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First report on Nucleolar Organizer Regions (NORs) polymorphism and constitutive heterochromatin of Moonlight Gourami, *Trichopodus microlepis* (Perciformes, Osphronemidae)

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Abstract. Nucleolar organizer regions (NORs) polymorphism, constitutive heterochromatin and chromosomal analysis of Moonlight gourami, *Trichopodus microlepis* in Thailand were firstly reported. Specimens were collected from the Chao Phraya and Mekong Basins, Thailand. The mitotic chromosomes were directly prepared from kidney tissues of ten males and ten females. Conventional staining, Ag-NOR banding and C- banding techniques were applied to stain the chromosomes. The results shown that the diploid chromosome number of *T. microlepis* was 2*n*=46 and the fundamental number (NF) was 46 in both males and females. The karyotype consisted of 46 telocentric chromosomes classifying as 14 large and 32 medium chromosomes. No heteromorphic sex chromosome was observed in *T. microlepis*. The results also showed that the interstitial nucleolar organizer regions (NORs) were clearly observed at the long arm of the chromosome pair 7. This is the first report on NORs polymorphism in *T. microlepis* that a heteromorphic NOR type in one female had a single NOR-bearing chromosome of the chromosome pair 7, whereas 10 males and nine females had two NOR-bearing chromosomes of the chromosome pair 7 with a homomorphic NOR type. Constitutive heterochromatin was located at all centromeres of all chromosome pairs. The karyotype formula of *T. microlepis* is 2*n* (46) = L^t_{14} + M^t_{32}.

Keywords: Moonlight gourami, *Trichopodus microlepis*, karyotype, Nucleolar Organizer Region, constitutive heterochromatin, chromosome.

INTRODUCTION

Trichopodus which was formerly included in *Trichogaster* (Peapke, 2009; Töpfer and Schlindler, 2009) is a genus of tropical freshwater labyrinth fish of the gourami or family Osphronemidae and subfamily Trichogastrinae found in Southeast Asia. Gouramis of the *Trichopodus* genus are closely related to those of *Trichogaster* (formerly *Colisa*), species of both genera have long and thread-like pelvic fins (known as "feelers" in the aquarium trade) used to sense the environment. However, *Trichopodus* species have shorter dorsal fin base and, when sexually mature, are much larger (Peapke, 2009; Töpfer and Schlindler, 2009). There are currently six recognized species in this genus including *Trichopodus cantoris*, pearl gourami (*T. leerii*), moonlight gourami (*T. microlepis*), snakeskin gourami (*T. pectoralis*), *T. poptae* and three spot gourami (*T. trichopterus*) (Peapke, 2009). The moonlight gourami is a labyrinth fish native to the Mekong River in Cambodia, Vietnam and the Chao Phraya Basin, Thailand (Vidthayanon 2005). These fish are silvery coloured with a slightly greenish hue similar to the soft glow of moonlight (Fig. 1). The moonlight gourami's concavely sloped head distinguishes it from other gourami varieties. This peaceful, attractive species is a popular aquarium fish.

Although the gourami fishes are importance for national economy of Thailand, there were quite scarce of cytogenetics in these fishes especially banding analysis in fish chromosomes. The study on fish chromosomes is the basic knowledge which can be applied for the several fields such as classification, evolution, heredity, systematic (Gold et al. 1990, Ueda et al. 2001, Barat et al. 2002, Barat and Sahoo 2007, Supiwong et al. 20019), breeding, rapid production of inbred lines and cytotaxonomy (Kirpichnikov 1981). Furthermore, cytogenetic studies on fish have also been used as biological indicator to determine the ecological toxicology (Klinkhardt 1993, Promsid et al. 2015) and cytogenetic techniques have been widely applied to improve farmed stocks in many aquaculture species in the World (Beardmore et al. 2001, Desprez et al. 2003, Pradeep et al. 2012). An important characteristic of Nucleolar Organizer Regions (NORs) in fish is related to that it has inter- and intra-species polymorphism. NORs characters can be a cytogenetic marker for cytotaxonomic studies and also have been used for studying of phylogenetic relationships among the Cyprinid fishes (Amemyia and Gold 1988, Galetti Jr 1998, Almeida-Toledo et al. 2000). Constitutive heterochromatin distributions on the chromosomes were widely studied in some fish groups (Brinn et al. 2004, Vicari et al. 2006, Mesquita et al. 2008, Takai 2012). Generally, most constitutive heterochromatins locate at centromeric/pericentromeric regions of the chromosomes. Some cases, these heterochromatins can be revealed at interstitial regions in some Pomacentrid fishes to support that the chromosomal evolution in this family is related to the chromosome fusion (Takai 2012). Moreover, constitutive heterochromatin is also highly accumulated on the W chromosome in *Parodon hilarii* (Parodontidae) (Moreira-Filho et al. 1993), *Characidium* fish (Crenuchidae) (Vicari et al. 2008) and *Lignobrycon myersi* (Triportheidae) (Rodrigues et al. 2016).

