แคริโอไทป์และอิดิโอแกรมมาตรฐานของปลาการ์ตูนส้มขาว (*Amphiprion ocellaris* Cuvier, 1830)

Standardized Karyotype and Idiogram of False Clown Anemonefish, *Amphiprion ocellaris* Cuvier, 1830 (Amphiprioninae, Perciformes)

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บทคัดย่อ

การวิจัยครั้งนี้มีจุดมุ่งหมายเพื่อศึกษาโครโมโซมเครื่องหมายและแคริโอไทป์ของปลาการ์ตูนส้มขาว (*Amphiprion ocellaris* Cuvier, 1830) ใช้ตัวอย่างปลาเพศผู้และเพศเมียอย่างละ 5 ตัว เตรียมโครโมโซมโดยวิธีทางตรงจากไตด้วยวิธีการสับให้ละเอียด ย้อมสีโครโมโซมแบบธรรมดาด้วยสีเกียมซ่า และย้อมแถบสีแบบนอร์ ผลการศึกษาพบว่าปลาการ์ตูนส้มขาวมีจำนวนโครโมโซมดิพลอยด์เท่ากับ 48 แท่ง มีจำนวนโครโมโซมพื้นฐานเท่ากับ 94 ทั้งเพศผู้และเพศเมีย ตรวจไม่พบความแตกต่างของโครโมโซมเพศในปลาเพศผู้และเพศเมีย แคริโอไทป์ประกอบด้วยโครโมโซมเมทาเซนทริกขนาดใหญ่ 6 แท่ง ซับเมทาเซนทริกขนาดใหญ่ 14 แท่ง อะโครเซนทริกขนาดใหญ่ 2 แท่ง เทโลเซนทริกขนาดใหญ่ 2 แท่ง เมทาเซนทริกขนาดกลาง 10 แท่ง ซับเมทาเซนทริกขนาดกลาง 6 แท่ง และอะโครเซนทริกขนาดกลาง 6 แท่ง เป็นรายงานครั้งแรกที่แสดงตำแหน่งโครโมโซมเครื่องหมายนอร์บนแขนข้างสั้นบริเวณปลายเทโลเมียร์ของโครโมโซมคู่ที่ 19 เป็นโครโมโซมชนิดอะโครเซนทริกคู่ใหญ่สุด ปลาการ์ตูนส้มขาวมีสูตรแคริโอไทป์ ดังนี้ $2n\ (48) = L^{m}_{6} + L^{sm}_{14} + L^{a}_{2} + L^{t}_{2} + M^{m}_{10} + M^{sm}_{6} + M^{a}_{8}$

คำสำคัญ: ปลาการ์ตูนส้มขาว โครโมโซม แคริโอไทป์ อิดิโอแกรม

Abstract

The first chromosomal characteristics of nucleolar organizer region/NOR and karyotype analysis of false clown anemonefish, *Amphiprion ocellaris* (Cuvier, 1830) were studied. Kidney cell samples were taken from five male and five female fishes. Mitotic chromosome preparations were conducted using follow standard protocol. Conventional staining and Ag–NORs banding techniques were applied to stain the chromosomes. The results showed that the diploid chromosome number of *A. ocellaris* was 2n = 48, the fundamental number (NF) was 94 in both males and females. Their karyotypes consisted of 6 large metacentrics, 14 large submetacentrics, 2 large acrocentrics, 2 large telocentrics, 10 medium metacentrics, 6 medium submetacentrics, and 8 medium acrocentrics

chromosomes. No heteromorphic sex chromosomes were found between male and female. The present study was the first report of NOR–bearing chromosome as the pair 19th, which showed clearly observable NOR. The karyotype formula is as follows:

$2n\ (48) = L^{m}_{6} + L^{sm}_{14} + L^{a}_{2} + L^{t}_{2} + M^{m}_{10} + M^{sm}_{6} + M^{a}_{8}$

Keywords: *Amphiprion ocellaris*, chromosome, karyotype, idiogram

Introduction

Thailand is one of the world's richest places for biodiversity, especially for marine fish species with more than 2,000 species recorded. Anemonefishes are member of the family Pomacentridae, one of the largest family in the order Perciformes, with approximately 325 species. Members of this family, commonly known as damselfishes are almost entirely marine and most species occur in tropical and to a lesser degree, subtropical latitudes. About 70% of damselfishes including anemonefishes are restricted to the vest Indo-West Pacific region. The genera *Amphiprion* and *Premnas* constitute the subfamily Amphiprioninae, one of four pomacentrid subfamilies. The only other damselfishes that sometimes dwell with anemone are *Dascyllus trimasculatus* and *D. albisells* which belong to another subfamily, the Chrominae (Fautin and Allen, 1992).

