COMPARATIVE MORPHOMETRY, MORPHOLOGY OF EGG AND ADULT SURFACE TOPOGRAPHY UNDER LIGHT AND SCANNING ELECTRON MICROSCOPIES, AND METAPHASE KARYOTYPE AMONG THREE SIZE-RACES OF *FASCIOLA GIGANTICA* IN THAILAND

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Abstract. Comparative morphometry of eggs and adults under light microscope, and morphology of adults under scanning electron microscope (SEM) were undertaken in the three size-races (< 25 mm, 25-35 mm, > 35 mm) of *Fasciola gigantica* (Thailand strain). Morphometric examination revealed intraspecific variation with respects to the dimensions of eggs and adults, whereas surface topography of the three size-race adults under SEM was morphologically similar. The observations on mitotic metaphase chromosomes of spermatogonial cells from testes of the three size-races revealed 2n=20 (diploid type), and no karyotypic difference was observed among them. The meiotic metaphase chromosome was 10 bivalents in primary spermatocyte in diplotene to diakinesis, and many mature spermatozoa were seen in the testicular preparations.

INTRODUCTION

The large liver fluke of bovine, *Fasciola gigantica*, is known as a hazardous parasite that causes substantial economic loss in livestock production in Thailand (Sukhapesna *et al*, 1994). It is also considered as a parasitic zoonosis, since several human cases have been reported (Chitchang *et al*, 1982; Miyazaki, 1991). In Thailand, this liver fluke has been found in wide range animals, *ie*, cattle, buffalo, sheep, goat, hog deer, antelope and chamois (Dissamarn, 1955; Usanakornkul and Sukhapesna, 1980; Loehr, 1982; Chompoochan *et al*, 1990, Suksaithaichana *et al*, 1991; Choompoochan and Achapet, 1992). The overall prevalence of the parasite in buffalo and cattle were 8.9% and 13.9%, respectively. The highest percentage of infection in Thailand was in the North (23.4%), while, the lowest one was found in the South (4%) (Sukhapesna *et al*, 1990).

Extensive study of other strains of *Fasciola spp* has been made by several previous investigators, *ie*, morphological study of various stages under light and scanning electron microscopes (Bennett, 1975a, b; Augot *et al*, 1998), karyotypic study of normal or diploid type and abnormal or triploid type that produces few sperms and/or exhibits parthenogenesis (Moriyama *et al*, 1979; Sakaguchi, 1980), isoenzyme study (Agatsuma *et al*, 1994), and molecular approach (mitochondrial ND1 and COI gene sequences) (Itagaki *et al*, 1998). Little is known about *F. gigantica* (Thailand strain) from the various morphotypes, and cytogenetic point of view, except the reports of surface topography and ultrastructure of tegument of adult *F. gigantica* (Sobhon *et al*, 1994), but no account was taken off various morphotypes and cytogenetics. In the light of above information and the evidence of a possible cryptic species in *F. gigantica*, therefore, investigation on morphometry, morphology under light and scanning electron microscopes of eggs and adults, and metaphase karyotypes of Thailand strain *F. gigantica* are presented herein.

MATERIALS AND METHODS

Collection of worms

The adults of *F. gigantica* were obtained from cattle at slaughter houses in Pathum Thani and

Nonthaburi provinces. The origins of cattle were randomly bought from various parts of Thailand, but mostly came from northern and northeastern Thailand. The recovered specimens were cleaned up with sterile 0.85% sodium chloride solution for three times, then kept in Hanks' balanced salt solution (HBSS) in an incubator at 37ºC until used.

Preparation of worms for studies

Since the main objective of the present study was aiming to search for the morphological and/or cytological variants of *F. gigantica* from the mixed, natural population, thus, prior to the processing of worms for further studies, the body length of live worms were measured, and tentatively classified into three size-races, *ie* < 25 mm (designated small race or SR), 25-35 mm (designated medium race or MR), > 35 mm (designated big race or BR).

Morphometrical study of eggs and adults

The fully differentiate eggs that recovered from uterus of the three size-races of gravid adult worms were smeared in 0.85% sodium chloride solution, and scrutinized under compound microscope, and the egg length and width were assessed using calibrated ocular micrometer.