As mention before, chromosomal analysis is very important and clearly exhibits the benefits. Moreover, the constitutive heterochromatin and polymorphism of NORs characteristics in the *T. microlepis* were not studied. Thus, the present study is the first report in *T. microlepis* from Thailand using Ag-NOR banding and C-banding techniques.

MATERIALS AND METHODS

Sample collection, chromosome preparation and chromosome staining

Ten male and ten female specimens of *T. microlepis* (Fig. 1) were obtained from the Chao Phraya River, Sing Buri Province, the central part of Thailand and the Mekong Basin, Nong Khai Province, Northeast of Thailand. Chromosomes were directly prepared *in vivo* as follows by Supiwong et al. (2013, 2017). Conventional staining was performed using 20% Giemsa's solution for 30 min (Rooney 2001). Ag-NOR banding was carried out following by Howell and Black (1980) and C-banding was performed following from the method of Sumner et al. (1972).

Figure 1. General characteristic of Moonlight Gourami, *Trichopodus microlepis* (Perciformes, Osphronemidae).

Chromosomal checks, karyotyping and idiograming

Chromosome counting was carried out on mitotic metaphase cells under light microscope for 30 cells per specimen to determine the diploid number (2*n*). Twenty clearly observable and well-spread metaphase cells from each male and female were selected and photographed. The short arm length (Ls) and the long arm length (Ll) of each chromosome were measured to calculate the total length of the chromosome for 20 well-spread metaphase cells. The chromosome types were classified from method of Turpin and Lejeune (1965) as metacentric, submetacentric, acrocentric and telocentric chromosomes. The karyotyping and idiograming methods were according to Turpin and Lejeune (1965) and Chaiyasut (1989).

RESULTS AND DISCUSSION

Diploid chromosome number, fundamental number and karyotype

The diploid chromosome number (2*n*) of *T. microlepis* was found as 46 (Figs. 2 and 3). This result is coincident with previous reports by Koref-Santibanez and Paepke (1994) and Seetapan and Khamma-Ai (2007). It is also the same 2*n* as in the other *Trichopodus* spp. (Abe 1975, Koref-Santibanez and Paepke 1994, Donsakul and Magtoon 1988, Seetapan and Khamma-Ai 2007, Magtoon et al. 2007, Supiwong et al. 2010), *Trichogaster chuna* (Koref-Santibanez and Paepke 1994) and *Trichogaster lalius* (Abe 1975, Rishi 1976, Koref-Santibanez and Paepke 1994). These species have the diploid chromosome number of 2*n*=46, which is an apparent modal diploid number of the *Trichopodus*. Accordingly, it can be concluded that chromosome number in this genus is conserved. However, it differs from the most species of the genus *Trichogaster* (*T. labiosa*, *T. fasciata*, *T. labiosus*, *T. sumatranus*) which had 2*n*=48 (Kaur and Srivastava 1965, Calton and Denton 1974, Abe 1975, Rishi 1975, Manna and Prasad 1977, Tripathy and Das 1981, Koref-Santibanez and Paepke 1994, Rishi et al. 1994, Sobita and Bhagirath 2007, Kushwaha et al. 2008) (Table 1).

