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Chromosomes of Japanese starfishes

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ABSTRACT—We developed a method for preparing starfish chromosomes from embryos. Blastulae were treated with colchicine (0.2–4.0 mg/ml), dissociated into single blastomeres by pippeting, swollen with 7% sodium citrate, and fixed with methanol: acetic acid (3:1). The fixed cells were dropped on a slide and air-dried. We examined the chromosome number in five species of asteroids belonging to 4 families (Luidiidae, Astropectinidae, Asterinidae, and Asteriidae), and all had a diploid number of 44. We analyzed the karyotype in 4 of the species, and all were different. We visualized the nucleolus organizer regions of an *Asterina* species and an *Asterias* species and found them to be quite different from each other.

Key words: karyotype, nucleolus organizer regions (NORs), asteroids, brittle stars, ophiuroids

INTRODUCTION

About 1800 species of starfishes have been identified (Hendler *et al.,* 1995; McEdward *et al.,* 2002). While extensive morphological (Fisher, 1911, 1928, 1930; Hayashi, 1940, 1943, 1973; Spencer and Wright, 1966; Fell, 1963; Heddle, 1967; Blake, 1987; Downey, 1973; Clark and Downey, 1992), developmental (Oguro *et al.,* 1976; Komatsu *et al.,* 1979; McEdward, 1992; Chia *et al.,* 1993; Byrne and Cerra, 1996), biochemical (Kubo, 1961; Schopf and Murphy, 1973; Mochizuki and Hori, 1980; Matsuoka, 1981, Matsuoka *et al.,* 1994), and molecular (Lafay *et al.,* 1995; Wada *et al.,* 1996; Knott and Wray, 2000) studies have been systematically carried out on starfishes, cytogenetic studies have not yet been applied to asteroid phylogeny.

Starfish chromosomes have been examined in early embryo sections since the start of the 20th century (Delage, 1901; Tennent and Hogue, 1906; Tennent, 1907; Jordan, 1910). In Japan, Makino and Niiyama (1947), examining testis sections showed that the diploid chromosome number of 4 species ranged from 38 to 50. Later studies with testes showed that the chromosome numbers of 8 species belonging to 4 families were 44 (Delobel, 1971; Colombera, 1974; Colombera *et al*., 1977; Colombera and Venier, 1980**;** Colombera and Tagliaferri, 1986). The constancy in chromosome number necessitates karyotypic comparison, but chromosomes from starfish testes are too small to permit karyotype analysis. Since I have been able to obtain clear chromosome preparations from sea urchin embryos by the air-drying method (Saotome, 1987), we thought it might be possible to modify the method and apply it to starfish embryos.

In this paper, we describe a method for preparing starfish chromosomes from embryos, and the results of karyotype and NOR analysis.

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MATERIALS AND METHODS

Materials

We used 5 species belonging to 4 families of asteroids and one species of ophiuroid (Table 1). We removed testes and ovaries from adults and immersed them in artificial sea water (Jamarin U, Jamarin Laboratory, Osaka, Japan). We treated the ovaries with 1–2 μM 1-methyladenine for 30 min (Sigma, St Louis, Mo., USA) to induce breakdown of germinal vesicles and obtain mature eggs (Kanatani, 1969). When the motility of sperm released in sea water was poor, we improved it by adding L-histidine (0.1 mM) (Wako Pure Chemical Industries, Osaka, Japan) to the sperm suspension. Eggs were inseminated before extrusion of the second polar body. The embryos (2000 per ml sea water) were cultured at room temperature and only *Asterias amurensis* embryos at 10–13°C. In *Asterina minor* and *Amphipholis kochii,* the several adults cultured at room temperature were transferred to small beakers (200–300 ml), kept at 4–10°C for 1–2 hr, and then returned to room temperature (Yamashita, 1983). We harvested the embryos, which were naturally born during overnight.

Chromosome preparation

Embryos (1×10^4) at the early blastula stage (250–500 cell stage) or wrinkled blastulae (20–30 embryos) in *A. minor* were treated with colchicine (0.2–4.0 mg/ml) (Sigma, St Louis, Mo., USA), sampled 4–5 times at 5–30 min intervals before or after a

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Table 1. Asteroid and ophiuroid species analyzed in this study.