The gravid adults of three size-races were fixed overnight in AFA solution, and stained with Semichon's acid carmine (Garcia and Ash, 1979). The slides were destained with 1% HCl in 70% ethyl alcohol, dehydrated in serial ethanol, cleared in methyl benzoate, and mounted in canada balsam. The stained adult worms were examined under dissecting microscope and dimensions of the body were assessed using a calibrated ocular micrometer.

SEM study

The gravid adult worms of three size-races were fixed overnight in 2.5% glutaraldehyde in phosphate buffer (PB) pH 7.4 at 4ºC, washed with PB (10 minutes, 2 changes), then post fixed (1 hour) in 1% osmium tetroxide at room temperature. Dehydration was by passage through a long ethanol series, *ie*, 35%, 50%, 70%, 80% (10 minutes), 95% (15 minutes, 2 changes) and followed by absolute ethanol (10 minutes, 2 changes). The adult worms were finally dried by the critical point dryer, mounted on stubs, sputter coated with gold, and examined at 42 KV in a Jeol Med JSM 840-A SEM.

Karyotype study

The testes of gravid adult worm of three size-races were excised in HBSS using dissecting blade, and immediately transferred to 0.5 ml of 1% colchicine in HBSS in test tube. After 30 minutes of incubation at 37ºC, the excised testis was transferred to 0.5 ml of 1% sodium citrate solution for 10 minutes. Fixation of the testis was then carried out in a fixative consisting of equal parts of 45% acetic acid and 95% ethanol for at least two minutes. Then it was transferred carefully ground in a glass homogenizer in 0.5 ml of 60% acetic acid. The suspension was dropped, using a Pasteur pipette, onto fat-free slides on a warming plate at about 45-50ºC. The dried slides were stained with 10% Giemsa in phosphate buffer at pH 7.0 for 30 minutes, rinsed with deionized water, air-dried at room temperature and mounted in Permount (Fisher). Slides were examined under a compound microscope using an oil immersion objective.

RESULTS

Morphometric measurement of eggs and karyotype study

Details of morphometric measurements of eggs and karyotype studies of the three size-races *F. gigantica* are demonstrated in Table 1.

For morphometric measurements of eggs, statistical analysis of the egg length and width among the three size-races using Kruskal-Wallis test manifested intraspecifically significant differences in all tests without related to the adult size-races, *ie*; egg length 155.61±10.48 μm (SR) > 150.91±8.03 μm (BR) > 150.53±9.11 μm (MR) ($H=149.62$, $p < 0.05$); egg width 86.27±4.64 μm (SR) > 87.33±4.33 μm (MR) > 87.98±5.16 μm (BR) ($H=56.19$, $p < 0.05$).

For mitotic and meiotic karyotypes studies of testicular tissues, the results of the studies revealed that the three size-races of *F. gigantica* (Thailand strain) had diploid chromosome numbers of 2n=20 with 10 bivalents of primary spermatocyte in diplotene to diakinesis. Observations on spermatogenesis revealed that all three size-races produced numerous, normal spermatozoa (Fig 1).

Morphometric measurement of adults

Details of morphometric measurements of the three size-race, gravid adults are shown in Table 2. Statistical analysis of body dimensions at various sites using Kruskal-Wallis test for all tests, and F-test for only body width at ventral sucker and anterior testis demonstrated that most of the cases, body dimensions of SR were significantly and gradu-

Table 1
Morphometric measurements of uterus eggs and metaphase karyotypes of three size-races of gravid *F. gigantica* obtained from cattle at slaughter houses, Pathum Thani and Nonthaburi provinces.

Experiments	Size-race[a]		
	SR	MR	BR
Measurements (μm)	X±SD (range)	X±SD (range)	X±SD (range)
Egg length	155.61±10.48 (125.00 - 188.80)	150.53±9.11 (125.00 - 181.20)	150.91±8.03 (122.50 - 175.00)
Egg width	86.27±4.64 (72.50 - 102.50)	87.33±4.33 (75.00 - 100.00)	87.98±5.16 (72.50 - 107.50)
Metaphase chromosomes			
2n = 20	30	30	30
3n = 30	-	-	-
Mixoploid	-	-	-
Sperms presented in testis	30	30	30

[a]Thirty samples for each size-race.

