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Malacological Studies on *Parafossarulus manchouricus* (Gastropoda: Prosobranchia) in Korea

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<**國文要約**>

韓國產 왜우렁(*Parafossarulus manchouricus*)의 貝類學的 硏究

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왜우렁(*Parafossarulus manchouricus*)은 우리 나라와 中國大陸, 日本, 臺灣等地에 널리 蔓延되어 있는 肝吸虫症(clonorchiasis)의 原因寄生虫인 肝吸虫(*Clonorchis sinensis*)의 第一中間宿主로서 Bithyniidae科에 屬하는 淡水卷貝의 一種이다.

우리나라의 清平, 晋州, 群山 및 日本, 臺灣等地에서 採集된 왜우렁과 公州地域에서 採集된 *Bithynia(Gabbia) misella*, 그리고 美國의 Michigan湖와 獨逸의 Bodensee近域에서 採集된 *Bithynia tentaculata*를 對象으로 이들의 產卵特性, 形態, 細胞學的 特性, 吸虫類의 自然感染實態와 棲息地의 生態等을 相互比較觀察하여 주로 우리나라에 分布되어 있는 왜우렁의 貝類學的 根據를 마련하고저 本 硏究가 遂行되었다.

왜우렁의 培養에 있어 重要한 要素는 먹이로서 幼貝인 경우 *Navicula*나 *Gomphonema*와 같은 Benthic diatoms가 必須的임을 알았고 알에서 孵化하여 成貝가 될 때까지는 54日, 그리고 產卵할 때까지는 約 150日이 所要됨을 알았다. 왜우렁뿐만 아니라 같은 Bithyniidae科에 屬하는 *B.(G.) misella*나 *B. tentaculata*도 年 1回 產卵함을 보았다.

왜우렁의 貝殼에는 螺線形 隆起(spiral ridges)가 있음이 他種과 區別되는 點이였고 *B.(G.) misella*의 成貝의 크기는 7.5 mm를 초과하지 않았다. 왜우렁 幼貝의 走査電子顯微鏡的 觀察에서 螺線形 주름만이 보였을 뿐이여서 Hydrobiidae科에 屬하는 卷貝類와는 相異한 形態를 나타내었다. 왜우렁의 舌齒는 *B. tentaculata*와 비슷한 形態를 보였으나 *B.(G.) misella*는 cusps가 일반적으로 크고 날카로웠으며 比較된 三種 모두의 齒型은 2 : 1 : 1 : 1 : 2이었다. 또한 韓國產 왜우렁과 臺灣產 왜우렁에 있어 殼高對殼口比가 서로 統計學的으로 有意하게 相異한 것은 地域的環境의 差異때문인 것으로 思料되었다.

왜우렁의 細胞分裂相은 他種과 差異가 없었으나 染色體數는 *B. tentaculata*와 마찬가지로 n=17이었고 *B.(G.) misella*는 n=18이었다. 왜우렁의 核型은 地域間에 그 差異를 볼 수 없었으며 性染色體는 確認할 수 없었다.

왜우렁의 肝吸虫幼虫感染率은 晋州產 0.14%, 群山產 1.25%이었으나 清平產에서는 0%였으며, 公州產 *B.(G.) misella*亦是 自然感染을 認定할 수 없어 肝吸虫의 中間宿主가 될 수 없음을 確認하였다.

왜우렁의 棲息處는 水流가 緩慢하거나 停滯된 水系였으나 比較的 汚染이 적고 溶存酸素量이 높은 곳에 棲息하고 있었으며 棲息處의 calcium ion量이 他地域보다 越等히 높았음을 알 수 있었다.

INTRODUCTION

The Bithyniidae are a family of freshwater prosobranch snails with a wide distribution. They are found sporadically throughout much of the world, including Eurasia, Africa and Australia. Their presence in the Western Hemisphere —in the North American Great Lakes—St. Lawrence region— is generally believed to be due to introductions by human agency. By malacological standards, the family is not a large one, in that any one geographical region (except Thailand) usually has only one or two species.

The Bithyniidae often have been considered to be an independent family, but just as frequently they have been placed with the Hydrobiidae, usually as a subfamily. Arguments may still be forthcoming to follow the latter placement. However, Taylor (1966) has convincingly argued that the bithyniids should be separated from the hydrobiids and transferred to the Viviparoidae (Ampullarioidea), and this procedure has been followed most recently by Burch (1982) in his revision of the North American freshwater gastropods. Ampullariod characters of bithyniids are their size (adult shells are more than 10mm long), calcareous operculum with paucispiral nucleus and concentric edges, nuchal lobes of the head-foot, relatively long, flexible and acute tentacles, yellow and orange skin pigment granules, spirally constructed fecal pellets, use of the ctenidium in food gathering, pallial inne vation of the penis, and dimorphic sperm (Taylor, 1966).