The fundamental number (NF) of *T. microlepis* was 46 in both males and females. The karyotype consisted of 46 telocentric chromosomes (all as mono-arm chromosomes). These results are agreeable with the previous reports of both *T. microlepis* and all *Trichopodus* species (Abe 1975, Donsakul and Magtoon 1988, Koref-Santibanez and Paepke 1994, Magtoon et al. 2007, Seetapan and Khamma-Ai 2007, Supiwong et al. 2010). Howev-

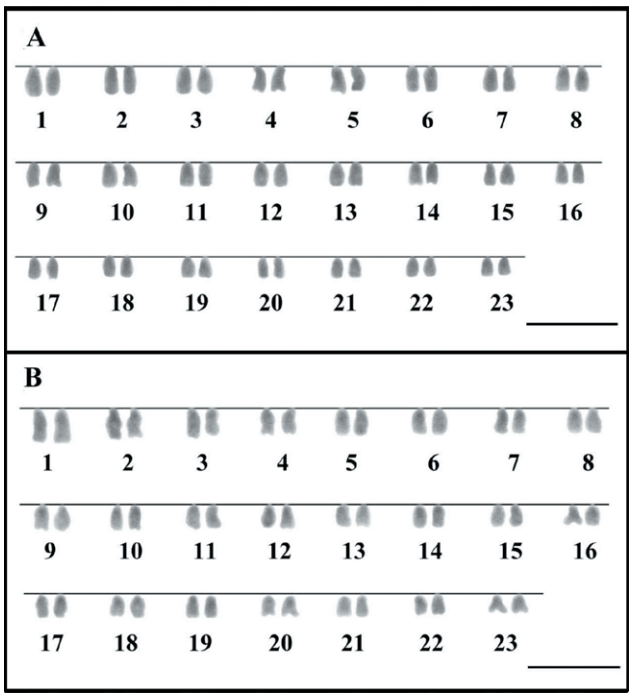

Figure 2. Karyotypes of male (A) and female (B) of *Trichopodus microlepis*, 2*n*=46 by conventional staining. Scale bars = 5 μm.

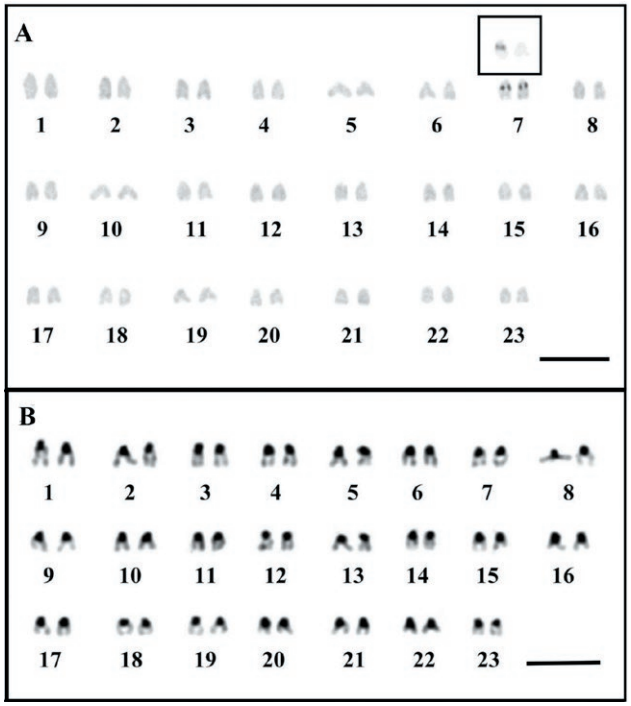

Figure 3. Karyotypes of *Trichopodus microlepis*, 2*n*=46 by Ag-NOR banding (A) and C-banding techniques (B). The chromosome pair 7 show Ag-NOR and heteromorphic Ag-NOR (inserted box). Scale bars = 5 μm.

Table 1. Karyotype characteristics of some species in the subfamily Trichogastrinae.