ally smaller than MR and BR, *ie*; body length 21.60±2.04 mm (SR), 30.77±3.33 mm (MR), 39.68±3.17 mm (BR) (H=84.83, $p < 0.05$); cephalic cone height 2.15±0.35 mm (SR), 3.09±0.70 mm (MR), 3.54±0.62 mm (BR) (H=54.53, $p < 0.05$); head to ventral sucker 2.79±0.28 mm (SR), 3.77±0.81 mm (MR), 4.20±0.74 mm (BR) (H=46.81, $p < 0.05$); head to anterior testis 6.61±0.64 mm (SR), 8.75±1.39 mm (MR), 9.91±1.43 mm (BR) (H=59.19, $p < 0.05$); head to posterior testis 16.08±1.65 mm (SR), 22.32±2.78 mm (MR), 26.27±3.13 mm (BR) (H=71.14, $p < 0.05$); diameter of ventral sucker 1,055.50±103.81 μm (SR), 1,418.20±302.97 μm (MR), 1,545.90±252.83 μm (BR) (H=48.67, $p < 0.05$). Intraspecific variations with respect to the reversion and/or patternless of the three size-races were cephalic cone base 2.79±0.31 mm (SR) < [3.84±0.75 mm (MR) = 3.99±0.66 mm (BR)] (H=45.13, $p < 0.05$); body width at ventral sucker 5.77±1.22 mm (MR) > [5.10±0.88 mm (SR) = 5.23±1.04 mm (BR)] (F=2.97, $p < 0.05$); body width at anterior testis 7.94±1.26 mm (SR) < [8.85±1.52 mm (BR) = 9.45±2.02 mm (MR)] (F=6.42, $p < 0.05$); body width at posterior testis 6.31±1.14 mm (SR) < [8.11±1.93 mm (MR) = 8.85±1.96 mm (BR)] (H=29.99, $p<0.05$); body width at widest point 8.45(1.33 mm (SR) < [9.88±1.87 mm (BR) = 10.27±2.35 mm (MR)] (H=16.43, $p < 0.05$); testis width 4.95±0.86 mm (SR) < 6.10±1.30 mm (BR) < 6.57±1.64 mm (MR), (H=21.34, $p < 0.05$); diameter of oral sucker 780.00±86.06 μm (BR) > [660.48±42.25 μm (SR) = 695.50±136.63 μm (MR)] (H=33.82, $p < 0.05$).

Surface topography of adults

The morphological features and surface topography of the three size-race adults *F. gigantica* were generally similar (Fig 2A-R), and no account of race-specific characteristics which could be used to differentiate the races under SEM. The adults were flat and leaf-like with a thick conic structure at the anterior end. The surface topography of the body was covered with numerous spines, and could be found throughout the entire area, except at the oral sucker, ventral sucker, and area anterior to ventral sucker or around the genital pore which very few or lacked for spines. Viewed throughout the body, the number, size and shape of spines varried according to the body regions, *ie*, dense, numerous number and small size at the anterior one-fourth, slightly dispersed and large size at the middle region; sparse, few number and muchly small size at the posterior one-fourth. Viewed ventrally, the oral sucker appeared as circular opening, surrounded by radially raised border of muscular rim (Fig 2A, B). At higher magnification (Fig 2C, D), both cluster and solitary of sensory papillae surrounded the oral sucker were clearly seen. It appeared as a small dome with a nipple-like protuberance. The spines on the anterior one-fourth were small size (base-width about 2 times height), serrated edge with approximately 7-10 sharp points (Fig 2E). Similar morphological

Table 2
Morphometric measurements of three size-races of gravid *F. gigantica* obtained from cattle at slaughter houses, Pathum Thani and Nonthaburi provinces.