Species	Collecting Place
Asteroids*	
Order Platyasterida	
Family Luidiidae	
Luidia maculata	The Amakusa Marine Biological Laboratory, Kyushu University, Kumamoto Prefecture
Order Paxillosida	
Family Astropectinidae	
Astropecten scoparius	The Tateyama Marine Biological Station, Ochanomizu University, Chiba Prefecture
Order Spinulosida	
Family Asterinidae	
Asterina pectinifera	The Misaki Marine Biological Station, University of Tokyo, Kanagawa Prefecture
Asterina minor	Hashirimizu, Kanagawa Prefecture Kushimoto, Wakayama Prefecture
Order Forcipulatida	
Family Asteriidae	
Asterias amurensis	Hashirimizu, Kanagawa Prefecture
Ophiuroids	
Order Myophiuroida	
Family Amphiuridae	
Amphipholis kochii	Abuta, Southwestern Hokkaido

* Classification is based on Spencer and Wright (1966).

division, collected by centrifugation (300×g×5 min), suspended in 1M urea, and dissociated into their component blastomeres by pipetting. The fertilization membranes were simultaneously removed. The colchicine concentration, sampling interval time, and sampling numbers varied with species. *A. minor* embryos, which have large eggs containing a lot of yolk, were suspended in 0.5 ml sea water in 35 mm dishes, beaten slightly with a bamboo spit (2 mm in diameter), and dissociated into their blastomeres. The dissociated blastomeres were collected by centrifugation, swollen with 7% sodium citrate for 10 min, fixed with methanol: acetic acid (3:1), and washed twice with the fixative. The fixed cells were air-dried (Saotome, 1982a), and the preparations were stained for 10 min with 3% Giemsa solution (Merck, Whitehouse Station, NJ, USA) diluted with phosphate buffer (pH 6.9). We used more than 6 pairs of adults and prepared chromosomes through 2–5 breeding seasons. Since we used only one pair and prepared chromosomes for only one season for *Luidia maculata* and *A. kochii*, their data were shown as preliminary ones.

Others

We visualized the nucleolus organizer regions (NORs) by the silver-staining method of Howell and Black (1978), with slight modifications. The preparations stained with Giemsa were destained with methanol after observation, and then stained with 50% silver nitrate solution for 2–3 min at 50–57°C (Saotome, 1991).

We classified chromosomes by arm ratio on the basis of the nomenclature of Levan *et al.* (1964) into metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t) chromosomes. We divided chromosomes into large-sized ones (L) and medium-sized ones (M).

RESULTS

Chromosome preparation

We examined stage of preparation, concentration of colchicine and hypotonic solution, dissociation into single blastomeres, and composition of fixative to establish preparing conditions. Good chromosome preparations could be obtained, when blastulae were treated with colchicine (0.2–4.0 mg/ml), dissociated into single blastomeres by pippeting, swollen with 7% sodium citrate, and fixed with methanol: acetic acid (3:1).

Chromosomes

Fig. 1a–e show typical metaphase chromosomes from starfish embryos of 5 species. The chromosomes spread over one layer, which made it possible to count them and to analyze the karyotype. The chromosomes were small, ranging in length from 1 μm to 5 μm. Fig. 1f indicates meiotic chromosomes from *Asterina minor* testes treated with colchicine by air-drying.

The modal diploid chromosome numbers in all 4 starfish species were 44 (Fig. 2a–d); the modal haploid number in *A. minor* testes was 22 (Fig. 2e). Preliminary data in *Luidia maculata* indicated that 39 (53%) out of 73 cells also had a diploid chromosome number of 44.

Karyotypes

Asteroids

Astropecten scoparius was characteristic in having 2 pairs of large subtelocentric chromosomes. The karyotype consisted of 9 pairs of metacentric, 7 pairs of submetacentric, and 6 pairs of subtelocentric chromosomes (Fig. 3). *Asterina pectinifera* had one pair of medium-sized metacentric, 10 pairs of metacentric, and 9 pairs of submetacentric chromosomes, and one pair each of subtelocentric and telocentric chromosomes (Fig. 4a). *A. minor* showed one pair of