Species	2*n*	NF	Karyotype	NOR	Reference
Trichogaster chuna	46	66	20m+26st/a	–	Koref-Santibanez and Paepke (1994)
T. labiosa	48	66	12m+6sm+12st+18a/t	–	Manna and Prasad (1977)
	48	68	20m+10st+18a/t	–	Koref-Santibanez and Paepke (1994)
T. lalius	46	70	24m/sm+22a/t	–	Abe (1975)
	46	–	26m+1sm/st+19a/t	–	Rishi (1976)
	46	66	20m+8st+18a/t	–	Koref-Santibanez and Paepke (1994)
T. fasciata	48	48	48a/t	–	Kaur and Srivastava (1965)
	48	74	14m+12sm+22a/t	–	Rishi (1975)
	48	78	8m+20sm+12st+8a/t	–	Manna and Prasad (1977)
	48	78	18m+12sm+18a/t	–	Tripathy and Das (1981)
	48	68	20m+12st+16a/t	–	Koref-Santibanez and Paepke (1994)
	48	80–81	16m+16sm+15a/t(16a/t)	–	Rishi et al. (1994)
	48	83	15m+16sm+4st+13a/t	6	Sobita and Bhagirath (2007)
	48	86	16m+16sm+6st+10a/t	2	Kushwaha et al. (2008)
T. sumatranus	48	48	48st/a	–	Calton and Denton (1974)
Trichopodus leeri	46	46	46a/t	–	Abe (1975) Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Seetapan and Khamma-Ai (2007)
T. microlepis	46	46	46a/t	–	Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Seetapan and Khamma-Ai (2007)
	46	46	46t	2	Present study
T. pectoralis	46	46	46a/t	–	Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Donsakul. and Magtoon (1988)
	46	46	46a/t	–	Seetapan and Khamma-Ai (2007)
T. trichopterus	46	46	46a/t	–	Abe (1975), Koref-Santibanez and Paepke (1994)
	46	46	46a/t	–	Magtoon et al. (2007)
	46	46	46t/t	2	Supiwong et al. (2010)

Remarks: 2*n* = diploid number, NF = the fundamental number, NOR = Nucleolar Organizer Region, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric, t = telocentric chromosomes and – = not available.

er, they are different from all of the genus *Trichogaster* (Kaur and Srivastava 1965, Calton and Denton 1974, Abe 1975, Rishi 1975, Manna and Prasad 1977, Tripathy and Das 1981, Koref-Santibanez and Paepke 1994, Rishi et al. 1994, Sobita and Bhagirath 2007, Kushwaha et al. 2008). The NFs of the genus *Trichogaster* range from 48 to 86 and karyotypes composed of both mono- and bi-arm chromosomes. Nirchio et al. (2002) proposed that species with high NF is advanced state or apomorphic character whereas one with low NF is a primitive state or plesiomorphic character. *T. microlepis* including all species of the genus *Trichopodus* have all mono-arm chromosomes in karyotype whereas most species of the genus *Trichogaster* display both mono- arm and bi-arm chromosomes (Table 1). Thus, the *Trichoprodus* seems to be more primitive karyotype than that in the *Trichogaster*. The *T. microlepis* karyotype consisted of 14 large telocentric and 32 medium telocentric chromosomes (Table 2). The karyotype formula for this species is $2n$ (46) = L^t_{14} + M^t_{32}. There is no evidence of differentiated sex chromosomes in this species which accord to all species of this genus (Abe 1975, Donsakul and Magtoon 1988, Koref-Santibanez and Paepke 1994, Magtoon et al. 2007, Seetapan and Khamma-Ai 2007, Supiwong et al. 2010). Similar to several gourami fishes, no cytologically distinguishable sex chromosome was observed.

Chromosome markers from Ag-NOR banding and C-banding

Present study was accomplished by using Ag-NOR staining and C-banding in *T. microlepis*. The NORs are used as makers to detect species specific character and indicate intra- and inter species chromosomal polymorphism in many groups of fishes (Ráb et al. 2008). The Ag-NOR positions were shown on the long arm near the centromere of the telocentric chromosome pair 7 (subcentromeric NOR) in 10 male and nine female fish (Fig.

Table 2 Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from 20 metaphases (both males and females) of the Moonlight gourami (*Trichopodus microlepis*) in Thailand, 2*n*=46

