

First Cytogenetic Study of Green Chromis, *Chromis viridis* (Perciformes, Pomacentridae) by Conventional Staining and Ag-NOR Banding Techniques

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ABSTRACT

This work represents the first cytogenetics analysis conducted on the green chromis, Chromis viridis (Cuvier, 1830) from Thailand. Mitotic chromosomes were prepared from the anterior kidney. The cell suspensions were harvested by in vivo colchicine treatment. The slides were prepared by conventional air-drying method. Chromosomes were analyzed after the conventional and Ag-NOR staining techniques. The results showed that C. viridis has 2n=48 (48t), the fundamental number (NF) was 48 and no heteromorphic sex chromosomes were indentified. NORs were located on subcentomeric region of long arms of the 6th telocentric chromosome pair.

Keywords: Chromosome, Karyotype, Nucleolar organizer region, Damsel fishes, Perciformes

INTRODUCTION

The family Pomacentridae belongs to order Perciformes which includes the damselfishes and clownfishes (Nelson, 2006). Around 385 species distributed in 28 genera are classified in this family (Allen, 1991, 1997). Pomacentrid fishes are usually found in tropical seas, with only a few species being found in temperate waters. The highest abundance of species is found on coral reefs in the Indo-West-Pacific region. Especially, the greatest concentration of species is found on the area from the Philippines to Australia hosts. The remaining species are found

in the Atlantic or eastern Pacific, while few ones are native species on freshwater or brackish water surroundings (Allen, 1991).

Table 1. Cytogenetic reviews of the subfamily Chominae (Pomacentridae).

No.	Subfamily/Species	2n	NF	NORs	Formula	Locality	Reference
1	<i>Chromis caerulea</i>	48	48	–	48t	Japan	Ojima and Kashiwagi (1981)
2	<i>C. chromis</i>	48	48	–	48t	Spain (Malaga)	Alvarez et al. (1980)
3	<i>C. chrysur</i>	48	50	–	2m+46t	Japan	Ojima (1983)
4	<i>C. flavicauda</i>	39	54	2	9m+6sm+24t	Brazil (ES)	Molina and Galetti (2002)
5	<i>C. insolata</i>	47	56	2	3m+6sm+38t	Brazil (ES)	Molina and Galetti (2002)
	<i>C. insolata</i>	46	56	2	4m+6sm+36t	Brazil (ES)	Molina and Galetti (2002)
6	<i>C. multilineata</i>	48	48	2	48t	Brazil (BA)	Molina and Galetti (2002)
7	<i>C. ternatensis</i>	48	48	–	48t	Pacific	Takai and Ojima (1999)
8	<i>C. viridis</i>	48	48	2	48t	Gulf of Thailand	Present study
9	<i>Dascyllus abudafur</i>	48	50	2	2a+46t	Gulf of Thailand	Getlekha, N. (2017)
10	<i>D. aruanus</i>	33	48	–	15m/sm+18t	S. Japan	Ojima and Kashiwagi (1981)
		32	48	–	16m/sm+16t	S. Japan	Ojima and Kashiwagi (1981)
		32	48	2	16m/sm+16t	S. Japan	Kashiwagi et al. (2005)
		31	48	–	17m/sm+14t	S. Japan	Ojima and Kashiwagi (1981), Kashiwagi et al. (2005)
		30	48	–	18m/sm+12t	S. Japan	Ojima and Kashiwagi (1981), Kashiwagi et al. (2005)
		30	50	2	18m+2st+10t	Gulf of Thailand	Getlekha et al. (2016)
		29	48	–	19m/sm+10t	S. Japan	Ojima and Kashiwagi (1981), Kashiwagi et al. (2005)
		28	48	–	20m/sm+8t	S. Japan	Ojima and Kashiwagi (1981)
		28	48	2	20m/sm+8t	S. Japan	Kashiwagi et al. (2005)
		27	48	–	21m/sm+6t	S. Japan	Ojima and Kashiwagi (1981)
11	<i>D. carneus</i>	48	50	2	2a+46t	Gulf of Thailand	Getlekha et al. (2016)
12	<i>D. melanurus</i>	48	48	–	48t	S. Japan	Ojima and Kashiwagi (1981)
		48	48	2	48t	S. Japan	Kashiwagi et al. (2005)
		48	50	2	2a+46t	Indian Ocean	Getlekha et al. (2016)