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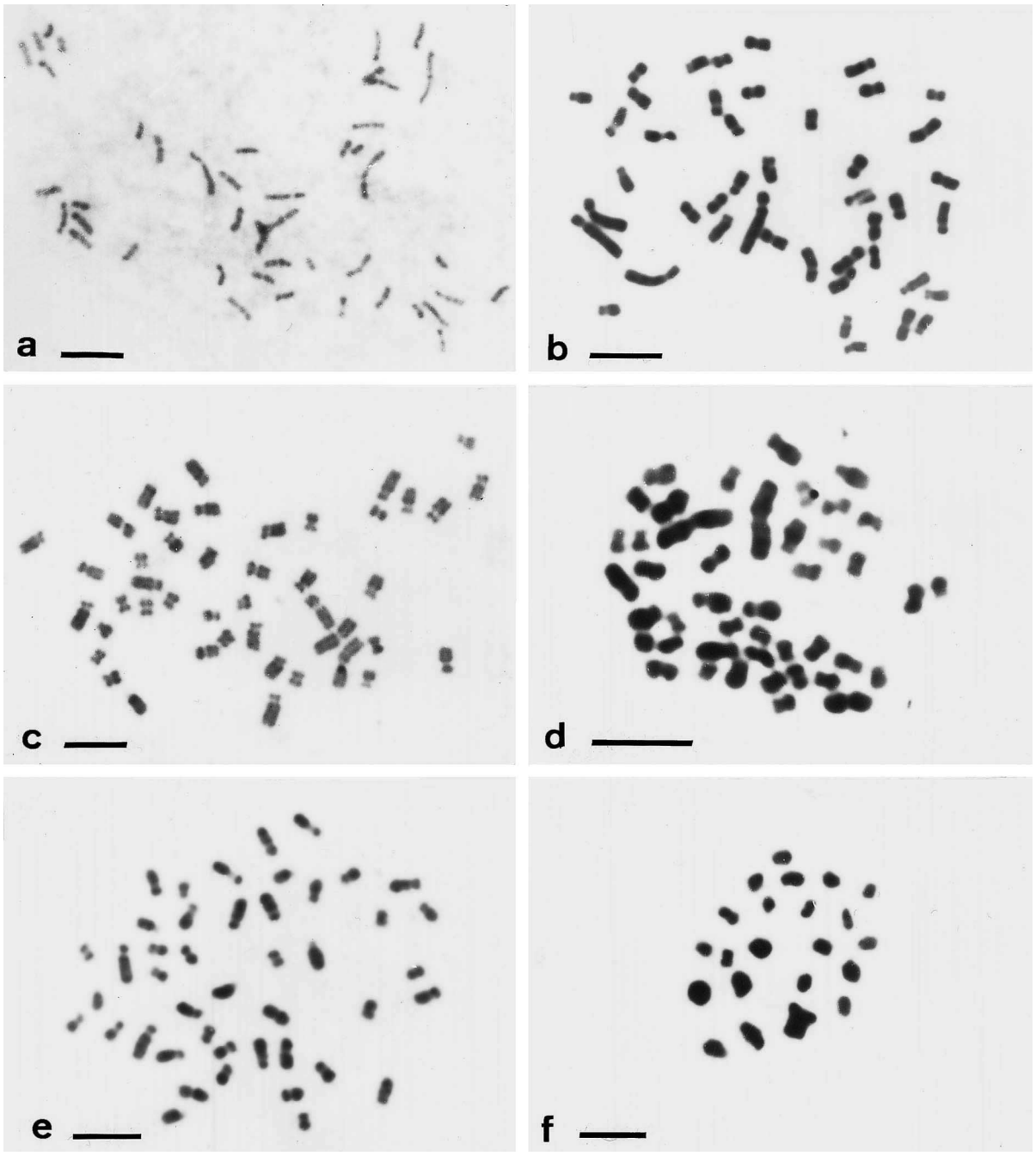


Fig. 1. Typical metaphase chromosomes from the embryos of 5 asteroid species. a) *Luidia maculata*, b) *Astropecten scoparius*, c) *Asterina pectinifera*, d) *Asterina minor*, e) *Asterias amurensis*, and f) typical meiotic chromosomes from *Asterina minor* testes. Bar, 5 μm.

medium-sized metacentric, 14 pairs of metacentric, 6 pairs of submetacentric, and one pair of telocentric chromosomes (Fig. 4b). *Asterias amurensis* had 12 pairs of metacentric, 7 pairs of submetacentric, and 3 pairs of telocentric chromosomes (Fig. 5). Preliminary data for *L. maculata* showed that it had about 6 pairs of metacentric, two pairs of large submetacentric, about 6 pairs of submetacentric and about 8 pairs of subtelocentric chromosomes.

Ophiuroids

We tried to prepare chromosomes from several ophiuroid species but succeeded only with *Amphipholis kochii* (Fig. 6). We made only three preparations because of the difficulty in getting mature adults and harvesting well-developed embryos. The preliminary results indicated that 42 (72%) out of 58 cells had a diploid chromosome number of 42, which had three pairs of metacentric, three pairs of submetacentric, 7 pairs of subtelocentric, and 8 pairs of telocentric chromosomes (Fig. 7).