Only a few cytogenetic were studied, four species of the anemonefishes (Amphiprioninae) have been reported. Each of them has a similar diploid chromosome number 2n=48, *Amphiprion polymnus* from Thailand (Tanomtong *et al*., 2012), *A. clarkii* from Japan and Indo-West Pacific (Arai and Inoue, 1976; Takai and Kosuga, 2007), *A. ocellaris* from Japan (Arai *et al*., 1976) and *A. frenatus*, from Japan and Philippines (Molina and Galetti, 2004; Takai and Kosuga, 2007).

The present study aimed to analyze the cytogenetic analysis of false clown anemonefish, (*A. ocellaris*) (Fig. 1) in which showed the standardization of karyotype and idiogram of *A. ocellaris*. We also first characterized the NOR-bearing chromosome in *A. ocellaris*. The result obtained can be used in cytotaxonomy and evolutionary relationships of these fishes. Moreover, it provides useful basic information for the conservation and breeding.

Materials and methods

1. Sample collection

The *A. ocellaris* (five males and five females) were obtained from Andaman Sea, Phuket, Thailand (Phuket Coastal Research and Development Centre). The fish were transferred to laboratory aquaria and were kept under standard condition for 7 days prior to the experiments.

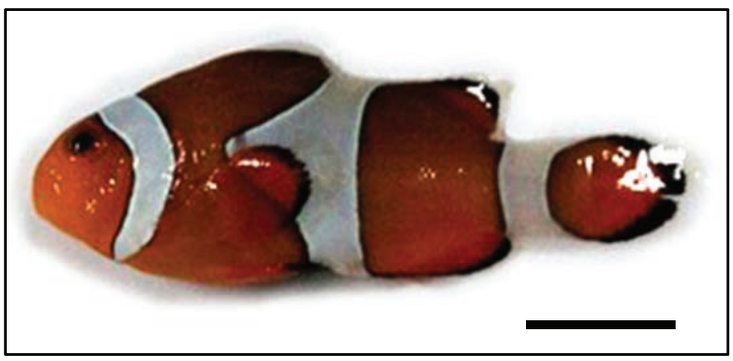

Figure 1 Generral charecteristics of the false clown anemonefish (*Amphiprion ocellaris*), scale bar indicates 1 cm.

2. Chromosome preparation

Chromosomes were directly prepared *in vivo*; follow the standard protocols (Chen and Ebeling, 1968; Nanda *et al*., 1995). Briefly, 0.3% phytohemagglutinin (PHA, Gibco 1163050) solution was injected to fish's abdominal. After 24 hours, 0.05% colchicine was injected to fish's abdominal (1 ml : 100 g body weight) and left it for 1–2 hours. Renal tissue were removed and cut into small pieces then gently mixed with 0.075 M KCl. After discarding all large piece tissues, 8 ml of cell sediments were transferred to centrifuge tube and incubated for 25–30 minutes. KCl was discarded from the supernatant after centrifugation again at 1,200 rpm for 8 minutes. Cells were fixed in fresh cool fixative (3 methanol: 1 glacial acetic acid) gradually added up to 7 ml before centrifuged again at 1,200 rpm for 8 minutes, then the supernatant was discarded (Supiwong *et al*. 2009). The fixation was repeated until the supernatant was clear and the pellet was mixed with 1 ml fixative. The mixture was dropped onto a clean and cold slide by micropipette followed by air-dry technique.

3. Chromosome staining

Conventional staining was performed by using 20% Giemsa's solution for 30 minutes. Ag-NOR banding (Howell and Black, 1980) was carried out by adding 2 drops of 50% silver nitrate and 2% gelatin on slides, respectively. The slides were then sealed with cover glasses and incubated at 60°C for 5 minutes. After that the slides were soaked in distilled water until the cover glasses were separated.