In Korea, three species of bithyniid snails have been recognized: *Parafossarulus manchouricus* (Bourguignat 1860), *Bithynia (Gabbia) kiusiuensis* (Hirase 1927) and *B. (G). misella* Gr-dler 1884. However, the generic-group names to which the species have been assigned have differed and the taxonomic hierarchial rank of

Table 1. Nomenclature used for Bithyniidae found in Korea

Name	References
Parafossarulus manchouricus	(Author)
Parafossarulus striatulus japonicus	Walker (1927)
Bithynia (Hydrobioides(section Parafossarulus))	Thiele (1929)
Bulimus [=Bithynia] (Parafossarulus)	Wenz (1938-44)
Parafossarulus manchouricus	Abbott (1948), Habe (1978), Kwon and Habe (1979)
Bithynia (Parafossarulus) manchouricus	Pace (1973)
Bithynia (Gabbia) misella	(Author)
Bithynia (Gabbia)	Thiele (1929)
Bulimus [=Bithynia] (Gabbia)	Wenz (1939)
Bulimus misellus	Abbott (1948)
Bithynia kiusiuensis	Hirase and Taki (1951), Habe (1977), Habe (1978)
Bithynia (Bithynia) misella	Pace (1973)
Bulimus kiusiuensis	Soh (1978)
Gabbia misella	Kwon and Habe (1979)

the various generic group names have not always been the same (Table 1). Also the distinction between *B. (G.) misella* and *B. (G.) kiusiuensis* has been questioned, with the two names sometimes considered to be synonyms.

The most medically important snail species of Korea is *Parafossarulus manchouricus*, a member of the freshwater prosobranch family Bithyniidae. The human parasite that this snail transmits is *Clonorchis sinensis*, the "Chinese liver fluke". This trematode worm is one of the main snail-borne parasites of the Orient, where it infects hundreds of thousands of people in China, Japan, Korea, Vietnam and presumably parts of India. *Parafossarulus manchouricus* is the main species, or perhaps the only species, of the generic group *Parafossarulus*. The distribution of this snail, as far as know, is the same as the human parasites it transmits. A second bithyniid species occurs in Korea, *Bithynia (Gabbia) misella*. This species has also been suggested as an intermediate host of *C. sinensis* (Soh, 1978). Critical information on *P. manchouricus* and *B. (Gabbia) misella*, as well as the whole family to which they belong, is very limited. There is no agreement on the number of species or genera in the family. Taxonomic opinions on the genera vary widely, and no one has made an attempt to catalogue or to evaluate the species. The dirth of information on these snails is indoubtedly partly responsible for the lack of control and eradication of clonorchiasis.

Clonorchiasis is the most serious and prevalent snail-borne disease in Korea. The disease occurs in nearly all provinces in South Korea, but it is especially prevalent in the southernmost provinces, where an estimated 20% of the people are infected. In spite of this, critical studies on Korean Bithyniidae are almost nonexistant. Therefore, this study was initiated to investigate aspects of the biology of the Korean Bithyniidae, *P. manchouricus* and *B. (Gabbia) misella*, and to compare Korean populations of these species with populations from other Far Eastern countries. Also, it was felt that a comparison of these two Korean species of Bithyniidae to *Bithynia tentaculata*, the most widely distributed and nomenclaturally important species of the family, was especially pertinent.

Prior to the initiation of present studies, bithyniid snails had never been cultured in the laboratory. However, independent of my own successful culture of bithyniid snails, Kruatrachue et al. (1982) cultured several Thai species, but with somewhat different techniques than I used. Previous observations on external morphology of Bithyniidae are those of Abbott (1942) on *Gabbia misella* of Japan, and Baker (1928) and Taylor (1966) on North American *B. tentaculata*. Chromosome numbers, all n=17, had been reported previously for four species of Bithyniidae, *B. tentaculata*, *B. leachi*, *B. usseriensis* and *Mysorella costigera*, but no karyotype studies had ever been published. Also, few information was available on parasites infesting Bithyniidae, other than *C. sinensis*.