Experiments	Size-races[a]		
	SR	MR	BR
Measurements (mm)	X±SD (range)	X±SD (range)	X±SD (range)
Body length	21.60 ± 2.04 (16.00 - 24.50)	30.77 ± 3.33 (25.00 - 35.00)	39.68 ± 3.17 (35.50 - 49.00)
Cephalic cone			
base	2.79 ± 0.31 (2.50 - 3.50)	3.84 ± 0.75 (2.50 - 5.00)	3.99 ± 0.66 (2.50 - 5.00)
height	2.15 ± 0.35 (1.50 - 3.00)	3.09 ± 0.70 (2.00 - 4.00)	3.54 ± 0.62 (2.50 - 4.50)
Body width at :			
ventral sucker	5.10 ± 0.88 (3.50 - 7.00)	5.77 ± 1.22 (3.00 - 7.50)	5.23 ± 1.04 (2.50 - 8.50)
anterior testis	7.94 ± 1.26 (6.00 - 11.00)	9.45 ± 2.02 (4.50 - 12.00)	8.85 ± 1.52 (5.50 - 12.00)
posterior testis	6.31 ± 1.14 (4.50 - 8.00)	8.11 ± 1.93 (4.50 - 10.50)	8.85 ± 1.96 (5.50 - 13.50)
widest point	8.45 ± 1.33 (6.50 - 11.50)	10.27 ± 2.35 (4.50 - 13.50)	9.88 ± 1.87 (6.00 - 14.00)
Testis width	4.95 ± 0.86 (4.00 - 7.00)	6.57 ± 1.64 (3.00 - 9.00)	6.10 ± 1.30 (4.00 - 9.00)
Head to :			
ventral sucker	2.79 ± 0.28 (2.50 - 3.50)	3.77 ± 0.81 (2.50 - 5.00)	4.20 ± 0.74 (2.50 - 5.50)
anterior testis	6.61 ± 0.64 (5.00 - 8.00)	8.75 ± 1.39 (6.00 - 11.00)	9.91 ± 1.43 (7.00 - 12.50)
posterior testis	16.08 ± 1.65 (12.50 - 19.50)	22.32 ± 2.78 (15.00 - 27.00)	26.27 ± 3.13 (20.00 - 35.00)
Diameter : (μm)			
oral sucker	660.48 ± 42.25 (572.00 - 728.00)	695.50 ± 136.63 (390.00 - 936.00)	780.00 ± 86.06 (624.00 - 1,066.00)
ventral sucker	1,055.50 ± 103.81 (806.00 - 1,274.00)	1,418.20 ± 302.97 (910.00 - 1,898.00)	1,545.90 ± 252.83 (1,040.00 - 2,028.00)

[a]Thirty samples for each size-race.

features were observed in ventral sucker, except the larger in size than oral sucker (Fig 2F, G). Spines on the middle region were large, elongate shape (height about 2 times of base-width), serrated edge with approximately 11-15 sharp points (Fig 2H, I). The tegument among spines appeared corrugated with transverse folds alternated with grooves. Observation througout the body tegument, both cluster and solitary of sensory papillae were occasionally seen. The cirrus was sausage-shaped with spinous dorsally, curved slightly ventrally, and fold markedly anteriorly (Fig 2J). At higher magnification (Fig 2K, L), the corrugation and/or pseudostriation of dorsal part of cirrus ornamented with variable size of sharp spines were clearly seen. It was rest up from its own socket, and approximately 1.50 x 2.50 μm to 6.33 x 50.00 μm in width and height. At posterior one-fourth region, the spines were much, smaller size without serrated edges (Fig 2M, N), and the markedly corrugated tegument with transverse folds alternated with grooves were obviously observed. Viewed dorsally similar surface topo-