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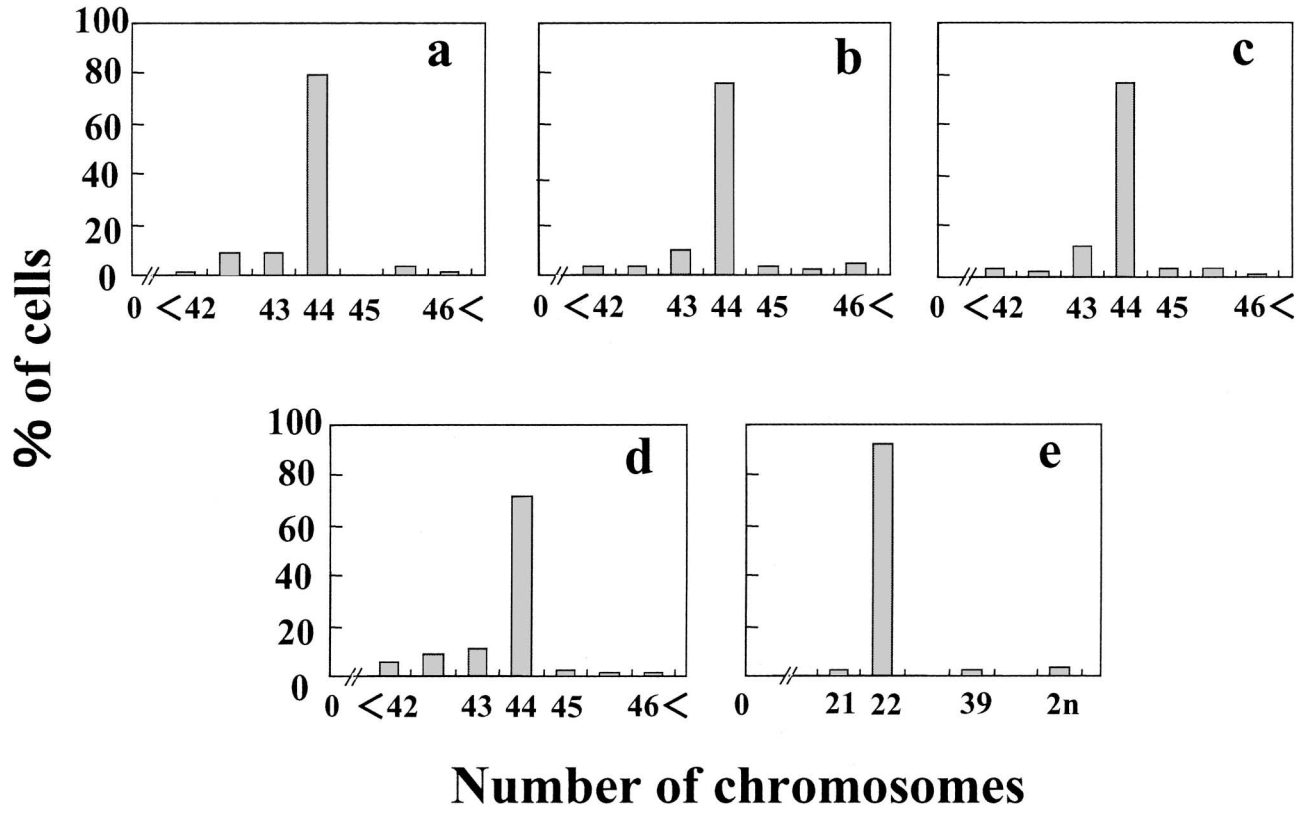


Fig. 2. Distribution of chromosome number in embryos of 4 asteroid species, a) *Astropecten scoparius* (n=72 cells), b) *Asterina pectinifera* (n=215 cells), c) *Asterias amurensis* (n=181 cells), d) *Asterina minor* (n=170 cells), and e) that in *Asterina minor* testes (n=56 cells).



Fig. 3. Karyotype of *Astropecten scoparius*. m=metacentric, sm=submetacentric, st=subtelocentric chromosome Bar, 5 μm

NORs

The NORs of *A. pectinifera* occurred on two regions. One was large (long arrow) and located on the long arm of a metacentric chromosome pair, while the other was small (short arrow) and located on a terminal position of a telocentric chromosome pair (Fig. 8a). The NORs of *A. amurensis* were located on a terminal position of three telocentric chromosome pairs (arrows in Fig. 8b).

DISCUSSION

In this paper, we reported a method for preparing starfish chromosomes from embryos. The timing of the preparation was critical because colchicine inhibited cytoplasmic division but did not stop nuclear division. Indeed, we observed many polyploid cells after long colchicine treatment, and were not able to obtain metaphase plates in those shortly after the stoppage of cleavage. Many metaphase plates could be prepared during the M phase at the early blastula stage (250 or 500 blastomeres). In *Asterina minor* eggs, which are large and contain a lot of yolk, wrinkled blastulae were good for preparation. Though we do not understand why colchicine did not stop nuclear division, we were not able to obtain good metaphase plates without it.