Note: 2n = diploid chromosome number, NF = fundamental number (number of chromosome arm), m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, NORs = nucleolar organizer regions and – = not available.

Table 1. Cont.

No.	Subfamily/Species	2n	NF	NORs	Formula	Locality	Reference
13	<i>D. reticulatus</i>	36	48	2	12m/sm+24t	S. Japan	Hardie and Hebert (2004), Kashiwagi et al. (2005)
		35	48	–	13m/sm+22t	S. Japan	Ojima and Kashiwagi (1981), Kashiwagi et al. (2005)
		34	48	–	14m/sm+20t	S. Japan	Ojima and Kashiwagi (1981)
14	<i>D. trimaculatus</i>	48	48	–	48t	S. Japan	Ojima and Kashiwagi (1981), Hardie and Hebert (2004)
		48	48	2	48t	S. Japan	Kashiwagi et al. (2005)
		47	48	–	1m+46t	Japan	Arai and Inoue (1976), Kashiwagi et al. (2005)
		48	50	2	2st+46t	Gulf of Thailand	Getlekha et al. (2016)
		48	50	2	2a+46t	Gulf of Thailand	Getlekha et al. (2017)

Note: 2n = diploid chromosome number, NF = fundamental number (number of chromosome arm), m = metacentric, sm = submetacentric, a = acrocentric, t = telocentric chromosome, NORs = nucleolar organizer regions and – = not available.

Out of 385 described species of Pomacentridae, only 49 species have been karyologically investigated (Arai, 2011). The study reports that less than 13% of the number of species of damselfishes has been currently analyzed, inclusion only 7 species of the genus *Chromis*, However, despite this few number, karyotypes involved in Robertsonian rearrangements with diploid chromosomes less than 2n=48 and large bi-armed chromosomes were found in two *Chromis* species (Molina and Galetti, 2002). In the other analyzed species, the diploid number were 46 and 47 in *C. insolata*, both the NF being 56, 2n=39 in *C. flavicauda*, the fundamental numbers (NF) presenting 54 while most of them (five species) had 2n=48 chromosomes and the NF ranged from 48 to 50 in *C. caerulea*, *C. chromis*, *C. chrysur*, *C. multilineata* and *C. ternatensis*. (Alvarez et al., 1980; Ojima and Kashiwagi, 1981; Ojima, 1983; Takai and Ojima, 1999; Molina and Galetti, 2002). Another three species of *Dascyllus* showed inter- and intraspecific Robertsonian polymorphism: the diploid chromosome numbers were 2n=47 and 48 in *D. trimaculatus*, 2n=34, 35, 36, and 37 in *D. reticulatus*, 2n=27, 28, 29, 30, 31, 32 and 33 in *D. aruanus*, and 2n=48 in *D. melanurus*, these NF presenting 48 in all specimens. (Ojima and Kashiwagi, 1981).

In this research, we analyzed the standard karyotype, idiogram and NORs sites in *C. viridis* population from Thailand. This species was chosen for this study to extend the existing information on the family Pomacentridae for support taxonomy of genus *Chromis*.

MATERIALS AND METHODS

Specimens collected

The green chromis species were analyzed: *Chromis viridis* (Cuvier, 1830) (8 males and 10 females) from the Gulf of Thailand (Pacific Ocean) (Figure 1). The specimens were caught using hand-net. After captured, the specimens were then kept in sealed plastic bags containing oxygen and transported to the research station. The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Department of Biology, Faculty of Science, Khon Kaen University.