The bithyniid snails including *P. manchouricus* were studied in regard to their culture, spawning and growth, external morphology and radula, karyology, parasites, and habitat ecology. The reasons for studying these aspects in particular are as follows. (1) Successful laboratory culture of the snails was necessary in order to have enough snails to pursue these studies. An outgrowth of the culture studies was the observations on spawning and growth. (2) Morphological characters are important for recognizing and separating species and for taxonomic analyses. (3) At the microscopic level, and in regard to genetics, chromosome analyses are important attributes of organisms, and these characteristics have been used exten-

Table 2. Bithyniid snail populations collected in Korea and various countries

Species	Localities collected	Date collected	Habitat
Bithynia tentaculata			
1. Michigan	Hamlin Lake, Ludington, Michigan, U.S.A.	August 18, 1981	Lake
2. German strain	Bodensee, Tübingen, Germany	Sept. 15, 1981	Lake
Parafossaulus manchouricus			
1. Chongpyung strain	Chongpyung, Korea	Sept. 5, 1983	Fish farm
2. Chinju strain	Chinju, Korea	August 10, 1983	Pond
3. Kunsan strain	Kunsan, Korea	July 15, 1983	Ditch
4. Japanese strain	Kurume, Japan	October 20, 1981	(not recorded)
5. Taiwan strain	Taipei, Taiwan	December 18, 1981	(not recorded)
Bithynia (Gabbia) misella	Gongju, Korea	August 20, 1983	Pond

sively by systematicists for assessing biological and taxonomic relationships. The use of karyotypes especially has had a long history in animal and plant systematics. (4) A trematode, *Clonorchis sinensis,* known to be carried by one of the bithyniid species, *P. manchouricus,* is a serious human parasite. (5) Ecological studies on the habitats of *P. manchouricus,* are basic to know biological aspects and to establish control measures of the snails.

MATERIALS AND METHODS

A) Sources of Snails

Three local strains of *Parafossarulus manchouricus* were collected at Chongpyung, Chinju and Kunsan, Korea. Other populations of *P. manchouricus* came from Japan and Taiwan. *Bithynia (Gabbia) misella* was collected at Gongju, Korea, and *Bithynia tentaculata* came from Michigan and Germany. The foreign snails were mailed in wet condition as soon as possible. Additional details about the snails are given in Table 2.

B) Culture Methods

Snails: Two local strains (Chongpyung and Kunsan, Korea) of *P. manchouricus,* one local strain (Gongju, Korea) of *B. (G.) misella,* and two geographical strains (U.S.A and German) of *B. tentaculata* were used mainly in the culturing studies, although other strains of bithyniids were also raised.

Containers

The snails were maintained in conventional aquaria. These were glass aquariua (20 x 25 x 35 cm) which held 7ℓ of water. A general view of the arrangement of the snail aquaria on the sheving unit is shown in Fig. 1.

Glass finger bowls and small plastic trays containing about 350 ml of water were used for the cultivation of egg masses and newly hatched snails.

Unbreakable commercial green plastic trays (7.5 x 20 x 30 cm) designed for plant seedlings were sometimes employed as aquaria, especially for the cultivation of medium-sized growing snails, since the trays were easy to handle and reasonable in price. Each tray can contain 1,500 ml of water.

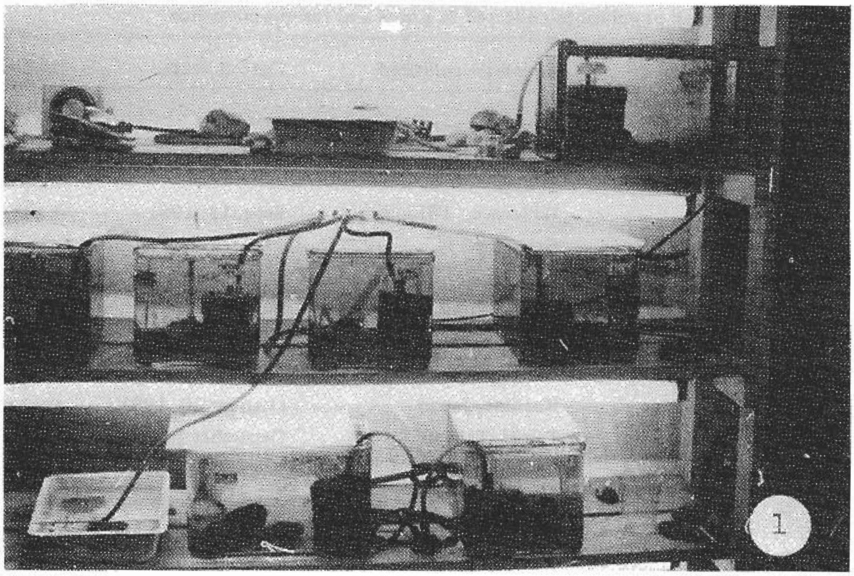

Fig. 1. General view of a shelving unit. The glass aquaria and the large and small green trays are beneath a "cool", white 15-Watt fluorescent light. A rubber tube runs to each container to provide aeration.