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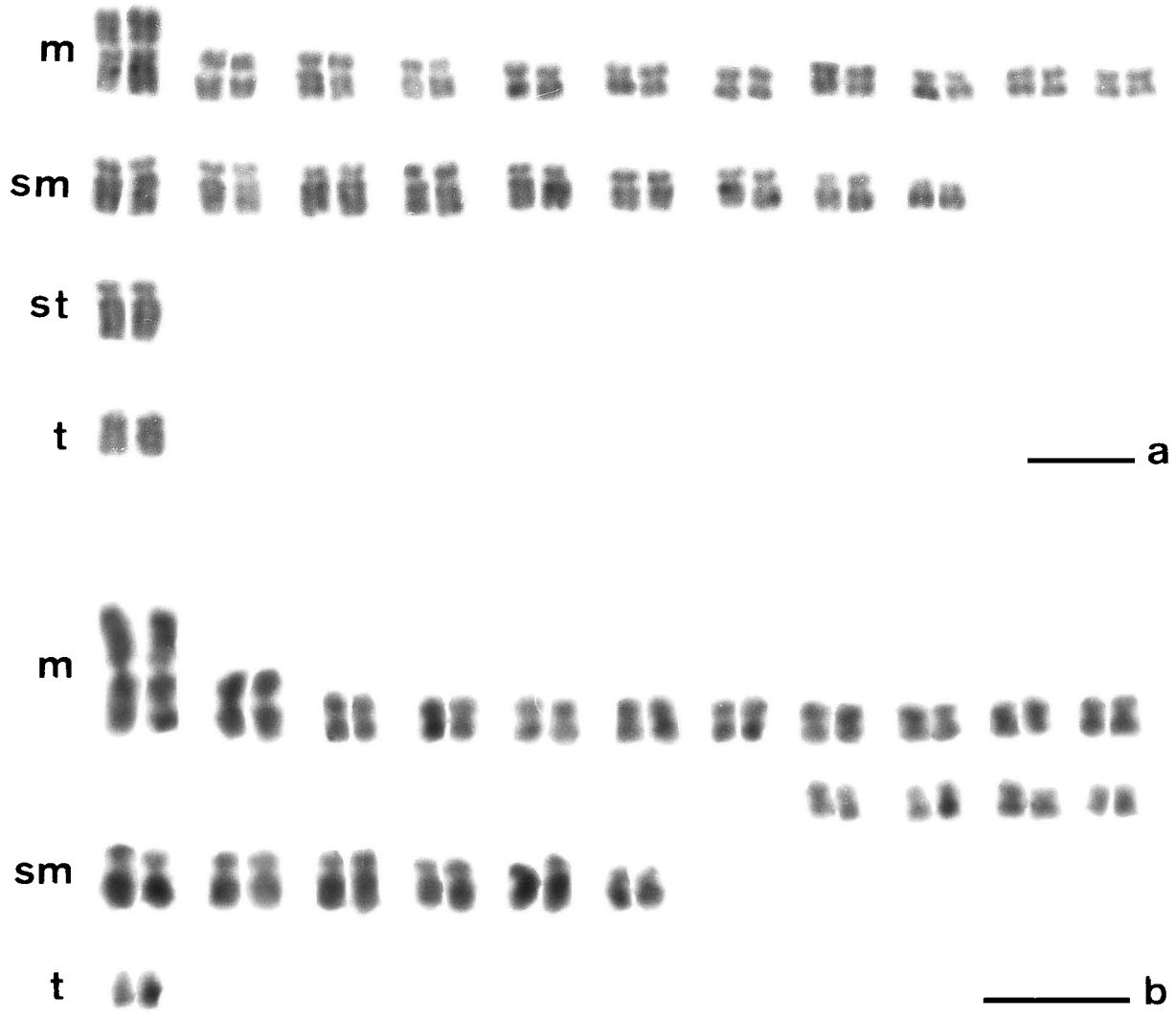


Fig. 4. Karyotypes of two Asterinidae species. a) *Asterina pectinifera* and b) *Asterina minor.* t=telocentric chromosome Bar, 5 μm