4. Karyotype analysis

Chromosome counting was performed on mitotic metaphase cells under light microscope. Twenty clearly observable and well-cells spread chromosomes plates of each male and female were

selected and photographed. The karyotype and idiogram were established following Turpin and Legene (1965).

Results and discussion

1. Diploid chromosome number, fundamental number and karyotype of *A. ocellaris*

The diploid chromosome number (2n, 2x) of *A. ocellaris* was 48 chromosomes in both males and females (Fig. 2 and 3). This is also found in other fish of the subfamily Amphiprioninae, *A. clarkia* (Arai and Inoue, 1976; Takai and Kosuga, 2007), false clown anemonefish (*A. ocellaris*) (Arai *et al*., 1976); tomato anemonefish (*A. frenatus*) (Molina and Galitti, 2004; Takai and Kosuga, 2007) and saddleback anemonefish (*A. polymnus*) (Tanomtong *et al*., 2012) (Table 1).

Table 1 Reviews of anemonefish cytogenetic reports in the subfamily Amphiprioninae.

Species (Anemonefish)	2n	NF	m	sm	a	t	NORs	Reference
False clown (*A. ocellaris*)	48	94	16	20	10	2	2 (a)	Present study
	48	84	14	22	12	-	-	Arai *et al*. (1976)
Saddleback (*A. polymnus*)	48	96	20	20	8	-	2 (a)	Tanomtong *et al*. (2012)
Clak's (*A. clarkii*)	48	78	14	16	18	-	-	Arai and Inoue (1976)
	48	86	12	26	10	-	2 (a)	Takai and Kosuga (2007)
Tomato (*A. frenatus*)	48	92	14	22	8	4	2 (a)	Molina and Gaetti (2004)
	48	86	12	26	10	-	2 (a)	Takai and Kosuga (2007)

Remarks: 2n = diploid chromosome number, NF = fundamental number, m = metacentric chromosome, sm = submetacentric chromosome, a = acrocentric chromosome, t = telocentric chromosome, NORs = nucleolar oganizer regions, and - = not available.

We found that the fundamental number (NF) of *A. ocellaris* was 94 in both males and females. The comparative studies with other fishes in the subfamily Amphiprioninae showed the different NF as those found in *A. clarkii*, NF = 86 (Takai and Kosuga, 2007); *A. ocellaris*, NF = 84 (Arai *et al*., 1976); *A. frenatus*, NF = 92 (Molina and Gaetti, 2004), and *A. polymnus*, NF = 96 (Tanomtong *et al*., 2012). In accordance to other species in the subfamily Amphiprioninae, no cytologically distinguishable sex chromosome was observed (Arai, 2011). From the Plasticity in sex differentiation is known to be common in teleost fishes, it suggests that in false clown anemonefish, sex

differentiation in each individual fishes is gradually determined by long-term social interactions (Iwata *et al*., 2008).

The karyotype of *A. ocellaris* consisted of 16 metacentric, 20 submetacentric, 10 acrocentric, and 2 telocentric chromosomes. It differs from the report of Arai *et al*. (1976) that found the karyotype of *A. ocellaris* consisting of 14 metacentric, 22 submetacentric, and 12 acrocentric (subtelocentric) chromosomes. Arai and Inoue (1976) reported the karyotype of *A. clarkii* that consists of 14 metacentric, 16 submetacentric, and 18 acrocentric chromosomes. Molina and Galetti (2004) reported that the karyotype of *A. frenatus* composes of 14 metacentric, 22 submetacentric, 8 subtelocentric, and 4 acrocentric chromosomes. Takai and Kosuka (2007) reported that karyotypes of *A.clarkii* and *A. frenatus* consist of 12 metacentric, 26 submetacentric and 10 acrocentric chromosomes while Tanomtong *et al*. (2012) reported the chromosome analysis of *A. polymnus* composing of 20 metacentric, 20 submetacentric, and 8 acrocentric chromosomes. The karyotype formula for *A. ocellaris* is as follows: $2n\ (48) = L^{m}_{6} + L^{sm}_{14} + L^{a}_{2} + L^{t}_{2} + M^{m}_{10} + M^{sm}_{6} + M^{a}_{8}$

