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The karyotype, C-bands and $AgNO_3$-bands of a lungless salamander from Korea: *Onychodactylus fischeri* (Boulenger) (Amphibia, Urodela)

K. Iizuka* and S. Yazawa[a]

Adachi 13th Junior High School, Adachi-ku, Tokyo 121 (Japan) and
[a]Shimura 5th Junior High School, Itabashi-ku, Tokyo 174 (Japan)
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Abstract. The karyotype of a lungless salamander, *Onychodactylus fischeri*, from Korea was analyzed and compared with that of the Japanese congeneric species, *O. japonicus*. In both species the diploid karyotype consists of *78* chromosomes, including 6 pairs of large chromosomes, 6 pairs of medium-sized ones, and the remaining 27 pairs of microchromosomes. The chromosome number of *O. fischeri*, 2n = 78, is, like that of *O. japonicus*, the largest so far reported in the order Urodela. C-banding showed that constitutive heterochromatin in *O. fischeri* was mainly in the centromeric regions and near the secondary constrictions of the large chromosomes. $AgNO_3$-bands were located in the secondary constrictions associated with C-band heterochromatin.
Key words. Onychodactylus fischeri; lungless salamander; karyotype; C-band; constitutive heterochromatin; $AgNO_3$-band.

The genus *Onychodactylus* included in the family Hynobiidae is considered to belong to the primitive salamanders together with the genera *Hynobius*, *Sallamandrella* and other genera found in eastern Asia. Members of *Onychodactylus* differ from the other genera in that they are lungless. The genus *Onychodactylus* consists of two species. *Onychodactylus japonicus* is found in Honshu and Shikoku in the Japanese islands, while *Onychodactylus fischeri* is widely distributed from Ussuri in Russia through the northeastern area of China to the Korean Peninsula. *O. fischeri* was first described by Boulenger[1] in 1886, based on a specimen from Ussuri. Dunn[2] treated the two species as different but he was doubtful of any clear taxonomic differences between them.

The karyotype of *O. japonicus* was first described by Morescalchi et al.[3] as having the diploid chromosome number of $2n = 58 \pm 2$. Ikebe et al.[4], however, showed that the correct chromosome number of this species is 2n = 78. Recently C-banding and R-banding analyses were performed in *Hynobius* and *Salamandrella*[5–13] and variations in repetitive DNA were examined in *Hynobius* and *Onychodactylus*[14].

There has been no report on the karyotype or chromosome banding pattern in *O. fischeri*. In this study we report the karyotype stained with Giemsa, C-banding and $AgNO_3$-banding pattern of *O. fischeri* from Korea and compare these karyotypes with those of *O. japonicus* and other hynobiid salamanders.

Materials and methods

The animals used for chromosome analyses were all larvae. The specimens comprised 2 individuals from Mt. Soraku, Kangwon-do and 5 individuals from Mt. Pukhan, Seoul, Kyonggi-do (these were collected in March 1991); 21 individuals from Mt. Chiri, Kyongsangnamu-do (August 1991) and 16 individuals from Mt. Songni, Chungchongbuku-do (July 1992). The chromosome preparations of specimens from Mt. Soraku and Mt. Chiri were performed in a field station laboratory.

Metaphase chromosomes were obtained by the methods described by Kezer and Sessions[15] and Iizuka et al.[16] from the intestinal epithelia. A colchicine solution (2 mg/ml) was injected intraperitoneally (0.1 ml/g of body weight). 24 to 48 h later, the animals were killed and the guts rinsed and kept in distilled water for 40 min before fixation in 3:1 methanol:acetic acid. Metaphase chromosome spreads were obtained by the air-drying method[17] and stained in 4% Giemsa solution (pH 7.0) for 20 min. C-banding was accomplished using the BSG technique of Sumner[18] with slight modification. The chromosome preparations made by the air-drying method were placed in 0.2 N HCl for 1 h at room temperature, and were treated with 5% $Ba(OH)_2$ for 5 min at 50 °C. Then they were incubated in 2 × SSC for 1 h at 60 °C and stained with 8% Giemsa solution for 40 min. $AgNO_3$-banding was performed according to the 1-step method of Howell and Black[19], i.e. treatment in 50% $AgNO_3$ solution for 1 h at 50 °C. Karyotype analyses were performed on 40 well-spread metaphase cells. Chromosome morphology is classified according to the system of Green et al.[20].