Environmental Parameters

1. Water. Initially, tap water was conditioned before use simply by adding 4-5 drops of 5% sodium thiosulfate solution to each aquarium for dechlorination.

This water was aerated for one week prior to use, and the pH of the conditioned water was 6.5-7.0.

2. Light. The aquaria were placed on shelves in a shelving unit (Fig. 2) and exposed alternately to 12 hr of artificial light (provided by a 15-Watt cool, white fluorescent tube) and 12 hr of darkness. An automatic timer controlled the cycling of the light.

3. Aeration. The water of all the glass aquaria, the finger bowls and the green plastic trays for the baby snails was continuously aerated by bubbling air from the aerators. The aerators in the glass aquaria consisted of a plastic container containing grains of charcoal covered with limestone and topped with cotton or glasswool.

4. Temperature. The temperature of the aquarium room was maintained by air-conditioning units during the summer and by controlled central heating during the winter. The water temperature for the aquaira ranged from 25°C to 27°C.

5. Food. Three kinds of food were used for snails reared and maintained in the laboratory: natural periphyton, powdered green leaves (commercial Ceralife®) and commercially prepared fishfood flakes (Tetra SML®, Tetra Co., D452 Melle, West Germany). Tetra SML flakes were supplied to the adult snails twice a week. Daily supply of food was avoided because an excess of food in the water caused a fungus bloom on the bottom of the aquarium. Such a bloom made conditions in the containers unfavorable. Ingredients and composition of Tetra SML are shown in Table 3.

Ceralife together with periphyton was used in rearing young snails until they became adults. The Ceralife powder was used by the snails

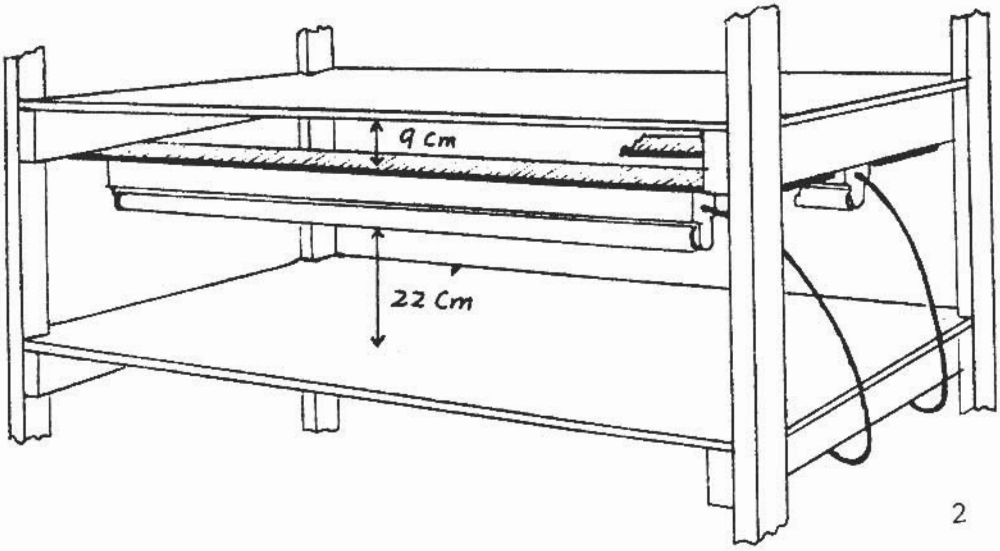

Fig. 2. Shelving unit for culturing bithyniid snails

Table 3. Ingredients and composition of "Tetra SML" large flakes for tropical fish*

Ingredients		Composition (as dry matter)
Fish meal	Plankton	Crude protein 46%
Oat gruel	Cyclops	Crude fat 5%
Roe	Agar agar	Crude fiber 8%
Shrimp meat	Wheatgerm oil	
Salmon eggs	Cod-liver oil	
Fishliver meal	Carotene	
Squid	Fishbone lime	

*Tetra Co., D 452 Melle, West Germany

to make food "sausages" prior to ingestion.

