The First Chromosomal Characteristics of Nucleolar Organizer Regions and Karyological Analysis of Clown Knife Fish, *Chitala ornata* (Osteoglossiformes, Notopteridae) by T-Lymphocyte Cell Culture

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**Summary** The first chromosomal characteristics of nucleolar organizer regions (NORs) of the clown knife fish, *Chitala ornata* (Gray 1831) from the Chi River in Roi-Et Province, northeast Thailand, were studied. Blood samples were taken from 4 male and 4 female fish. Standard whole blood lymphocytes were cultured at 27°C for 96 h in the presence of colchicines. Metaphase spreads were performed on microscopic slides and then air-dried. Conventional and Ag-NOR staining techniques were applied to stain the chromosomes. The results showed that the diploid chromosome number of *C. ornata* was 2n=42, and the fundamental number (NF) was 44 in both male and female. Chromosomes types were present as 8 large telocentric, 2 medium acrocentric, 14 medium telocentric, and 18 small telocentric chromosomes. No strange sized chromosomes related to sex were observed. The region adjacent to the short arm near the centromere of chromosome pair 11 showed clearly observable secondary constriction/NORs. The karyotype formula for *C. ornata* is as stated:

\[2n \text{ (diploid)} = L_8^1 + M_2^5 + M_1^4 + S_1^18\]

**Key words** *Chitala ornata*, Karyotype, Chromosome, Nucleolar organizer regions (NORs).

The information on diploid chromosome numbers (2n), fundamental number (NF), size and type of chromosomes which have been obtained from cytogenetic studies have aided in the understanding of evolutionary mechanisms in the groups investigated (Khan *et al.* 2000, Tripathy and Das 1988). These studies are also important steps towards the establishment of genetic improvement techniques involved in chromosome manipulations and chromosome determinations, such as polyploidy induction, gynogenesis and androgenesis, sex control and inter- and intra-specific hybridizations (Wu *et al.* 1986, Diter *et al.* 1993). These genetic techniques have been widely applied to improve farmed stocks in many aquaculture species in the world (Arai 2001, Beardmore *et al.* 2001, Desprez *et al.* 2003, Dunham 2007).

The clown knife fish, *Chitala ornata* (Gray 1831), is member of the class Actinopterygii (ray-finned fishes), order Osteoglossiformes (bony tongues), and family Notopteridae (knife fishes). At present, there are 4 genera: *Xenomystus*, *Papyrocranus*, *Notopterus* and *Chitala*. In Thailand, there
are only 4 species, namely: \textit{N. notopterus}, \textit{C. lopis}, \textit{C. ornata} and \textit{C. blanci} (Smith 1945, Roberts 1992). The commonly known clown knife fish, \textit{C. ornata}, belongs to one of the oldest groups of extant teleost freshwater fishes. It is wildly distributed in freshwater bodies in Asia, including the Mekong basin in Laos, Thailand, Cambodia and Vietnam and the Chao Phraya and Meklong basins in Thailand. The clown knife fish fetches a high market value as a food item. It is very distinct, normally silvery gray in colour with a long knife like body (laterally compressed) and a long anal fin that gives these fish their common name. Mature fish normally have 5–10 (or even more) black spots ringed with white that usually increase in number and size as the fish grows. They are nocturnal creatures and cruise during the twilight hours. They normally hunt live prey and will try any fish that fits into their mouths (Rainboth 1996).

Only a few cytogenetic studies, on 3 species, of the knife fishes (Notopteridae) have been reported. Each of them has a similar diploid number: \textit{N. notopterus}, 2\textit{n}=42 from Thailand (Srivastava 1964, Donsakul and Magtoon 1990), 2\textit{n}=48 from India (Nayyar 1965); \textit{C. ornata}, 2\textit{n}=42 from Thailand (Donsakul and Magtoon 1990), 2\textit{n}=48 from India (Nayyar 1965) and \textit{C. blanci} 2\textit{n}=42 from Thailand (Donsakul and Magtoon 1990).