Results

In the conventionally Giesma-stained karyotype, the diploid chromosome number of *O. fischeri* was found to

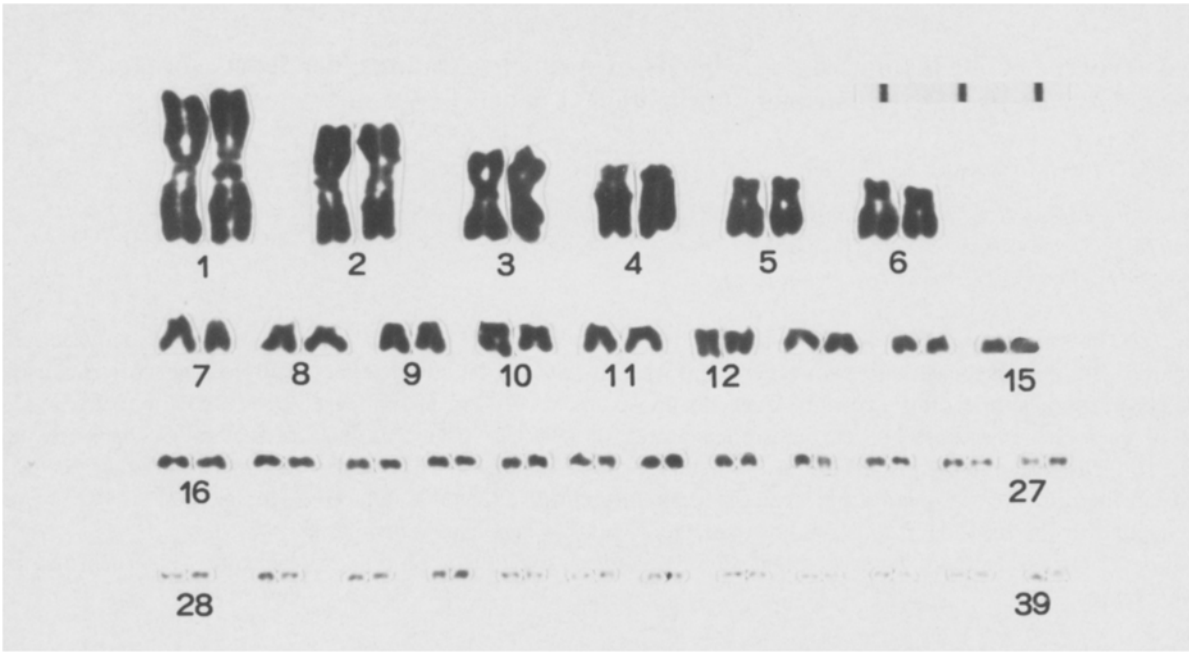

Figure 1. A karyotype of *Onychodactylus fischeri* stained with conventional Giemsa using intestinal epithelia of larva from Mt. Pukhan, Seoul, Korea (2n = 78). One section of scale bar denotes 10 μm.