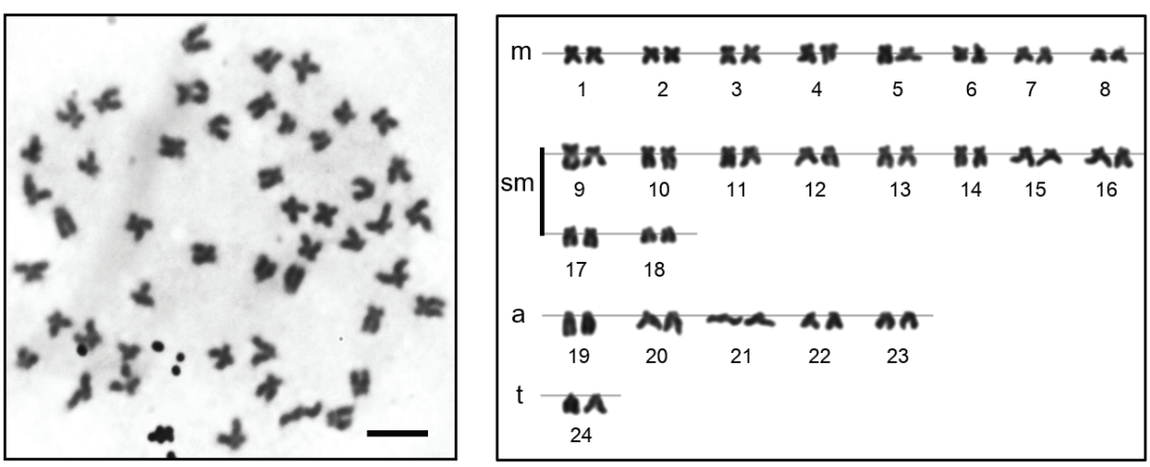

Figure 2 Metaphase chromosome plate (left) and karyotype (right) of male false clown (*A. ocellaris*), 2n (diploid) = 48 by conventional staining, scale bar indicates 5 μm. There is no observation of strange size chromosome related to sex.

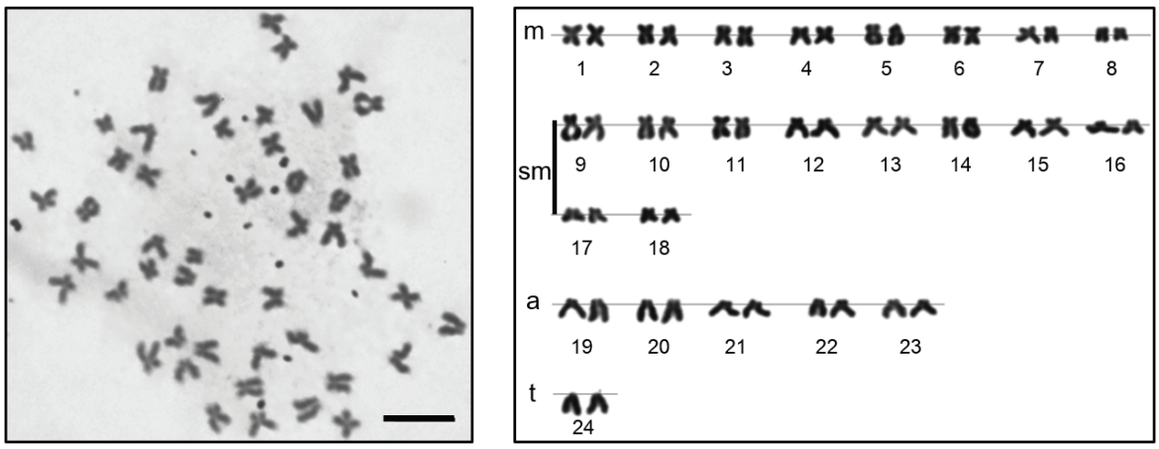

Figure 3 Metaphase chromosome plate (left) and karyotype (right) of female false clown (*A. ocellaris*), 2n (diploid) = 48 by conventional staining, scale bar indicates 5 μm. There is no observation of strange size chromosome related to sex.