The present study is the first report of a cytogenetic study on \textit{C. ornata} accomplished with the Ag-NOR staining technique. The results obtained can provide increasing cytogenetic information for future studies on taxonomy and the evolutionary relationships among these fishes. Moreover, it provides useful basic information for conservation and breeding practices, as well as studies on the chromosomal evolution of this fish.

Materials and methods

The \textit{C. ornata} samples were obtained from Chi River in Roi-Et Province, northeast Thailand. The fish were transferred to laboratory aquaria and were kept under standard conditions for 7 days prior to experimentation. Blood samples were collected from 4 males and 4 females from the caudal vain and subsequently kept aseptically. The samples were kept in 3 ml vacuum tubes containing heparin to prevent blood clotting and cooled on ice until to experimentation.

\textbf{Cell culture and harvest}

The whole blood microwculture technique, which we adapted from Fujiwara \textit{et al.} (2001) was used in the lymphocytes cell culturing. Five millilitres of DMEM medium was prepared by 4\% PHA (Phytohemagglutinin) as a mitogen and kept in blood culture flasks. A blood sample of 0.5 ml was dropped into a medium bottle and mixed thoroughly. The culture bottles were loosely capped, incubated at 27\textdegree C under 5\% of carbon dioxide environment and regularly shaken in the morning and evening. At the harvesting time, the 96th hour of incubation, colchicine was added and well mixed, followed by further incubation for 30 min.

The blood sample mixture was centrifuged at 3,000 rpm for 5 min and the supernatant was discarded. Ten millilitres of hypotonic solution (0.075 M KCl) was applied to the pellet then the mixture was incubated for 30 min. KCl was discarded from the supernatant after being centrifuged again at 3,000 rpm for 5 min. Cells were fixed in fresh cool fixative (3:1 methanol:glacial acetic acid) and gradually added up to 8 ml before centrifuged again at 3,000 rpm for 5 min. After that the supernatant was discarded, the fixation was repeated until the supernatant was clear and then the pellet was mixed with 1 ml fixative. The mixture was dropped onto a clean and cold slide by micropipette following by the air-dry technique. The slide was conventionally stained with 20\% Giemsa’s solution for 30 min.

\textbf{Ag-NOR staining method}

The 2 drops of 50\% silver nitrate and 2\% gelatine were added on slides, respectively. Then the
slides were sealed with cover glasses and incubated at 60°C for 5 min. After that the slides were soaked in distilled water until cover glasses were separated. The slide was stained with 20% Giemsa’s solution for 1 min (Howell and Black 1980).

**Chromosomal checks, karyotyping and idiogramming**

Chromosome counting was performed on mitotic metaphase cells under a light microscope. Twenty clearly observable and well spread chromosomes of each male and female were selected and photographed. The length of the short arm chromosome (Ls) and the length of long arm chromosome (Ll) were measured and calculated to the length of total arm chromosome (LT, LT = Ls + Ll). The relative length (RL), the centromeric index (CI) and the standard deviation (SD) of RL and CI were estimated (Chaiyasut 1989). The CI (q/p+q) between 0.50–0.59, 0.60–0.69, 0.70–0.89 and 0.90–0.99 were described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. Fundamental number (number of chromosome arm, NF) was obtained by assigning a value of 2 to metacentric, submetacentric and acrocentric chromosomes and 1 to telocentric chromosome. All parameters were used in karyotyping and idiograming.

**Results and discussion**

**Diploid chromosome number, fundamental number and karyotype of C. ornata**

The diploid number (2n) found in C. ornata, was 42 chromosomes in both males and females (Fig. 1). This is in accordance with the previous study of Donsakul and Magtoon (1990). However, it is different from the previous study by Nayyar (1965), who reported that C. ornata from India had 2n=48. The NF was 44 in both males and females which is different from the report of Donsakul and Magtoon (1990), who detected an NF of 42. This difference was due to different criteria used for the classification of chromosome type. In comparison with the genus Chitala from Thailand, the diploid chromosome number of C. blanci from Thailand was 2n=42 (Donsakul and Magtoon 1990).