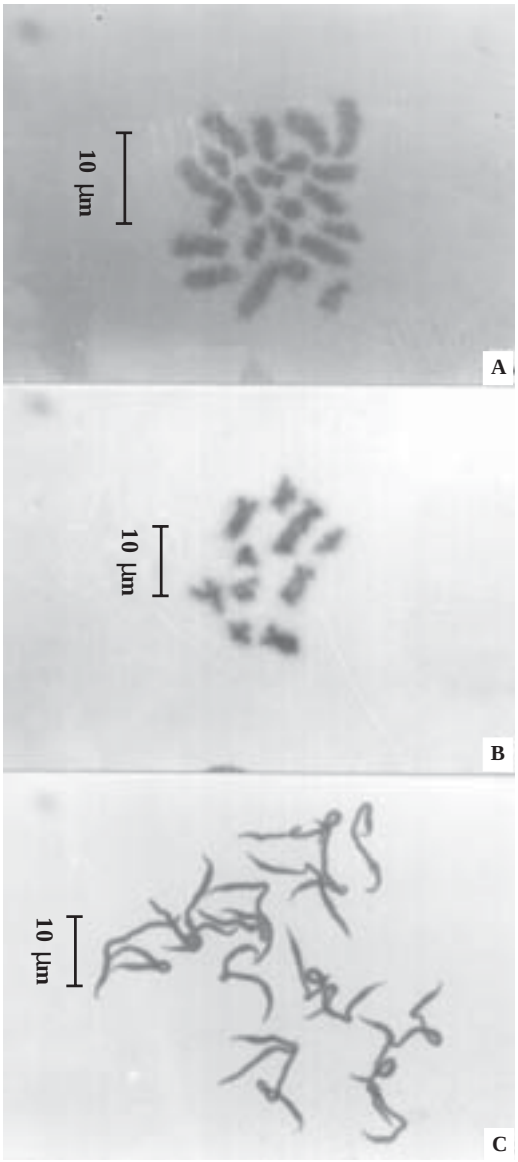

Fig 1–Mitotic metaphase with 20 chromosomes from spermatogonial cells of small race *F. gigantica* (A). Primary spermatocyte in diplotene to diakinesis with 10 bivalents of medium race (B). Numerous and normal spermatozoa found in the testis of big race (C).

graphy as ventral surface was observed, except the spines appears less in number and smaller in size than ventral spines (Fig 2D, P, Q, R). Additionally, the corrugation tegument with transverse folds alternated with grooves were obviously more than ventral surface.

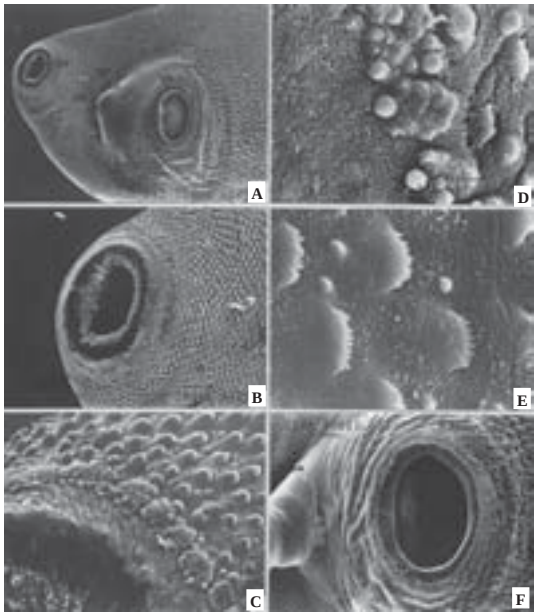

Fig 2–Surface topography of the three size-race adult *F. gigantica*. Note all of body surface topography characteristics of the three size-race adults were morphologically similar. [A] Ventral aspect of anterior part, showing oral sucker (OS), ventral sucker (VS), and genital pore (GP) with slightly protruded of cirrus (CR). [B] Oral sucker, showing circular opening surrounded by radially raised border of muscular rim. [C] Showing the cluster and solitary of sensory papillae (sp) around the oral sucker. [D] A higher magnification of sensory papillae, showing the small dome with nipple-like protuberance. [E] Spines at anterior one-fourth of ventral part, note base-width approximately 2 times of height with 7-10 sharp points, corrugated tegument with transverse folds (f) alternated with grooves (g). [F] Ventral sucker which was similar morphological features to oral sucker. Note very few or lack for spines on the area anterior to ventral sucker, or posterior to genital pore.

DISCUSSION

Biometry, karyotype and surface topography studies of various stages of helminthes to assess specific morphological, cytological and ultrastructural differences have been the efficient and reliable multi-disciplinary tools for differentiation varieties or cryptic species of mixed, natural population of helminthic parasites and/or other biological organism. For biometric studies; nematodes, *eg, Gnathostoma spinigerum* (Rojekittikhun and Pubampen, 1998), *Wuchereria bancrofti* (Jitpakdi *et*