2. Chromosome markers of *A. ocellaris*

The region adjacent to the telomere of the short arm on the largest acrocentric chromosome pair 19 has observable NORs (Fig. 4). In all species (four species) of the subfamily Amphiprionidae investigated to date, the single NOR-bearing acrocentric chromosome pair (telomeric region) is conserved (Molina and Galetti, 2004; Takai and Kosuka, 2007; Tanomtong *et al*., 2012). Normally, most fishes have only one pair of NOR (single NOR) on chromosome. However, some fishes have more than 2 NORs, which have caused by Robertsonian translocation (Molina and Gaetti 2004). Furthermore, NOR is usually located at the telomere (telomeric NOR) of the chromosome arm (Sharma *et al*., 2002).

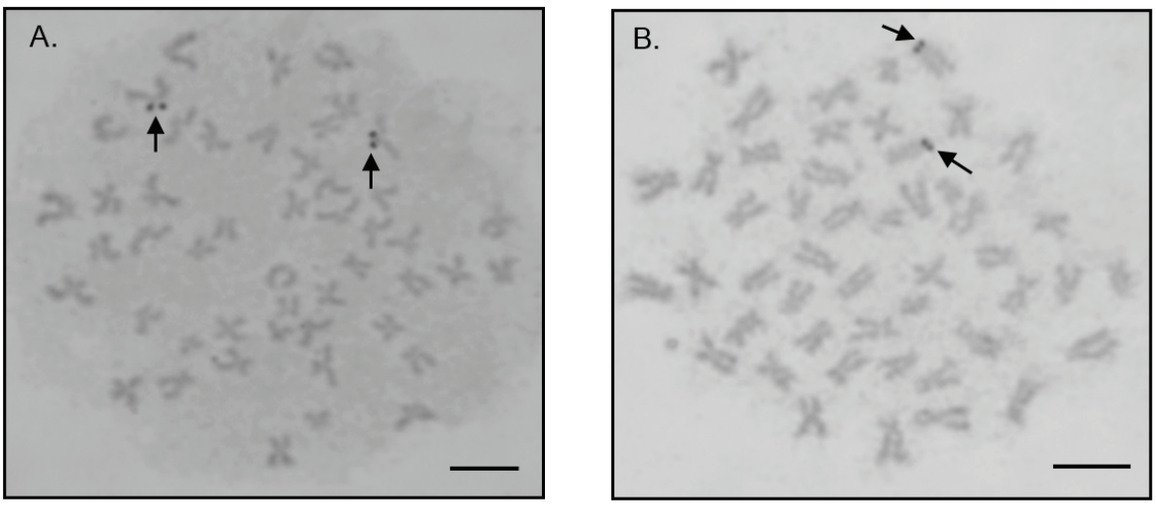

Figure 4 Metaphase chromosome plates of male (A.) and female (B.) false clown (*A. ocellaris*), 2n (diploid) = 48 by Ag-NORs staining technique, scale bars indicate 5 μm. The region adjacent to the short arms near telomere of largest acrocentric chromosome pair 19 showed clearly observable nucleolar organizer regions/NORs, (arrows).

The asymetrical karyotype of *A. ocellaris*, and the four types of chromosomes (metacentric, submetacentric, acrocentric and telocentric chromosomes), in which we found indicated the advanced karyotype. The idiogram showed continuous length gradation of the chromosomes. The largest and smallest chromosomes show size differenes (approximatly 2 folds). The chromosome markers of *A. ocellaris* are the chromosome pair 9 which are the largest submetacentric chromosome and chromosome pair 23 which are the smallest acrocentric chromosomes. Data of chromosomal checks on mitotic metaphase cells of *A. ocellaris* is shown in Table 2. Fig. 5 shows the idiograms of *A. ocellaris* obtained by conventional staining and Ag-NORs banding tachniques.

Acknowledgments

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Table 2 Mean length of short arm chromosome (Ls), length of long arm chromosome (LI), length of total chromosomes (LT), relative length (RL), centromeric index (CI) from 20 metaphases of male and female false clown anemonefish (*A. ocellaris*), 2*n* (diploid) = 48.