Small stones covered with natural periphyton were collected. The slimy mucilage film discharged by diatoms on the stones was a good natural food source, especially for growing baby snails. Permanent slides of diatoms were prepared for taxonomic identification. The diatoms included mainly *Navicula* spp. and *Gomphonema* spp., and other as yet unidentified taxa. The stones were placed in the finger bowls, in the green plastic trays and glass aquaria. The stones were replaced every two weeks.

6. Aquarium environment. The periphyton-covered stones served not only as food but also as substrate for the snails. No mud was added as a substrate. Long established aquaria usually became filled with filamentous green algae (mainly *Rhizoclonium hieroglyphicum*, but also *Oedogonium* sp., *Ulothrix* sp. and *Closterium* sp.). These algae originated from the field-collected snial shells as well as from the stones. Sometimes aquatic plants such as *Elodea*, *Myriophyllum* and *Sagittaria* were placed in the aquaria in order to stabilize the aquarium environment. No definite relationship between the presence of particular plants and the survivorship of

snails were observed in the present study. However, death of snails in aquaria with dense filamentous green algae was occasionally observed.

Snail Maintenance

The number of snails per tank did not exceed 20 for the larger snails (7-10 mm shell length) or 50 for the 3-7 mm shell size class. As many as several hundred newly hatched snails were maintained in each 7ℓ aquarium.

After the snails had laid eggs in the aquaria, egg masses were scraped from the aquaria walls with a razor blade and placed into 350-ml glass finger bowls or small plastic trays with conditioned water. The water in the containers was supplied with continuous aeration until the baby snails hatched. The egg masses were maintained under constant fluorescent illumination at temperatures between 25°C and 27°C. The eggs of *P. manchouricus* and *B. tentaculata* hatched approximately two weeks after being laid and the eggs of *B. (G.) misella* in about three weeks (Table 7). The newly hatched snails were then transferred with a Pasteur pipette to plastic culturing trays. The young were fed on powdered Ceralife (powdered green leaves) and periphyton. During cultivation of the young snails, the lengths and widths of their shells were measured every three days with a dissecting microscope equipped with an ocular micrometer.

C. Egg-laying Characteristics

Egg mass data were obtained by observing four laboratory populations: two strains of *P. manchouricus*, *B. (G.) misella* and German strain of *B. tentaculata*. In each of the tanks of *P. manchouricus* (Chongpyung and Kunsan strains) and *B. (G.) misella*, there were 20 snails; 10 females and 10 males. In the German strain of *B. tentaculata*, there were 15 snails; 10 females and 5 males. The egg masses were counted every third day.

For *P. manchouricus* (Kunsan strain), *B. (G.) misella* and *B. tentaculata* (Michigan strain), egg masses were compared as to number eggs per egg mass, size of eggs and incubation period. For the latter, the egg masses were transferred to finger bowls and observed every day.

D. Morphological Studies

Morphological studies included shells, opercula, radulae and egg masses. Observations were made on shell shape, size ratios of various parts, characteristics of whorls, sutures, peritreme, columella and surface sculpture (including microsculpture). These characteristics were observed with the use of a stereoscopic microscope, and in some cases with a compound light microscope and with a scanning electron microscope.

In order to gather additional information on the external morphologies of the bithyniid snails used in this study, the shell and aperture lengths of the snails were measured with a dial caliper and expressed in terms of the relative spire height, which is defined as the ratio of the shell over aperture length of the adult shells (Vermeij, 1973). The relative spire height values were calculated by the formula

$$H_{sp} = \frac{1_{sh}}{1_a}$$

where H_{sp}=relative spire height, 1_{sh}=the length of the shell, and 1_a=length of the aperture. Morphological differences (relative spire height values) between pairs of populations were tested for statistical significance by use of the Student's *t* test.

Measures of the intensity of external shell sculpture were expressed by assigning integral consecutive ranks, starting with Rank 1 as the smoothest specimen in the series being compared

(Vermeij, 1973) (Table 6).