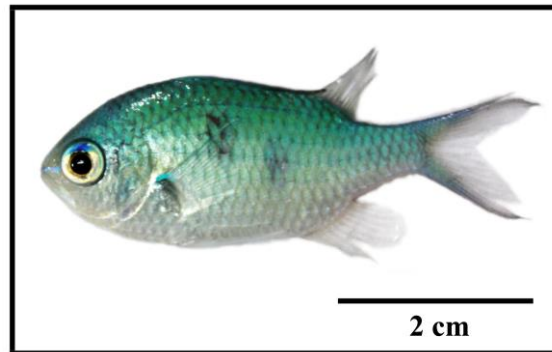


Figure 1. General characteristic of male green chromis, *Chromis viridis*.

Chromosome preparation

All the experiments followed ethical protocols and anesthesia with ice prior to sacrifice. Chromosomes were prepared from the cell suspensions of the anterior kidney. Briefly, the 0.01% colchicine was injected into its abdominal cavity for 1 h. The kidney tissue was taken off and gently minced in hypotonic solution (0.075M KCl) and then incubated for 30 min. The cell suspensions were then fixed in Carnoy's fixative solution (ratio 3 methanol: 1 acetic acid). The cell suspensions were dropped on the slide (Getlekha et al., 2017; Kasiroek et al., 2017; Supiwong et al., 2017).

Chromosome staining

Conventional staining technique. The slides were dipped into a copling jar containing 20% Giemsa's solution and left for 10 minutes. After that, they were rinsed in distilled water and left for air-drying (Rooney, 2001).

Ag-NOR banding technique. The 50% silver nitrate and 2% gelatin (ratio 2: 1, respectively) were dropped on slides. The slide was mounted with cover glass and incubated at 60°C for 8 min. The slide was then rinsed with distilled water until cover glass is seceded. After that, the slides were left for air-drying (Howell and Black, 1980).

Chromosome checking, karyotyping and idiogramming

Image processing, to determine the diploid chromosome numbers, karyotype and idiogram structures were made based on at least 20 metaphase

spreads. Images were taken using a light microscope connects with Canon EOS 550D DSLR camera and were organized using Photoshop. Chromosomes were classified as metacentric (m), submetacentric (sm), acrocentric (a) and telocentric (t) according to their arm ratios (Turpin and Lejeune, 1965).

Idiogram is a diagram representation of a chromosomal karyotype of a haploid set (n) which includes autosomes and sex chromosomes, showing the number and morphological characteristics of chromosomes of a species. The average length of chromosomes, the type of chromosome and the centromere region were used for idiogram structuring (Tanomtong, 2011).

RESULTS

Cytogenetic analyses were conducted on *C. viridis* from the Gulf of Thailand (Indo-Pacific). The $2n$ of *C. viridis* is 48 chromosomes in all specimens, with karyotypes predominantly formed by telocentric chromosomes (Figure 2, 3 and Table 2). The NF of *C. viridis* was 48 in both sexes and the karyotype comprises 48 telocentric chromosomes (48 mono-armed chromosomes) (Figure 4). Ag-NOR sites were detected on the subcentromeric portion of the long arms of the no. 6th telocentric pair in *C. viridis* (Figure 3). The karyotypes of *C. viridis* by Geimsa's staining and Ag-NOR staining techniques are depicted in Figures 2 and 3. The idiogram of *C. viridis* represent gradually declining length of the chromosomes (Figure 4). The important karyotype feature is the predominately with telocentric chromosome, which was found in all telocentric chromosomes. The karyotype formula of *C. viridis* is as follow: $2n (48) = 48t$