Chromosome pairs	Ls	LI	LT	CI	RL	Chromosome sizes	Chromosome types
1	3.3218	3.5141	6.8359	0.5141	0.0497	Large	Metacentric
2	2.7335	3.8579	6.5914	0.5853	0.0480	Large	Metacentric
3	3.1680	3.2162	6.3842	0.5038	0.0464	Large	Metacentric
4	2.5709	2.9075	5.4784	0.5307	0.0399	Medium	Metacentric
5	2.1154	2.8708	4.9862	0.5758	0.0363	Medium	Metacentric
6	1.9880	2.9465	4.9344	0.5971	0.0359	Medium	Metacentric
7	2.2707	2.3737	4.6445	0.5111	0.0338	Medium	Metacentric
8	1.9650	2.6090	4.5740	0.5704	0.0333	Medium	Metacentric
9	2.9299	4.8596	7.7895	0.6239	0.0567	Large	Submetacentric
10	2.1154	4.3710	6.4864	0.6739	0.0472	Large	Submetacentric
11	2.3639	4.0814	6.4452	0.6332	0.0469	Large	Submetacentric
12	1.9708	4.3172	6.2880	0.6866	0.0457	Large	Submetacentric
13	2.2707	3.9659	6.2366	0.6359	0.0454	Large	Submetacentric
14	2.1207	4.0591	6.1798	0.6568	0.0450	Large	Submetacentric
15	1.9880	3.9314	5.9194	0.6642	0.0431	Large	Submetacentric
16	1.9708	3.7965	5.7672	0.6583	0.0420	Medium	Submetacentric
17	1.6642	3.3184	4.9826	0.6660	0.0362	Medium	Submetacentric
18	1.4287	3.0101	4.4388	0.6781	0.0323	Medium	Submetacentric
19*	1.2225	4.9816	6.2041	0.8030	0.0451	Large	Acrocentric
20	1.5139	3.9659	5.4797	0.7237	0.0399	Medium	Acrocentric
21	1.0357	4.3736	5.4093	0.8085	0.0394	Medium	Acrocentric
22	1.1049	4.0703	5.1751	0.7865	0.0376	Medium	Acrocentric
23	1.0734	2.9645	4.0379	0.7342	0.0294	Medium	Acrocentric
24	0.0000	6.1920	6.1920	1.0000	0.0450	Large	Telocentric