The radulae were prepared for scanning electron microscopy (SEM) by macerating fresh buccal masses in a 10% potassium hydroxide (KOH) solution for 24 hours at room temperature and then removing the radulae from the disintegrating muscle tissues and adherent membranes of the buccal mass. Each radula was then placed in a vial containing 70% ethanol and cleansed in an ultrasonic cleaner for 5-15 sec. Each radula was then placed on double sticky tape on a SEM stub. A drop of double distilled water was first applied on the tape, and the radula was transferred to the stub with a pipette. Under the dissecting microscope, the radula was spread with the aid of microforceps and fine brush. When the radula was in the proper position on the stub, excess water was removed with a brush or capillary tube. After fixing the radula, it was washed first with 70% ethanol and then with water in order to spread the radula on the tape. The completely dried radulae were stored in a dust-free container until the gold coating was applied. The gold-coated radulae were observed with a JEOL JSM-U3 scanning electron microscope. All SEM pictures were taken using Polaroid film (Type 55/positive-negative).

A juvenile shell and operculum were selected for SEM study with the same procedure as used for the radulae. A juvenile was used because its sculpture would be less affected than older snails by abrasion and erosion.

E. Chromosome Cytology

The snail specimens used in this study were *B. tentaculata* (Michigan and German strains), *P. manchouricus* (Chongpyung strain) and *B. (G.) mesella*. Since cell divisions occur more frequently in reproductive tissue, and meiosis occurs there as well, the organs used for chromosome studies were the gonads. The cytological preparations included treatment with a spindle inhibitor, tissue fixation and acetic-orcein squash procedures for temporary and permanent mounts. The methods followed were those of Patterson and Burch (1978), with some minor modifications.

The shell of each snail was gently crushed with a scalpel or with a laboratory rubber hose clamp so as not to damage the vital organs. The pieces of shell were removed with forceps. Ten to fifteen animals were then pooled together in a square watch glass, a small Petri dish or a small glass vial containing an adequate amount of conditioned water.

The conditioned water was soon removed with a syringe or a Pasteur pipette, and the residue removed by absorbent paper. A known volume (15 ml in this study) of 0.00025% Velban® (vinblastine sulfate, Eli Lilly and Co., Indianapolis, Indiana) in an isotonic solution of 0.04% NaCl was added to the container. The snails were left in this solution for 3 hrs at room temperature. Velban was used as an inhibitor of spindle formation in the dividing cells. The solution to which the snails were exposed was prepared from a stock solution of 1 mg/ml of vinblastine sulfate dissolved in bacteriostatic sodium chloride injection solution. This stock solution could be kept in a refrigerator at 2-8°C for 30 days without losing its potency (Kitikoon, 1982). Then, half of the total volume of the solution was withdrawn using a syringe and replaced with the same amount of 0.02% NaCl solution. The snails were kept in this solution for 30 min before it was withdrawn. The entire fluid was completely withdrawn again, and a known volume (15 ml in this study) of ¼ dilution of 0.00025% Velban (0.0000675%) in 0.02% NaCl solution was added. The snails were kept in this hypotonic solution for 30 min at room temperature. The hypotonic solution helped to keep the chromosomes loose and facilitated their spread and separation when the squash procedure was performed.

All the crushed snails treated with the spindle inhibitor were alive and in apparent good condition before being fixed. The snails were fixed by withdrawing the hypotonic solution from the snail containers and immediately replacing it with Carnoy's fixative (glacial acetic acid, chloroform and 95% ethanol, 1:3:6 parts by volume) or modified Carnoy's fixative (glacial acetic acid and 95% ethanol, 1:3 parts by volume). In case of female snails, they were treated with Carnoy's fixative first for 15 min and then with modified Carnoy's fixative. This procedure was performed in order to get rid of lipids in the ovary by the chloroform in the Carnoy's fluid. The amount of fixative solution was at least 10 times greater in volume than the tissue. Although the fixing time varied according to the size and condition of the tissue, the snails used in this study were usually stained approximately an hour after fixation. The fixative in which the snails were to be stored for future use was changed after 24 hrs and kept in the refrigerator at 2° to 10°C.

Acetic-orcein was used for staining the chromosomes. The staining solution was made by placing 2.2 gm of Certified Synthetic Orcein (Matheson Coleman and Bell, Narwood, Ohio) in 100 ml glacial acetic acid and boiling gently for 10 min with constant stirring or with the addition of glass beads. In order to dissolve the orcein completely, 2.2 gm of orcein was first dissolved in 20 to 30 ml of 95% ethanol or methanol before adding 100 ml of glacial acetic acid. Methanol (or ethanol) evaporated during boiling. This stain gave excellent results. Following boiling, the mixture was cooled and filtered through glass wool to remove undissolved stain.