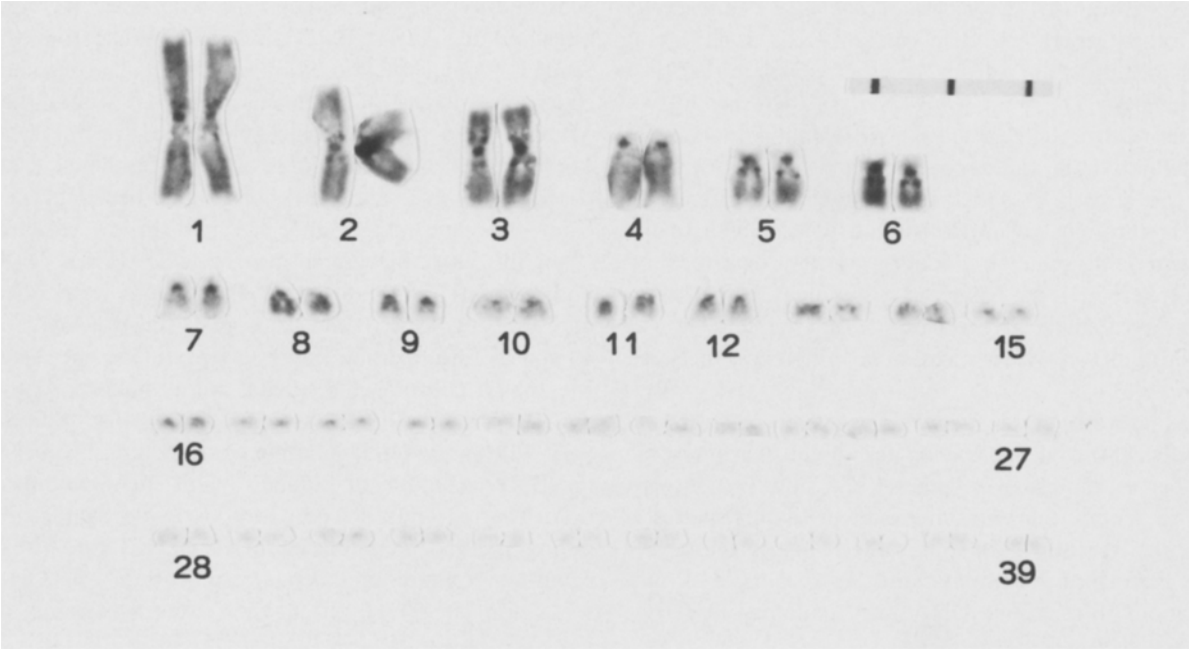

Figure 2. A C-banding karyotype of *O. fischeri* from Mt. Pukhan.

be 2n = 78 (fig. 1). The 6 homologous pairs of chromosomes consisted of 3 pairs of metacentric chromosomes (nos 1, 2 and 3) and 3 pairs of subtelocentric elements (nos 4, 5 and 6). Secondary constrictions were observed in both arms of chromosome nos 1, 2 and 3 and in the long arms of chromosome nos 5 and 6. Six pairs of medium-sized chromosomes were submetacentric or subtelocentric in shape, while the remaining 27 pairs of microchromosomes were all telocentric. The fundamental number was therefore 102.

In the C-banded preparations, the centromeres of all 6 large pairs were deeply stained, and the positions corresponding to the secondary constrictions in 5 pairs of the large chromosomes also stained darkly (fig. 2). Some weak interstitial C-bands with small amounts of heterochromatin were discernable on the arms

