *M. favus* because both have reticulated forms on the body and similar ranges of countable characteristics. Therefore, only morphology data is not suitable for species identification. However, in the present study, the cytogenetic data such as the karyotype, the NOR- bearing chromosome pairs, and NOR positions are different between *M. armatus* and *M. favus*. Thus, these findings confirm that cytogenetic data (karyotype structure and NOR characteristic) can be used for species identification in the case of *M. armatus* and *M. favus*. The results are agreeable with the report by Duong et al. (2020), who studied the differences in Cytochrome C oxidase I (COI) gene sequences and morphology of fishes in the family Mastacembelidae in the Mekong Delta, Viet Nam. They revealed that genetic distance based on COI sequences between *M. armatus* and *M. favus* was 12.4%, indicating that two species can be classified by DNA barcoding. As mentioned above, cytogenetics and molecular genetics are power tools for species identification in this genus.

In conclusion, data from conventional staining and Ag-NOR staing techniques gave a deeper understanding of the karyotypic structures and marker chromosomes of *Mastacembelus* species. The results showed that despite having a shared 2n = 48 diploid number, karyotype formula and the Ag-NOR sites vary among species. Cytogenetic data revealed species-specific patterns in the genus *Mastacembelus*. Our further studies will focus on exploring the genetic diversity among these species including other genera in this family by using Fluorescence In Situ Hybridization (FISH) method to make phylogeny for the group and discuss the chromosomal evolution of this family.

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