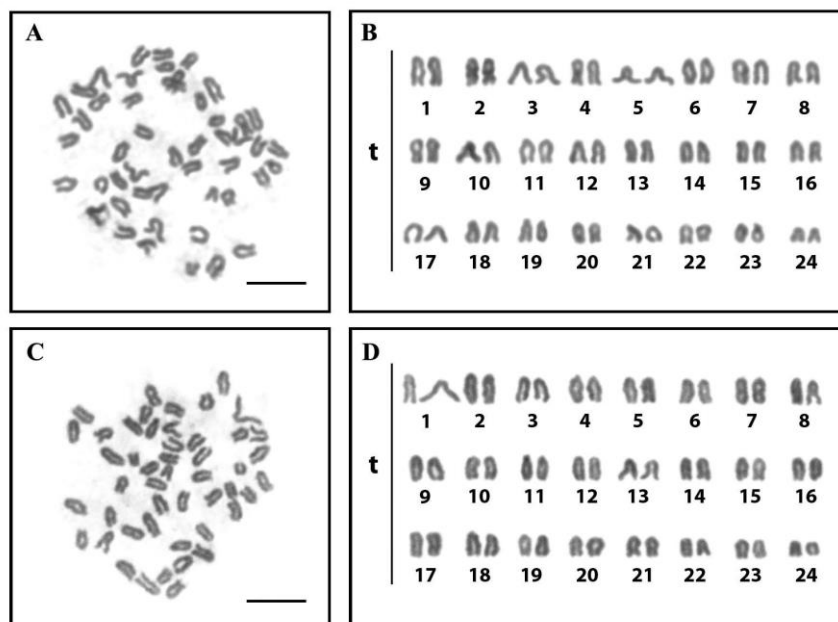


Figure 2. Metaphase chromosome plates and karyotypes of male (A and B) and female (C and D) of the green chromis (*Chromis viridis*, $2n=48$) by conventional staining (scale bars indicate 10 μ m).

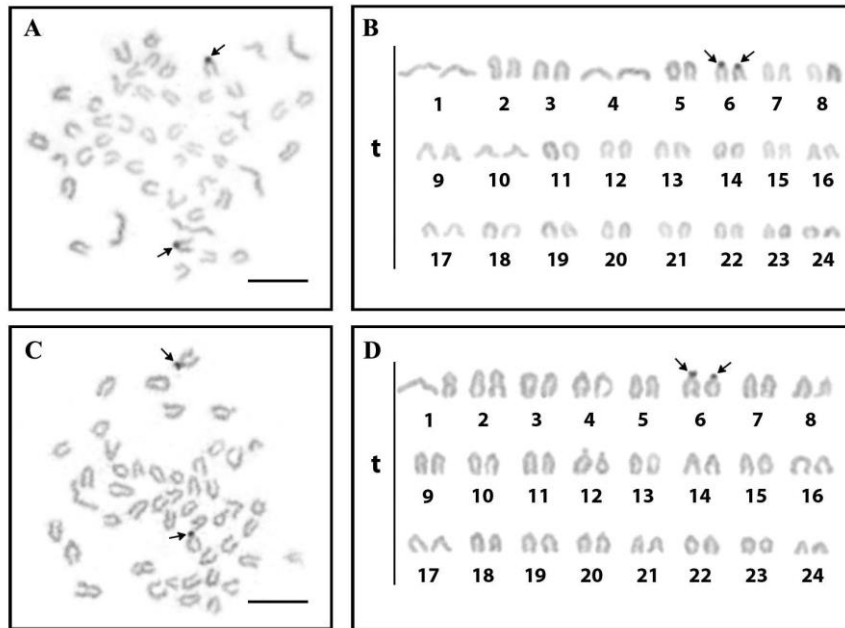


Figure 3. Metaphase chromosome plates and karyotypes of male (A and B) and female (C and D) of the green chromis (*Chromis viridis*, $2n=48$) by Ag-NOR banding technique; scale bars indicate 10 μm . The subcentromeric region of telocentric chromosome pair 6 showed clearly observable nucleolar organizer regions (NORs) (arrows).