Chromosome pair	Ls	Ll	LT	RL±SD	CI±SD	Type	Size
1	0.000	0.755	0.755	0.0306±0.0026	1.000±0.000	telocentric	L
2	0.000	0.682	0.682	0.0276±0.0015	1.000±0.000	telocentric	L
3	0.000	0.647	0.647	0.0261±0.0011	1.000±0.000	telocentric	L
4	0.000	0.621	0.621	0.0251±0.0008	1.000±0.000	telocentric	L
5	0.000	0.603	0.603	0.0243±0.0007	1.000±0.000	telocentric	L
6	0.000	0.589	0.589	0.0237±0.0006	1.000±0.000	telocentric	L
7*	0.000	0.578	0.578	0.0232±0.0005	1.000±0.000	telocentric	L
8	0.000	0.567	0.567	0.0228±0.0004	1.000±0.000	telocentric	M
9	0.000	0.559	0.559	0.0225±0.0004	1.000±0.000	telocentric	M
10	0.000	0.549	0.549	0.0221±0.0004	1.000±0.000	telocentric	M
11	0.000	0.538	0.538	0.0217±0.0004	1.000±0.000	telocentric	M
12	0.000	0.529	0.529	0.0213±0.0004	1.000±0.000	telocentric	M
13	0.000	0.521	0.521	0.0209±0.0004	1.000±0.000	telocentric	M
14	0.000	0.513	0.513	0.0206±0.0004	1.000±0.000	telocentric	M
15	0.000	0.506	0.506	0.0203±0.0004	1.000±0.000	telocentric	M
16	0.000	0.498	0.498	0.0201±0.0004	1.000±0.000	telocentric	M
17	0.000	0.491	0.491	0.0197±0.0005	1.000±0.000	telocentric	M
18	0.000	0.479	0.479	0.0193±0.0006	1.000±0.000	telocentric	M
19	0.000	0.470	0.470	0.0189±0.0006	1.000±0.000	telocentric	M
20	0.000	0.457	0.457	0.0184±0.0005	1.000±0.000	telocentric	M
21	0.000	0.442	0.442	0.0178±0.0007	1.000±0.000	telocentric	M
22	0.000	0.425	0.425	0.0170±0.0009	1.000±0.000	telocentric	M
23	0.000	0.397	0.397	0.0159±0.0015	1.000±0.000	telocentric	M

Remarks: * = NOR-bearing chromosome, L=large, and M=medium.

3A). The single pair of NOR is the same as in *T. trichopterus* (Supiwong et al. 2010) and *T. fasciata* reported by Kushwaha et al. (2008) but there is difference in *T. fasciata* which had three pairs of NORs (Sobita and Bhagirath 2007) and *Betta splendens* which had two pairs of NORs (Furgala-Selezniow et al. 2008). Gold and Amemiya (1986) suggested that the occurrence of multiple NORs in fishes was considered to be apomorphic or advance condition whereas single pair of NORs was considered to be plesiomorphic or a primitive condition. Considering for NOR loci between *T. microlepis* and *T. trichopterus*, although both species had the single NOR pair, the NOR positions are difference. The present results revealed that *T. microlepis* had interstitial NORs on the chromosome pair 7 whereas *T. trichopterus* had telomeric NORs (region adjacent to the telomere) on the chromosome pair 2 (Supiwong et al. 2010). Therefore, the NOR-bearing chromosome markers can be used as a tool for classification in this fish group. In addition, intraspecific NOR heteromorphism between the homologous chromosomes of pair 7 was also displayed in one female specimen (Fig. 3A, inserted box). This phenomenon is common event found previously in several fishes in Thailand such as *Puntioplites proctozysron* (Supiwong et al. 2012), *Lutjanus johnii* (Phimphan et al. 2013), *Pterapogon kauderni* (Kasiroek et al. 2017) and *Hemibagrus wyckii* (Supiwong et al. 2017).

Constitutive heterochromatic blocks were observed at centromeric and pericentromeric regions of all chromosomes and with no clear interstitial and telomeric positive C-bands (Fig. 3B). It indicates that the chromosomes of *T. microlepis* are conserved and non-related to chromosomal fusion or an increase in heterochromatin during evolution. Present result is similar to some species in another family of the order Perciformes such as *Geophagus brasiliensis* and *C. facetum* in the Cichlidae family (Vicari et al. 2006), *Plectroglyphidodon lacrymatus*, *Chrysiptera leucopoma*, *C. rex* and *Neoglyphidodon melas* in the Pomacentridae family (Takai 2012). However, there are several species which presented the complex types of positive C-bands. *Symphysodon haraldi, S. aequifasciatus* and *S. discus* (Cichlidae) had heterochromatic blocks on the pericentromeric regions of all chromosomes and the proximal regions of both arms of some