The karyotype consisted of 8 large telocentric, 2 medium acrocentric, 14 medium telocentric, and 18 small telocentric chromosomes. This result is similar to the report of Donsakul and Magtoon (1990), which revealed that C. ornata had the following chromosomes; 2 subtelocentric and 40 acrocentric chromosomes. No cytologically distinguishable sex chromosomes were observed which is similar to the results from Donsakul and Magtoon (1990) and the other knife fish (Nayyar 1965, Donsakul and Magtoon 1990). It is possible that the fish’s sex-chromosomes are dependent on an initiation of differentiation. Therefore, chromosomes containing sex-determination gene cannot be found by cytogenetic analyses. The origin and development of sex-chromosome has been reported on Neotropical fish in Brazil (Bertollo et al. 2004). This present study is the first report of its chromosome size, karyotype formula, and standardized idiogram. The karyotype formula for C. ornata is as follows:

\[ 2n = L_8^1 + M_2^a + M_4^b + S_1^18 \]

**Chromosome markers of C. ornata**

Our present study, was accomplished by using the Ag-NOR staining technique. The objective of this technique was to present nucleolar organizer regions (NORs) representing the location of genes (loci) that function in ribosome synthesis (18S and 28S ribosomal RNA). NORs produce numerous gene expressions and contain more non-histone protein than other chromosome regions. Therefore, the specific dark band was induced by the reduction of organic silver by these proteins, which change form silver to dark (Sharma et al. 2002). The region adjacent to the centromere of the short arm on chromosome pair 11 (medium acrocentric chromosomes) has observable NORs
Fig. 1. Metaphase chromosome plates and karyotypes of the clown knife fish (*Chitala ornata*) 2n=42 by conventional (A) and Ag-NOR straining techniques (B). The arrows indicate NOR–bearing chromosome pair 11, scale bars 5μm.

Fig. 2. Metaphase chromosome plates of male (A and B) and female (C and D) of the clown knife fish (*Chitala ornata*) diploid (2n)=42 from Northeast Thailand by Ag-NOR staining technique, arrows indicate nucleolar organizer regions; NORs (scale bars 5μm).
The extra characteristic of short arms near the centromere of chromosome pair 11 could be representative of a chromosome marker. More than 200 species of fish have been examined with the Ag-NORs staining technique. In addition, the amount and location of NOR could provide an explanation for the evolution of each chromosome.

Normally, most fish have only 1 pair of small NORs on the chromosome. However, some fish have more than 2 NORs, which may be caused by the translocation between some parts of chromosomes with a having NOR and another chromosome. Furthermore, NORs are usually located close to the telomere of the chromosome arm. If a NOR appears between the centromere and telomere (interstitial NOR), it may be the result of tandem fusion between this chromosome with a NOR and another one. However, it may be caused by the centric fusion or pericentric inversion between 2 telocentric chromosomes that one chromosome has a NOR at the telomere (Sharma et al. 2002).

The asymmetrical karyotype of *C. ornata*, and the 2 types of chromosomes (acrocentric and telocentric chromosome) that we found are important chromosome markers. The idiogram shows the continuous length gradation of chromosomes. The largest and smallest chromosomes show size differences (approximately 3 fold). The chromosome markers of *C. ornata*, are chromosome pair 1 which are the largest telocentric chromosomes and chromosome pair 21 which are the smallest telocentric chromosomes. Data on chromosomal checks on the mitotic metaphase cells of *C. ornata* are shown in Table 1. Figure 3 shows the idiogram of *C. ornata* fish obtained by conventional staining.