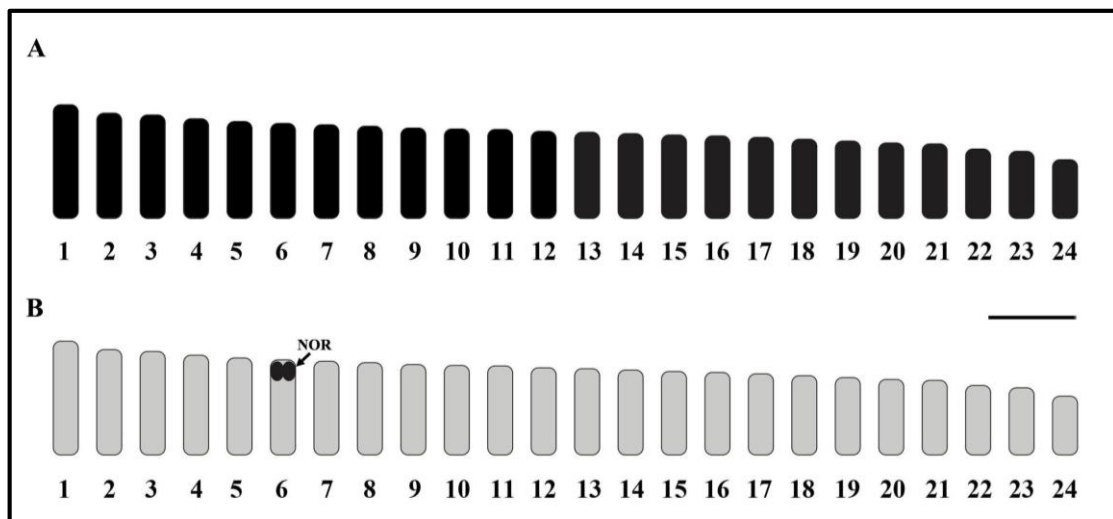


Figure 4. Standardized idiogram showing lengths and shapes of chromosomes of the green chromis (*Chromis viridis*, $2n=48$) by conventional staining and Ag-NOR banding techniques; scale bars indicate 2 μm . The arrow indicates nucleolar organizer regions.

Table 2. Mean length of short arm chromosome (Ls), length long arm chromosome (Ll), length total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphase cells of the male and female the green chromis (*Chromis viridis*), $2n=48$.

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Chromosome type
1	0.00	2.44	2.44	0.0558±0.0043	1.0000±0.0000	telocentric
2	0.00	2.26	2.26	0.0519±0.0030	1.0000±0.0000	telocentric
3	0.00	2.22	2.22	0.0507±0.0028	1.0000±0.0000	telocentric
4	0.00	2.14	2.14	0.0481±0.0013	1.0000±0.0000	telocentric
5	0.00	2.08	2.08	0.0478±0.0013	1.0000±0.0000	telocentric
6*	0.00	2.04	2.04	0.0469±0.0013	1.0000±0.0000	telocentric
7	0.00	2.01	2.01	0.0452±0.0014	1.0000±0.0000	telocentric
8	0.00	1.98	1.98	0.0444±0.0011	1.0000±0.0000	telocentric
9	0.00	1.94	1.94	0.0447±0.0006	1.0000±0.0000	telocentric
10	0.00	1.92	1.92	0.0431±0.0005	1.0000±0.0000	telocentric
11	0.00	1.91	1.91	0.0439±0.0008	1.0000±0.0000	telocentric
12	0.00	1.87	1.87	0.0420±0.0008	1.0000±0.0000	telocentric
13	0.00	1.85	1.85	0.0414±0.0010	1.0000±0.0000	telocentric
14	0.00	1.82	1.82	0.0419±0.0010	1.0000±0.0000	telocentric
15	0.00	1.79	1.79	0.0401±0.0009	1.0000±0.0000	telocentric
16	0.00	1.77	1.77	0.0406±0.0010	1.0000±0.0000	telocentric
17	0.00	1.74	1.74	0.0391±0.0013	1.0000±0.0000	telocentric
18	0.00	1.70	1.70	0.0382±0.0009	1.0000±0.0000	telocentric
19	0.00	1.66	1.66	0.0373±0.0012	1.0000±0.0000	telocentric
20	0.00	1.62	1.62	0.0363±0.0010	1.0000±0.0000	telocentric
21	0.00	1.60	1.60	0.0367±0.0016	1.0000±0.0000	telocentric
22	0.00	1.49	1.49	0.0334±0.0015	1.0000±0.0000	telocentric
23	0.00	1.44	1.44	0.0321±0.0022	1.0000±0.0000	telocentric
24	0.00	1.26	1.26	0.0281±0.0026	1.0000±0.0000	telocentric