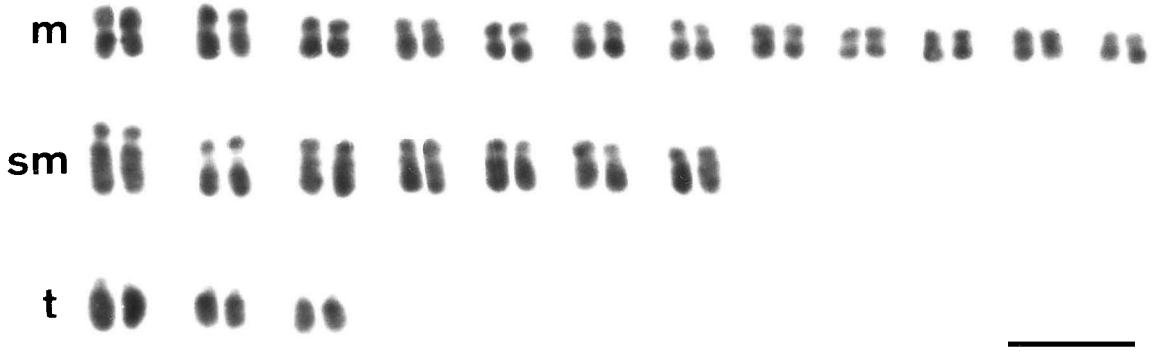


Fig. 5. Karyotype of *Asterias amurensis*. Bar, 5 μm

Colchicine may affect chromosome morphology. We could prepare well-spread chromosomes from *A. minor* testes (Fig. 1f) and count clearly chromosome number of 22 as a haploid number (Fig. 2e), but not analyze karyotype owing to chromosome smallness. Though karyotype analysis might be also difficult in the metaphase plates from testes of other species (Colombera, 1974; Colombera *et al*., 1977; Colombera and Venier, 1980; Colombera and Tagliaferri, 1986), we found that chromosome preparations from embryos were good to analyze karyotype.

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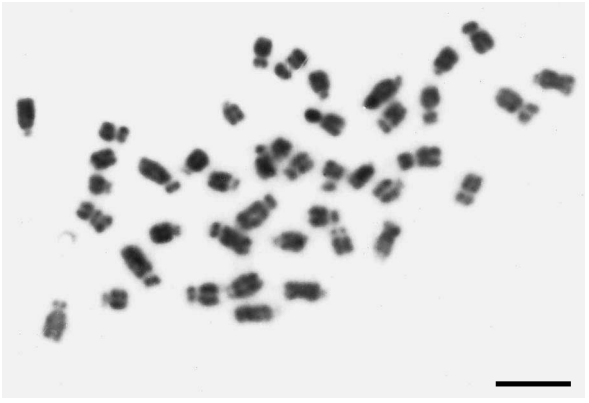


Fig. 6. Preliminary metaphase chromosomes from *Amphipholis kochii* embryos. Bar, 5 μm

The diploid chromosome number was 44 in all 5 asteroid species we studied. Since all 8 species (*Astropecten bispinosus, Astropecten arantiacus, Ophidiaster ophidianus, Hacelia attenuata, Asterina gibbosa*, *Asterina pectinifera, Asterina coronata japonica,* and *Echinaster sepositus*) belonging to 4 families reported until now also had a diploid chromosome number of 44, (Delobel, 1971; Colombera, 1974; Colombera *et al*., 1977; Colombera and Venier, 1980; Colombera and Tagliaferri, 1986), the basic diploid chromosome number for asteroids may be 44. The high aneuploidy frequency (17%–22%) we observed may have been caused by contamination of embryos showing polyspermy, which induces irregular cleavage and results in abnormal chromosome separation (Saotome, 1982b). When adults matured fully, good chromosome preparations could be obtained owing to the high rate of germinal vesicle breakdown and fertilization, and synchronous cleavage. When they did not mature fully, however, the fertilization rate was low and cleavage was not synchronous. In those cases, the addition of more concentrated sperm solution was apt to induce polyspermy. The cases showing polyspermy, of course, were not used for chromosome preparations.

The karyotypes from 5 species were compared on the basis of chromosome size and arm ratio. The karyotype of *Astropecten scoparius* (chromosome formula; (9 m+7 sm+(2 L+4) st) belonging to Astropectinidae, differed from the karyotypes of *Asterina pectinifera* ((1M+10) m+9 sm+1 st+1 t) and *Asterina minor* ((1M+14) m+6 sm+1 t) (both Asterinidae) as well as *Asterias amurensis* (12 m+7 sm+3 t) (Asteriidae) (Fig. 3, 4, 5). Astropectinidae is distinct from Asterinidae and Asteriidae morphologically (Hayashi, 1974) and immunologically (Mochizuki and Hori, 1980), which is consistent with the karyological difference. The *A. scoparius* karyotype may be close to the karyotype of *L. maculata* (about 6 m+(2L+about 6) sm+about 8 st), belonging to Luidiidae. Paleontological evidence suggests that the order Platyasterida (Luidiidae) comprises the more primitive starfish from which the Paxillosida (Astropectinidae) diverged (Fell, 1963; Spencer and Wright, 1966). Moreover, *A. scoparius* resembles *L. maculata* in its nonbrachiolarian mode of development (Oguro *et al.,* 1976). These relationships may be consistent with the similarity in their karyotypes, but clear karyotype analysis is needed to clarify them. The karyotypes of *A. pectinifera* and *A. minor* were similar but differed in number of meta- and submetacentric chromosomes. Though *A. pectinifera* and *A. minor* belong to the same genus, they differ in the size and yolk content of eggs and in type of larval development– indirect (*A. pectinifera*; Komatsu, 1984) VS. direct (*A. minor* ; Komatsu *et al,* 1979). Immunological data indicate that *A. pectinifera* differs considerably from *A. minor* compared with other species belonging to genus *Asterina* (Mochizuki and Hori, 1980; Matsuoka, 1981). Those developmental and immunological differences may be correlated with karyological differences. Structural changes, such as inversion may have occurred between meta- and submetacentric chromosomes in the course of evolution. The karyotype of *A. amurensis* was quite different from that of other three species we examined.