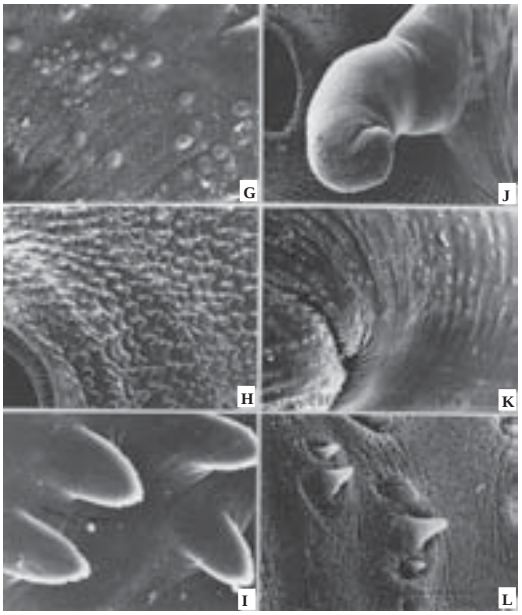

Fig 2–[G] Showing solitary and cluster of sensory papillae around the ventral sucker. [H] Spines on the area posterior to ventral sucker. [I] A higher magnification of spines at middle region of ventral surface, note height approximately 2 times of base-width with 11-15 sharp points. [J] The sausage-shaped cirrus, showing spinous dorsally, curved slightly ventrally, and fold markedly anteriorly. [K] A higher magnification of cirrus, showing pseudostriation of dorsal part ornamented with variable size of sharp spines, and lack for spines on ventral area. [L] A higher magnification of spines, showing variable size of spines rest up from its own socket.

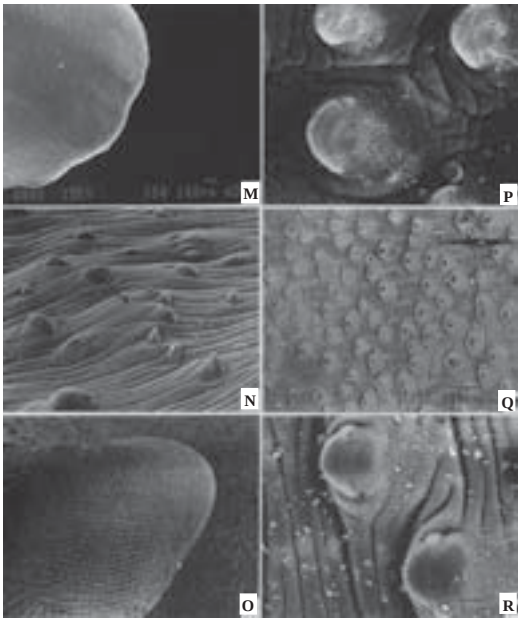

Fig 2–[M] Ventral aspect of posterior one-fourth, note few number of spines. [N] A higher magnification of posterior one-fourth area, showing much small spines without serrated edges (s), and markedly corrugated tegument with transverse folds alternated with grooved. [O] Dorsal surface of anterior part, showing the pattern of rings of spines. [P] A higher magnification of spines at anterior one-fourth of dorsal part, note base-width approximately 2 times of height which were similar to ventral surface-spines. [Q] Middle region of dorsal surface, showing disperse, smaller size of spines than ventral surface. [R] A higher magnification of spines at middle regions, note base-width approximately 2 times of height.

al, 1999); trematodes, *eg, Schistosoma haematobium* (Kechemir and Theron, 1997), *Fasciola hepatica* (Augot *et al*, 1998); cestodes, *eg, Taenia saginata* (Fan *et al*, 1992), *Echinococcus granulosus* (Lymbery, 1998). For karyotypic studies; nematodes, *eg, Dirofilaria immitis* (Sakaguchi *et al*, 1980), *Loa loa* (Post and Pinder, 1995); trematodes, *eg, Paragonemus westermani* (Terosaki, 1980), *Rubenstrema exasperatum* (Mutofova and Kanev, 1996); cestodes, *eg, Diphyllobothrium ditremum* (Petkeviciute, 1992), *Cyathocephalus truncatus* (Petkeviciute, 1996). For scanning electron microscopic studies (SEM); nematodes, *eg, Brugia malayi* (Choochote *et al*, 1987), *W. bancrofti* (Ratanatham *et al*, 1997); trematodes, *eg, F. hepatica* (Bennett, 1975), *F. gigantica* (Sobhon *et al*, 1994); cestodes, *eg, Echinococcus granulosus* (Warren *et al*, 1995), *Siluratemia siluri* (Scholz *et al*, 1999).