Note: * NOR-bearing chromosome.

DISCUSSION

Chromosome number, fundamental number and karyotype of *C. viridis*

The diploid chromosome number of *C. viridis* is 48 chromosomes with chromosomes predominantly formed by telocentric chromosomes similar to the other species in the genus *Chromis*, and the morphologically differentiated sex chromosome could not be observed (Alvarez et al., 1980; Ojima and Kashiwagi, 1981; Ojima, 1983, Takai and Ojima, 1999; Molina and Galetti, 2002) (Table 1). Such chromosome pattern represents the basal form of the order Perciformes and it has been detected on most species of this order (Ohno, 1970). In addition, it differs from reports of Molina and Galetti (2002) that reported the $2n$ of 46-47 and 39 in *C. insolata* and *C. flavicauda*, respectively (Table 1), indicating the

occurrence of chromosomal rearrangements during the karyotype evolution of these groups.

The fundamental number of *C. viridis* was 48 (48 mono-armed chromosomes) agreement with the data of the genus *Chromis* in the previous studies that pointed a NF=48 in *C. caerulea*, *C. chromis*, *C. multilineata* and *C. ternatensis* (Alvarez et al., 1980; Ojima and Kashiwagi, 1981; Takai and Ojima, 1999; Molina and Galetti, 2002) demonstrating a highly conserved karyotype occurring in these fishes. The other species in the genus *Chromis* have higher NF, for example *C. chrysur* with NF=50 (Ojima, 1983), *C. flavicauda* with NF=54 and *C. insolata* with NF=56 (Molina and Galetti, 2002). The occurrence of higher NF suggests the occurrence of pericentric inversion events.

Chromosome markers of *C. viridis*

Nucleolar organizer region is the secondary constriction being found on chromosomes in addition to primary constriction (centromere) and includes the genes for rRNA synthesis (18S, 5S and 28S). This constriction stained by the silver staining in order to visualize the nucleolar organizing regions (Ag-NORs), indicating NORs. The NORs of sharing at the subcentromeric region on telocentric chromosomes of *C. viridis* rather resembled to the scattering form that spotted in other Chrominae species (Takai and Ojima, 1995). The result differences from NORs of the *C. insolata*, the NOR regions are located in non-homologous chromosomes in both species (Molina and Galetti, 2002). Moreover, in *C. flavicauda*, NOR sites were present on the telomeric portion of distinct chromosomal pairs. One site was detected on the long arms of a small submetacentric chromosome no. 7, while another one was located on the short arms of a small acrocentric chromosome no. 18. This may reveal that chromosomal rearrangement may have occurred on these fishes, leading to widespread chromosomal polymorphism. (Molina and Galetti, 2002) (Figure 5).