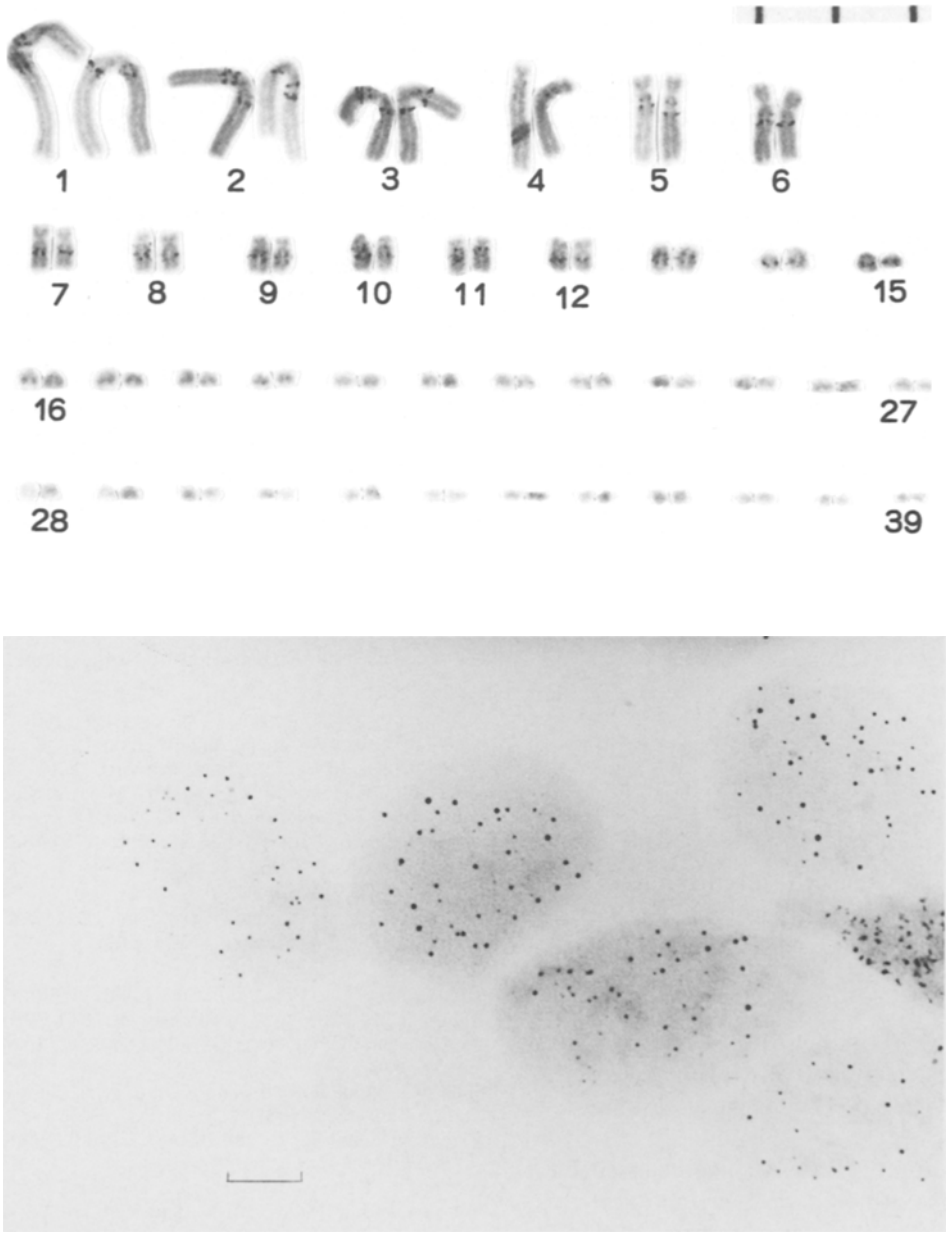

Figure 4. Interphase nuclei of *O. fischeri* from Mt. Pukhan. The number of Ag-spots by $AgNO_3$-stain was about 40.

of all large chromosomes. In all the medium-sized ones C-bands were observed on the centromeres, but we could not find clear C-bands on any of the micro-chromosomes. $AgNO_3$-staining bands were clearly observed on the secondary constrictions associated with C-band heterochromatin (fig. 3). We recognized the same number of Ag-spots on chromosomes as in interphase nuclei (fig. 4). The number of Ag-spots on silver-stained chromosomes was about 40. No heteromorphic pairs were observed in Giemsa-stained, C-banded or $AgNO_3$-banded karyotypes.

Discussion

The karyotype of *O. fischeri* is almost identical in chromosome number and morphology to that of *O. japonicus*[4]. Sessions et al.[21] reported the chromosome number of *O. fischeri* to be $2n > 66$ as their unpublished observation. In our result, however, the chromosome number

of *O. fischeri* was clearly determined as 2n = 78, which is much larger than that reported by Sessions et al.[21]. It is hard to define the reason for this disagreement, since the details of the unpublished results of Sessions et al.[21] are not available. We, however, consider that the difference is artifactual rather than a biological difference between the samples, since a similar discrepancy is found between the reports on the chromosome number of *O. japonicus* by Morescalchi et al.[3] and Ikebe et al.[4]. The chromosome number of the genus *Onychodactylus*, 2n = 78, is the largest so far reported in the order Urodela[4].