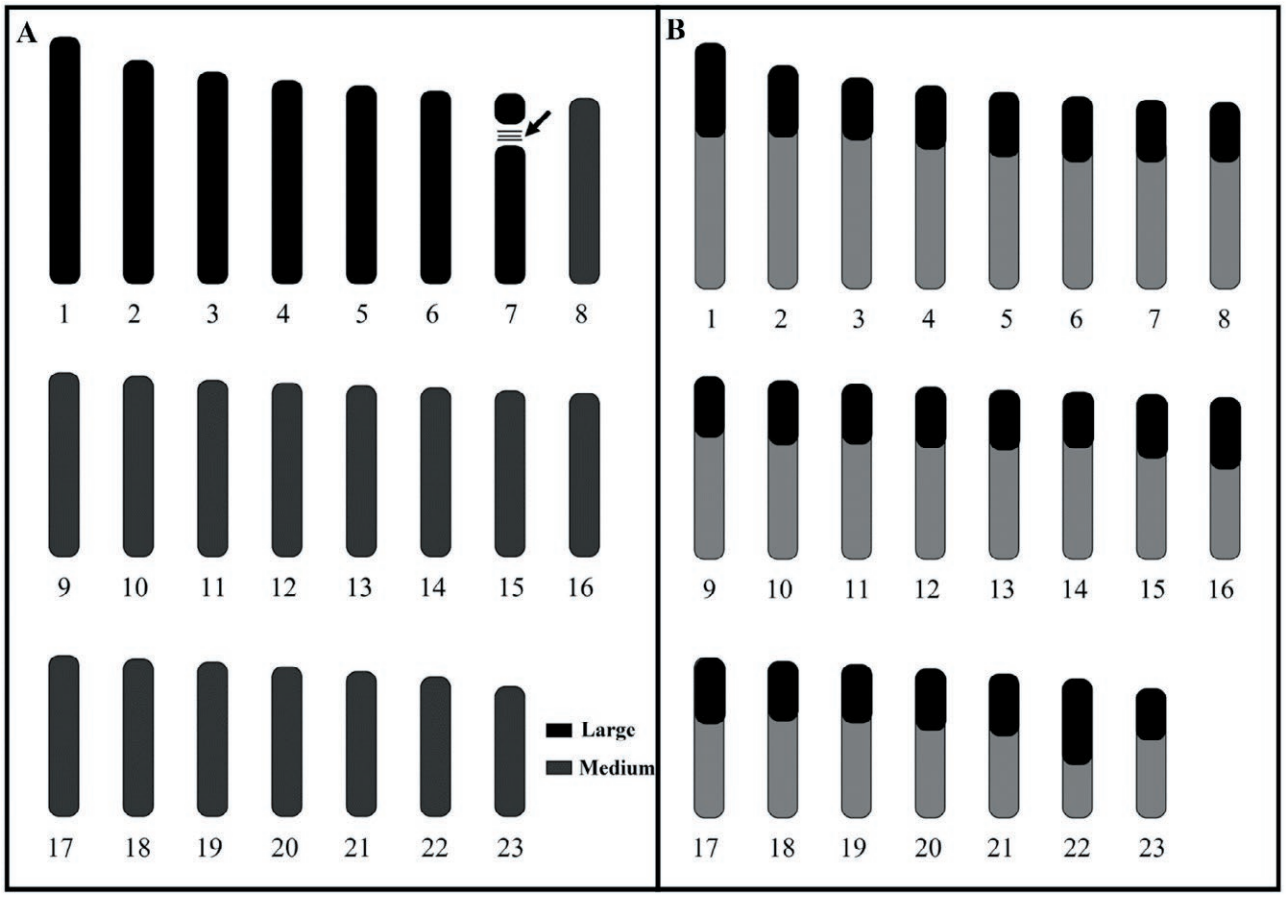

Figure 4. Idiograms showing shape and length of chromosome of Moonlight Gourami, *Trichopodus microlepis* (Perciformes, Osphronemidae) represented the haploid set (*n*=23) by conventional staining (A) and C-banding (B). Arrow indicates secondary constriction/NOR on the long arm of the telocentric chromosome pair 7.

chromosomes (Mesquita et al. 2008), while *N. nigroris* (Pomacentridae) exhibited the distribution of positive C-bands in most centromeric regions and including many terminal and interstitial regions (Takai 2012).

The idiogram shows a continuous length gradation of chromosomes. Approximately two-fold of the size differences between the largest and smallest chromosomes were revealed. The marker chromosomes are the chromosome pair 1, which is the largest telocentric and the chromosome pair 23 is the smallest telocentric. The data of the chromosome measurement on mitotic metaphase cells (from all specimens) are shown in Table 2. Idiograms by conventional staining and C- banding are shown in Fig. 4. In conclusion, NOR phenotype and constitutive heterochromatin patterns on the chromosomes are specific to species in the genus *Trichopodus*. For more information about the chromosomal diversity and chromosomal evolution in this genus, more species and techniques should be further studied.

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