Chromosome preparations were made using the acetic-orcein squash technique. A small piece of gonadal tissue was removed under the dissecting microscope using two fine dissecting forceps. During removal of reproductive tissues, the snails were pinned to the paraffin in containers containing Carnoy's fixative, so as to keep tissues always wet. The mantle or epidermis of the hepatopancreatic area, where the snail's gonad is located, was carefully peeled off so that the stain could penetrate more rapidly. A small piece of the gonadal tissue (testis or ovary) was placed in the well of a depression slide to which one or two drops of stain were added. Although proper staining usually requires 15-30 minutes, the tissue was allowed to stain for 1 or 2 hrs at room temperature, which always gave good results. The stained tissue was then removed to a clean slide and a few drops of 45% acetic acid were added to rinse off excess stain. The tissues were divided into about 6 pieces of equal size (ca. 1mm^3) using spear-head dissecting needles. After rinsing off excess stain with 45% acetic acid, the pieces of stained tissue were moved equidistantly apart, a drop of clear 45% acetic acid added and then covered with a coverslip (22 x 40mm). The slide was placed between absorbent papers of a bibulous paper packet and squashed with tumb pressure. Care was taken to prevent the coverslip from slipping on the slide and to prevent entrapment of air bubbles around the tissues. Finally, bees wax and vaseline were applied as a temporary sealant to the edges of the coverslip.

Some temporary preparations were made permanent as reference slides. In this procedure, coverslips were wiped with silicon eye-glass paper prior to making the squash. This helped keep tissue from adhering to the coverslip when it was removed for making permanent mounts. The temporary sealant was first removed with a razor blade, and the slide was frozen briefly on a block of dry ice. Then the coverslip was raised by gently prying under one end with a single-edged razor blade. After being separated, the slides and coverslips were dehydrated in 95% and 100% ethanol and mounted with Euparal®

Observations were made with Nikon (Nippon Kogaku) compound microscopes using 100x (n.a. 1.25) oil immersion objectives and 10x and 30x oculars. The chromosomes were drawn with the aid of a camera lucida and reproduced at a table top magnification of about 5,000x. Photographs were taken using a 10x ocular, oil immersion objective, a Kodak 57A green filter and Kodak Technical Pan Film.

Karyotypes of the chromosomes were prepared from camera lucida drawings and photographs. The camera lucida drawings were especially helpful in determining the exact position of centromeres of the mitotic metaphase chromosomes and in matching the pairs of homologues. A dial caliper was used in measuring chromosome arm lengths and total lengths. The chromosomes were divided into three groups according to the position of their centromeres. Chromosomes whose short arm was less than 1/20th of the total chromosome length (TCL) (or with an arm ratio > 19) were called "acrocentric" chromosomes; chromosomes with an arm ratio between 19 and 2 were called "submetacentric"; and chromosomes with an arm ratio between 2 and 1 were referred to as "metacentrics" (White, 1973). Chromosome lengths were standarized as a percentage of the total haploid complement length (% TCL) (Paris Conference, 1971). The chromosomes in each karyotype were arranged in descending order by size and according to the positions of their centromeres.

For the detection of so-called Q-banding patterns on the chromosomes, after smearing the reproductive tissues of each snail on a clean slide by the squashing technique (Patterson and Burch, 1978), the slides were fixed in 95% methanol or 50:50 alcohol-ether for 30 to 60 min, and were then air-dried. The dried slide preparations were immersed in MacIlvaines citric acid-phosphate buffer at pH 4.0-4.5 for 3 to 5 min and stained in 0.5% (w/v) freshly prepared aquaous solution (deionized water) of quinacrine mustard or quinacrine dihydrochloride ("Atebrin") for 5 min. They were then washed for 3 min in running tapwater or distilled water, rinsed in MacIlvaines buffer (pH 5.5) and mounted in buffer (pH 5.5) or, for more permanent preparations, in buffered glycerol. After sealing the cover glasses, fluorescent-microscopic observations were carried out.

The light source was an "HBO 200" mercury vapour lamp with a 1.5mm "BG 12" excitor filter and a 530 nm barrier filter. All observations were made using oil immersion objectives (Pearson *et al.*, 1970; Cheng *et al.*, 1978).

F. Natural Infections of Trematode Cercariae

Field-collected specimens of three populations of *P. manchouricus* (Chongpyung, Chinju and Kunsan strains) and one local population of *B. (G.) misella* (Gongju, Korea) were checked for natural infections of trematode cercariae.