Morescalchi[22] suggested that Hynobiidae (2n = 56 to 78) and Cryptobranchidae (2n = 60) have asymmetrical bimodal karyotypes with high chromosome numbers and microchromosomes, and he regarded this as a characteristic of primitive karyotypes in salamanders. Thus, according to this suggestion, *O. fischeri* should have a karyotype dominated by primitive features. Iizuka and Kakegawa[9] reported the fundamental numbers of Taiwanese and Japanese lotic-breeding type hynobiids (2n = 58) as 90 to 102. *Hynobius* has biarmed microchromosomes, in contrast, *Onychodactylus* possesses all uniarmed microchromosomes[23]. Similarity of the fundamental number value between *Hynobius* and *Onychodactylus* would suggest that both genera derived from a common ancestor through a reduction in the number of microchromosomes[21].

Yang[24] reported that *Batrachuperus karlschmidti*, a hynobiid of the genus *Batrachuperus* from Sichuan Province in China, has a karyotype with 68 chromosomes, and a similar karyotype composition as *Onychodactylus* except for the number of microchromosomes. This might support the morphological evidence[25] of close phylogenetic relationships between *Onychodactylus* and *Batrachuperus*. However, Kuro-o et al.[14] suggested that, on the evidence of Southern blot hybridization using repetitive DNA, *O. japonicus* is phylogenetically very remote from *Hynobius*, *Salamandrella* and *Batrachuperus* (Kuro-o et al., personal communication).

O. fischeri had constitutive heterochomatin in all the centromeric positions of its 6 large pairs and near the secondary constrictions of 5 large pairs of chromosomes. The C-banding patterns of *O. fischeri* were different from those of *Hynobius* species and *Salamandrella keyserlingii*[5–11]. The karyotypes of *Hynobius* species and *Salamandrella keyserlingii* have centromeric C-bands on all the chromosomes and numerous, interstitial, weak C-bands on the large ones. However, we could not find such multiple, intersititial C-bands on any chromosomes of *O. fischeri*. Dissimilarity of C-banding pattern among *O. fischeri*, *Hynobius* species and *Salamandrella keyserlingii* must arise from the ancestral karyotype through chromosomal rearrangements by fusions, inversions, and/or addition and loss of highly repetitive DNA in the process of their evolution. Multiple $AgNO_3$-bands were observed at the same positions as C-band heterochromatin except for centromeric heterochromatin. The number of Ag-spots in silver-stained chromosomes was about 40. The positions stained by the $AgNO_3$-technique have been considered as the nucleolus organizer regions associated with 18S and 28S ribosomal DNA genes[26]. Although such an extremely large number of Ag-spots is very rarely found in vertebrates[26], Iizuka and Kakegawa also observed multiple $AgNO_3$-bands in the karyotype of lotic-breeding type *Hynobius*[9]. These facts suggests that in the order Urodela the heterochromatic regions are closely related to the Ag-NORs. However, it is quite possible that the NORs are not specifically stained but protein associated with the heterochromatic regions of the chromosomes. The fact that the same number of Ag-spots is also present in the interphase nuclei does not exclude this artifact. In future we shall investigate the number of nucleolar organizers in *O. fischeri* by in situ hybridization with ribosomal DNA because in the order Urodela a positive Ag-staining is not always synonymous with the presence of NORs[27]. In situ hybridization of 18S and 28S ribosomal DNA to the metaphase chromosomes of newts and salamanders is a much reliable technique to demonstrate the actual number and location of NORs.

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* To whom reprint requests should be addressed at: 5-10-8 Sendagi, Bunkyo-ku, Tokyo 113 (Japan).

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