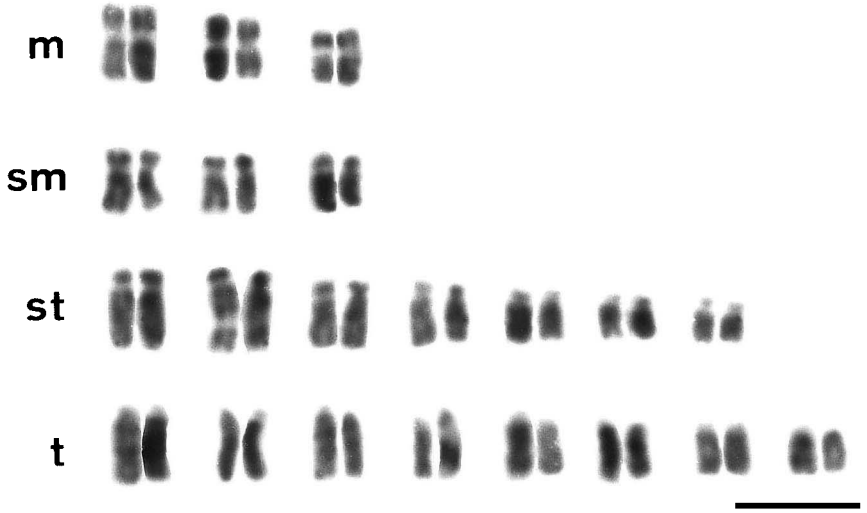


Fig. 7. Preliminary karyotype from *Amphipholis kochii* embryos. Bar, 5 μm

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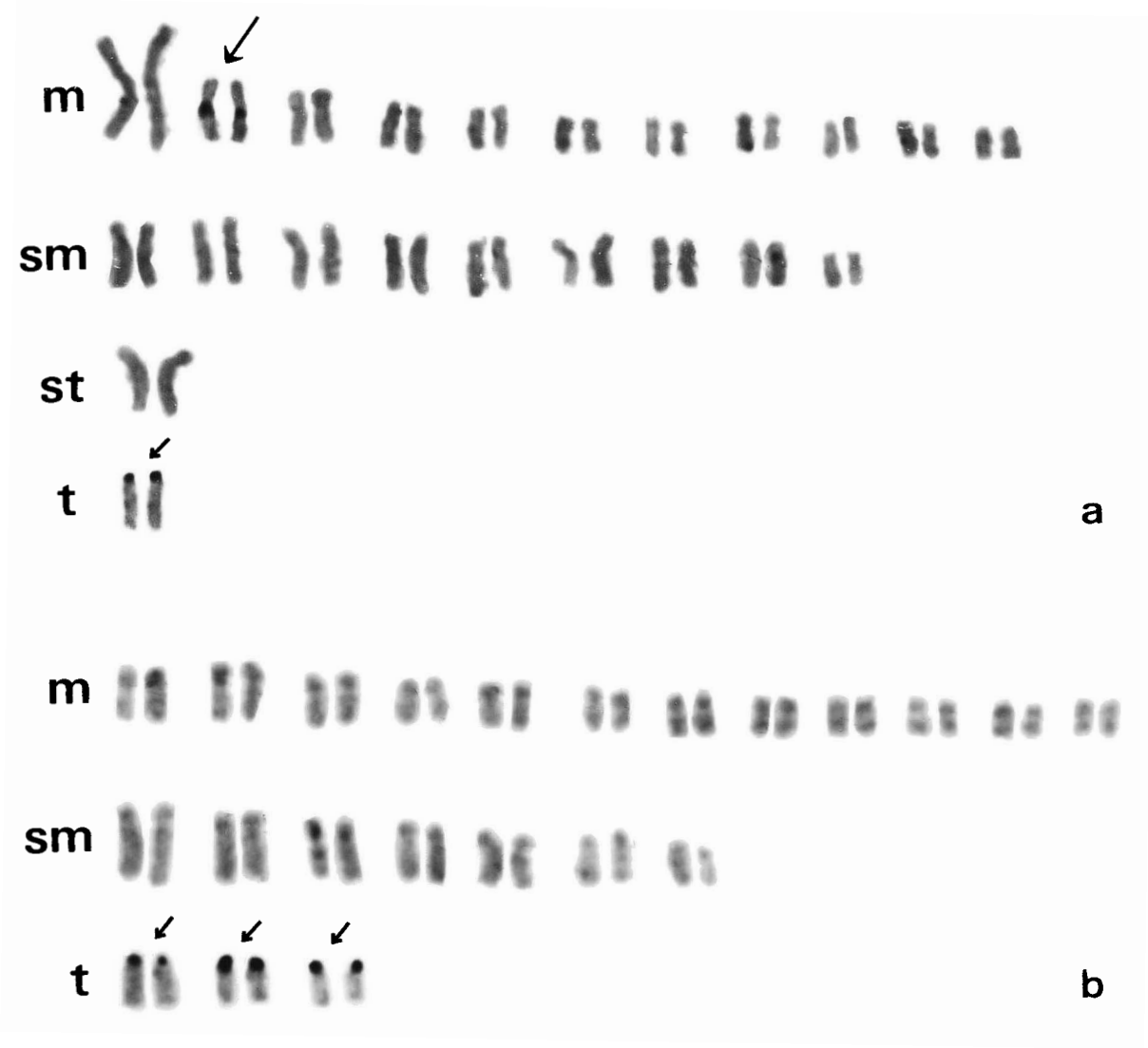