The search for varieties and/or sibling species in *F. gigantica* has been the subject of investigations for more than 2 decades, and extensive studies have been affirmed by several Japanese investigators. Based on karyotypic studies, *F. gigantica* (Japanese strain) could be classified into three cytological races, *ie*, triploid race with 3 sets of 10 basic chromosomes (3n=30, n=10), diploid race with 2 sets of 10 basic chromosomes (2n=20, n=10), and mixoploid and/or mosaic race which simultaneously having two kinds of cells showing the chromosome number of 3n=30 and 2n=20 in a single individual (Sakaguchi and Nakogawa, 1975; Sakaguchi and Yoneda, 1976; Moriyama *et al*, 1979). The proportion of appearances of three types of cells to total number of cells observed were 75.8-76.7% triploid, 16.7-17.1% diploid, and 6.2-7.5% mixoploid races, respectively (Moriyama *et al*, 1979; Sakaguchi, 1980).

Additionally, the chromosomes of both primary spermatocytes and oocytes from triploid and diploid races were completely asynaptic with either 30 or 20 univalents, consequently, spermatogenesis and oogenesis were entirely aberrant (Sakaguchi, 1977, 1980). No spermatozoa were found in the cytoplasm of oocytes at any stages of oogenesis, even in young eggs from the two races. Based on these finding evidences, the author proposed that the reproduction of Japanese *F. gigantica* was of parthenogenetic nature, and differed from other strains of ordinary *F. gigantica*. Furthermore, the comparative studies of nucleotide sequences of mitochondrial NADH dehydrogenase subunit 1 (ND) and cytochrome *c* oxidase subunit I genes between triploid and diploid *F. gigantica* revealed nearly identical and/or very low intraspecific variation, but were different from that of *F. hepatica* (Itagaki *et al*, 1998). Since the marked variation in adult dimensions of mixed, natural population of Thailand strain *F. gigantica*, thus, the comparative morphometry of eggs and adults, surface topography of adult under SEM, and testicular metaphase karyotypes of the provisionally classified races, *ie*, body length < 25 mm (designated small race), 25-35 mm (designated medium race), > 35 mm (designated big race) were carried out in the present studies to search for some intraspecific differences and/or variants among the three tentative races. The intraspecific variation of egg dimensions [egg length 155.61±10.48 μm (SR) > 150.91±8.03 μm (BR) >150.53±9.11 μm (MR); egg width 87.98±5.16 μm (BR) > 87.33±4.33 μm (MR) > 86.27±4.64 μm (SR)], and some adult structures and dimensions [body width at ventral sucker 5.77±1.22 mm (MR) > 5.23±1.04 mm (BR) > 5.10±0.88 mm (SR), body width at anterior testis 9.45±2.02 mm (MR) > 8.85±1.52 mm (BR) > 7.94±1.26 mm (SR), body width at widest point 10.27±2.35 mm (MR) > 9.88±1.87 mm (BR) > 8.45±1.33 mm (SR), testis width 6.57±1.64 mm (MR) > 6.10±1.30 mm (BR) > 4.95±0.86 mm (SR)] without related to the adult size-races, the morphological similarity of adult surface topography (oral sucker, ventral sucker, genital pore, cirrus, tegument, spine, sensory papilla) under SEM, the pure diploid type (2n=20) of mitotic chromosomes of spermatogonial cells with synaptic 10 bivalent of primary spermatocytes in diplotene to diakinesis, and normal spermatogenesis with numerous spermatozoa in all size races indicated that the variably morphological races of Thailand *F. gigantica* did not exhibit presence of a possible species complex. These appear to be the result of reaction norm (or plasticity), *ie*, similar genotypes are giving birth to several phenotypes (Schmalhausen, 1949). Similar results were also found in adult dimensions of *S. haematobium* (Kechemir and Theron, 1997), and *F. hepatica* eggs obtained from different types of host (Abrous *et al*, 1998). Nonetheless, the present results furnish impetus for further investigation of assortative mating (Paterson, 1980) among different size-races to elucidate the pre-mating barrier by using biochemical and molecular genetics. These investigations are in progress and will be published soon.

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