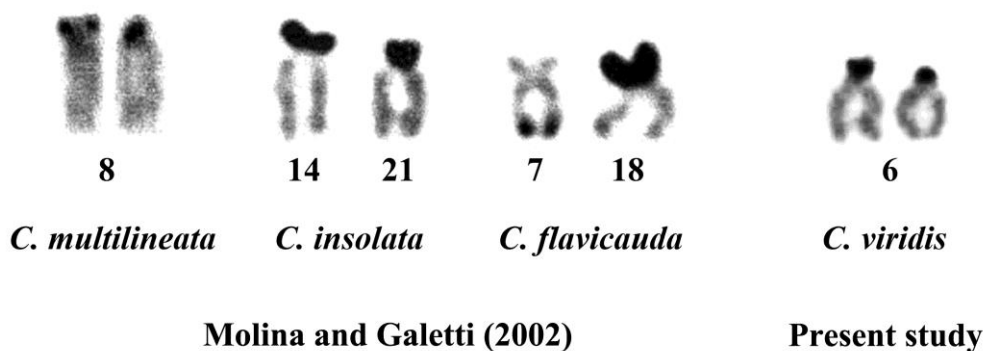


Figure 5. A comparison of the nucleolar organizer regions (NORs) of *Chromis* species.

Chromosome evolution of the Chrominae subfamily

Chromosomal rearrangements represent the main cause of karyotype diversification among several Perciformes species (Molina and Galetti, 2002). Most Pomacentridae fishes display a conserved $2n=48$, but vary in their fundamental number (NF=48-90) possibly due to the occurrence of pericentric inversions (Takai and Ojima, 1987). On the other hand, Chrominae displays a particular tendency concerning Robertsonian rearrangements such as those observed in *D. trimaculatus* ($2n=47-48$), in *D. reticulatus* ($2n=34-37$) and in *D. aruanus* ($2n=27-33$) (Ojima and Kashiwagi, 1981). In common, the possibility of different cytotypes in among these fishes is related to the presence of multiple sex chromosomes because they are asynchronic hermaphrodites (Ojima and Kashiwagi, 1981).

Fish species of inter-oceanic distribution, from the Caribbean reefs and from the Gulf of California in the Pacific Ocean, had a Pleistocene radiation (Shulman and Bermingham, 1995; Ward, 1995), demonstrating that the changes that occurred during this time are strict enough to affect distinct groups depend on a wide or narrow ability of migration (Goodbred and Graves, 1996).

From the previous cytogenetic study. The conservation of karyotype patterns among these fishes can be revealed on Table 1. The centric fusion is the main cause of the reduction of the $2n$ and the occurrence of distinctive metacentric chromosomes in *C. insolata* and *C. flavicauda* (Molina and Galetti, 2002). In Chrominae species may present karyotypic orthoselection that polymorphism could be temporary and represent an evolutionary process in each step (White, 1973). Other fish species represent chromosomal pattern similar to *Chromis*. In the interphase period, may be configured that could have help chromosomal exchange among non-homologous chromosomes during a later arrangement of these chromosomes (Aguilar et al., 1998).

CONCLUSION

Based on the chromosome study of the green chromis (*C. viridis*) using conventional staining and NOR banding techniques can verify diploid chromosome, fundamental number and size of chromosome. The results showed that *C. viridis* has $2n=48$ predominantly with telocentric chromosome, the fundamental number (NF) was 48. The Ag-NORs sites were located on the subcentromeric region of long arms of the telocentric chromosome pair no. 6th. The idiogram of *C. viridis* represent gradually declining length of the chromosomes. The important karyotype feature is the predominately with telocentric chromosome, which was found in all telocentric chromosomes like a common ancestor of marine fish concern with high ability of distribution, as a result high gene flow and low chromosome evolution. The karyotype formula of *C. viridis* is as follow: $2n (48) = 48t$

Up to the present, there are 8 species of the Genus *Chromis* cytogenetically analyzed. *Chromis* species provides remarkable karyotype features for chromosome evolutionary discussion. Further studies of other species as well as additional information and molecular techniques for chromosome analyses are expected to clarify and explain the reasons to support the karyotype polymorphism and chromosome evolution in these fishes.

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