Fig. 8. NORs in a) *Asterina pectinifera* and b) *Asterias amurensis*. Arrows indicate Ag-NORs.

Asteriidae is a well- characterized family, and well-defined by the possession of both straight and crossed pedicellariae and compressed ambulacrals, by which it is distinguished from all other asteroids (Fisher, 1911; Clark and Downey, 1922; Hyman, 1955; Spencer and Wright, 1966; Blake, 1987; Gale, 1987). Asteriidae is also well differentiated from the other families immunologically (Mochizuki and Hori, 1980). These morphological and immunological differences are consistent with the karyological one. *A. pectinifera* and *A. amurensis* are similar in larval development (indirect type), but differ morphologically. We were also able to distinguish them by karyotype and NORs (Fig. 8). We could visualize NORs in only 2 species out of 5 asteroid species. There was variation in number of NORs. 10 cells (45.5%) out of 22 cells had 4 Ag-NOR sites in *A. pectinifera* and cells of 57% (4/7 cells) had 6 Ag-NOR sites in *A. amurensis*. The NORs on one pair of telocentric chromosomes were common to *A. pectinifera* and *A. amurensis,* but NORs on one pair of metacentric chromosomes was specific for *A. pectinifera*. Further data about NORs in other species may make it possible to discuss asteroid phylogeny. We, furthermore, have to compare karyotypes in many species belonging to each order to discuss phylogeny at the order level.

The karyotype figures in this paper show paired chromosomes but we also observed a pair of heterotypic chromosomes, which might represent sex chromosomes. In *A. pectinifera,* about half of karyotypes examined had heterotypic chromosomes (data are not shown). More detailed examination by several banding and FISH methods is needed to determine whether the asteroids indeed have sex chromosomes.

Some palentological evidence suggests that the ophiuroids are more closely related to the asteroids than to the echinoids (Fell, 1963; Spencer and Wright, 1966). Our preliminary data showed that the ophiuroid *A. kochii* (Amphiuridae) had a diploid chromosome number of 42 (Fig. 6). Since diploid chromosome number of *Ophiomyxa pentagona* (Ophiomyxidae) and *Macrophiothrix longipeda* (Ophiot-

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richidae) has been reported to be 42, and that of *Ophiothrix fragilis* (Ophiotrichidae), *Ophioderma longicauda* (Ophiodermatidae), and *Ophiocomina nigra* (Ophiocomidae) is 44 (Colombera, 1974; Colombera and Tagliaferri, 1986), chromosome numbers of 42 and 44 may exist in ophiuroids. *A. kochii* had many subtelocentric and telocentric chromosomes (Fig. 7), which contrasted with the karyotypes of asteroids. Since we found considerable difference in karyotype between asteroids and ophiuroids, karyotypic comparison may be an important phylogenetic tool.

To elucidate chromosome evolution in asteroids and ophiuroids, we need chromosomal data from many species. This method described here is applicable to species from which we can consistently obtain mature eggs and sperm, fertilized eggs, and well-developed embryos.

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