### Acknowledgments

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### Table 1. Mean length of the short arm chromosome (Ls), long arm chromosome (Ll), and total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes in 20 cells of the clown knife fish (*Chitala ornata*) 2n=42

<table>
<thead>
<tr>
<th>Chromosome pairs</th>
<th>Ls</th>
<th>Ll</th>
<th>LT</th>
<th>RL±SD</th>
<th>CI±SD</th>
<th>Chromosome size</th>
<th>Chromosome type</th>
</tr>
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<tr>
<td>1</td>
<td>0.000</td>
<td>1.000</td>
<td>1.000</td>
<td>0.084±0.000</td>
<td>1.00±0.000</td>
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<td>Large</td>
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<td>2</td>
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<td>0.892</td>
<td>0.076±0.000</td>
<td>1.00±0.000</td>
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<td>Large</td>
</tr>
<tr>
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<td>0.000</td>
<td>0.802</td>
<td>0.802</td>
<td>0.068±0.000</td>
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<td>Telocentric</td>
<td>Large</td>
</tr>
<tr>
<td>4</td>
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<td>0.732</td>
<td>0.732</td>
<td>0.061±0.000</td>
<td>1.00±0.000</td>
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<td>Large</td>
</tr>
<tr>
<td>5</td>
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<td>0.668</td>
<td>0.668</td>
<td>0.056±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Medium</td>
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<tr>
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<td>0.637</td>
<td>0.637</td>
<td>0.053±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Medium</td>
</tr>
<tr>
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<td>0.612</td>
<td>0.051±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Medium</td>
</tr>
<tr>
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<td>0.578</td>
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<td>0.049±0.000</td>
<td>1.00±0.000</td>
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<td>Medium</td>
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<tr>
<td>9</td>
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<td>0.553</td>
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<td>1.00±0.000</td>
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<td>Medium</td>
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<tr>
<td>10</td>
<td>0.000</td>
<td>0.536</td>
<td>0.536</td>
<td>0.045±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Medium</td>
</tr>
<tr>
<td>11*</td>
<td>0.120</td>
<td>0.402</td>
<td>0.522</td>
<td>0.044±0.000</td>
<td>0.770±0.069</td>
<td>Acrocentric</td>
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<tr>
<td>12</td>
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<td>0.043±0.000</td>
<td>1.00±0.000</td>
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<td>Medium</td>
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<tr>
<td>13</td>
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<td>0.496</td>
<td>0.496</td>
<td>0.042±0.000</td>
<td>1.00±0.000</td>
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<td>Small</td>
</tr>
<tr>
<td>14</td>
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<td>0.479</td>
<td>0.479</td>
<td>0.040±0.000</td>
<td>1.00±0.000</td>
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<td>Small</td>
</tr>
<tr>
<td>15</td>
<td>0.000</td>
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<td>0.464</td>
<td>0.039±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
<tr>
<td>16</td>
<td>0.000</td>
<td>0.451</td>
<td>0.451</td>
<td>0.038±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
<tr>
<td>17</td>
<td>0.000</td>
<td>0.437</td>
<td>0.437</td>
<td>0.037±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
<tr>
<td>18</td>
<td>0.000</td>
<td>0.421</td>
<td>0.421</td>
<td>0.035±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
<tr>
<td>19</td>
<td>0.000</td>
<td>0.395</td>
<td>0.395</td>
<td>0.033±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
<tr>
<td>20</td>
<td>0.000</td>
<td>0.367</td>
<td>0.367</td>
<td>0.031±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
<tr>
<td>21</td>
<td>0.000</td>
<td>0.336</td>
<td>0.336</td>
<td>0.028±0.000</td>
<td>1.00±0.000</td>
<td>Telocentric</td>
<td>Small</td>
</tr>
</tbody>
</table>

Remarks: *=satellite chromosome (NORs).
Fig. 3. Idiogram showing lengths and shapes of chromosomes of the clown knife fish (*Chitala ornata*) diploid (2n)=42 from Northeast Thailand by conventional staining technique. The arrow indicates NOR–bearing chromosome pair 11.

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References


Chaiyasut, K. 1989. Cytogenetics and Cytotaxonomy of the Family Zephyranthes. Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok.


Donsakul, T. and Magtoon, W. 1990. A chromosome study on three species of featherbacks, Notopterus chitala (Hamilton), N. blanci (D’Aubenton) and N. notopterus (Pallas), from Thailand. Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok.