Remark: * NORs bearing chromosomes

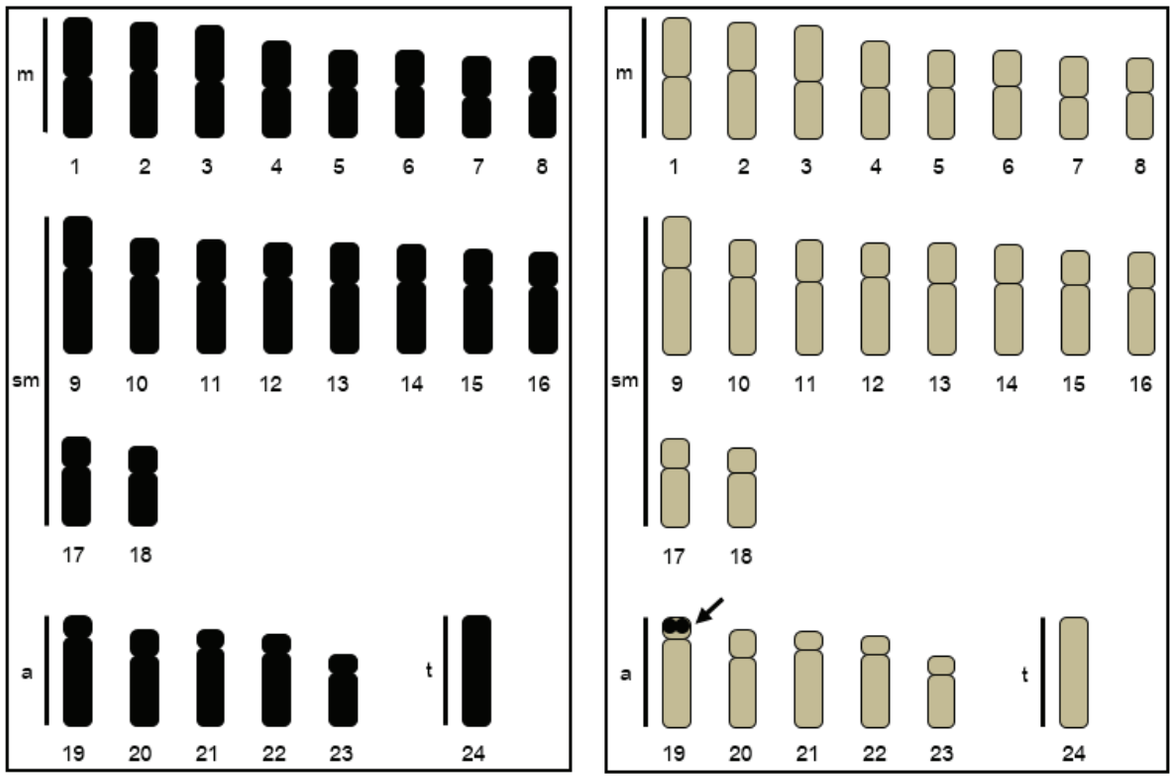

Figure 5 Standard idiograms showing length and shape of chromosome of false clown anemonefish (*A. ocellaris*) demonstrated the haploid set (n=24) by conventional staining (left) and Ag-NORs staining techniques (right), arrow indicates NORs location.

References

Arai, R. 2011. Fish Karyotype a Check List. Springer, Japan, pp. 28-215.

_____ and Inoue, M. 1976. Chromosome of seven species of Pomacentridae and two species of Acanthuridae from Japan. Bull. Natl. Mus. Ser. A. (Zool) 2: 73–78.

_____ , _____ and Ida, H. 1976. Chromosome of four species of coral fishes from Japan. Bull. Nat. Sci. Mus. 2: 137.

Chen, T.R. and Ebeling, A.W. 1968. Karyological evidence of female heterogamety in the mosquito fish, *Gambusia affinis*. Copeia 1: 70–75.

Fautin, D.G. and Allen, G.R. 1992. Field guide to anemonefish and their host sea anemones. California Academy of Sciences University of Kansa and Western Australian Museum.

Howell, W.M. and Black, D.A. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015.

Iwata, E., Nagai, Y., Hyoudou, M. and Sasaki, H. 2008. Social environment and sex differentiation in the false clown anemonefish, Amphiprion ocellaris. Zoological Science 25(2): 123–8.

Kavaco, K.F., Pazza, R., Bertollo, L.A.C. and Moreira-Filho, O. 2005. Molecular cytogenetics of *Oligosarcus hepsetus* (Teleotei, Chareciformes) from two Brazilian locations. Genetica 124: 85–91.

Molina, W.E. and Galetti, P.M. 2004. Karyotypic changes associated to the dispersive potential on Pomacentridae (Pisces, Perciformes). Journal of Experimental Marine Biology and Ecology 309: 109-119.

Nanda, I., Schsrtl, M., Feichtinger, W., Schlupp, I., Parzefall, J. and Schmid, M. 1995. Chromosomal evidence for laboratory synthesis of triploid hybrid between the gynogenetic teleost *Poecilia formosa* and its host species. Journal of Fish Bio. 47: 619–623.

Sharma, O.P., Tripathi, N.K. and Sharma, K.K. 2002. A review of chromosome banding in fishes. In: Sobti, R.C. (ed). Some aspects of chromosome structure and functions. New Narosa Publishing House, Delhi.

Sola, L., De Innocentiis, S., Gornung, E., Papalia, S., Rossi, A.R., Marini, G., De Marco, P. and Cataudella, S. 2000. Cytogenetic analysis of *Epinephelus marginatus* (Pisces: Serranidae), with the chromosome localization of the 18S and 5S rRNA genes and of the (TTAGGG) on telomere sequence. Marine Biology 137: 47–51.

Supiwong, W., Jearranaiprepameand, P. and Tanomtong, A. 2009. A new report of karyotype in the chevron snakehead fish, *Channa striata* (Channidae, Pisces) from Northeast of Thailand. Cytologia 74(3): 317–322.

Takai, A. and Kosuka, S. 2007. Karyotypes and banded chromosomal features in two anemonefishes (Pomacentridae, Perciformes). Chromosome Science 10: 71–74.

Tanomtong, A., Supiwong, W., Chaveerach, A., Khakhong, S., Tanee, T. and Sanoamuang, L. 2012. Frist report of chromosome analysis of saddleback anemonefish, *Amphiprion polymnus* (Perciformes, Amphiprioninae) in Thailand. Cytologia 77(4): 441–446.

Turpin, R. and Lejeune, J. 1965. Les Chromosomes humains. Cornell University: Gauthier-Villars.