Each animal was placed in a glass vial (15 x 17 mm) containing 2 ml of aerated tap water, kept under a fluorescent light for several hours and the vial examined for the presence of shed cercariae. Later, the snails were crushed for further inspection for cercariae. In order to examine the ventral side of the cercariae, a drop of the solution containing the cercariae was placed on a cover slip. A slide glass was placed very slowly over the loaded cover slip until the two made contact. The glass slide then was turned right side up and the excess mounting fluid between the cover slip and the glass slide blotted by a strip of filter paper at the end of a cover slip. This preparation was sealed with vaseline around the cover slip to prevent evaporation of mounting fluid for the duration of observation.

G. Water Chemistry in the Habitats of the Snails

Stream temperatures were recorded at every visit and maximum summer water temperatures were measured with a maximum-minimum thermometer. Water samples were collected for physical and chemical analyses in late summer. Water samples were refrigerated and returned for analysis of 5-day biochemical oxygen demand (BOD), pH and heavy metal ions. Dissolved oxygen (DO) and BOD were analysed by Winkler's method described in Standard Methods for the Examination of Water and Waste Water, APHA, AWWA and WPCF, 1981.

Cadmium, lead and copper ions were quantitatively measured in 300 ml of water samples pre-treated with nitric acid by atomic absorption spectrometry, after extraction of heavy metals by DDTC-MIBK (Japanese manual for The Examination of Public Health Science, 1980). Zinc, manganese, calcium and magnesium ions were also detected in 300 ml of water samples pre-treated with nitric acid by direct method using the atomic absorption spectrometer (Standard Methods for the Examination of Water and Waste Water, 1981). Mercury ions were measured in 200 ml of water samples by cold vapor atomic absorption spectrometry (Standard Methods for the Examination of Water and Waste Water, 1981).

RESULTS

A. Egg-laying Characteristics and Snail Growth

Laboratory culture of bithyniid snila has been considered previously to be extremely difficult.

In this study, the various bithyniid species studied were cultured and maintained very successfully with the care they received, so more elaborate experiments were not performed. Egg-laying characteristics and snail growth were experimented.

The adult snails of the Chongpyung strain of *P. manchouricus* were collected in September, 1983, and cultured in an aquarium. The snails did not lay eggs for 6 months and started laying eggs in march, 1984. The snails then stopped laying eggs in May, 1984 (Table 4). Culture of the Kunsan strain of *P. manchouricus* was initiated in September, 1983, and although only one egg mass was found in October and November, The majority of their egg masses were found from March through April of the next year.

The snails of the Gongju strain of *B. (G.) misella* were placed in conventional aquaria in September 1983. The egg masses were found from March through April 1984, similar to that observed for *P. manchouricus*.

The German strain of *B. tentaculata* exhibited the same trend as the other bithyniids, i.e., they laid the majority of their eggs in March of the next year (Table 4).

Twenty-three baby snails hatched from an egg mass of *P. manchouricus* (Kunsan, Korea strain) in October, 1983. The width and height of the shells were measured every three days up to day 108 and then measured once a week up to day 192. The baby snails were transferred from the finger bowls to green plastic trays at day 24. Two trays were employed; one tray contained 10 young snails, the other 13. Data obtained are shown in Table 5. The shell length of newly hatched snails averaged about 0.9 mm. On day 156, they had reached an average shell length of 9.18 mm, and had laid 9 egg masses. The young snails grew very fast at the beginning, and attained almost full adult size within 54 days. A steady increase in shell sizes was observed until the snails began laying eggs.

Table 4. Number of egg masses laid by the Chongpyung and Kunsan strains of *Parafossarulus manchouricus*, the Gongju strain of *Bithynia (Gabbia) misella*, and the German strain of *Bithynia tentaculata*

		Number of egg masses			
		P. manchouricus		*B. (G.) missella*	*B. tantaculata*
Year	Month	Chongpyung St.*	Kunsan St.*	Gongju strain*	German strain**
1983	Sep.	–	–	–	–
	Oct.	–	1	–	–
	Nov.	–	1	–	–
	Dec.	–	–	–	–
1984	Jan.	–	–	–	–
	Feb.	–	–	–	–
	Mar.	8	15	9	38
	Apr.	18	19	10	–
	May	–	–	–	–
	Jun.	–	–	–	–
	Jul.	–	–	–	–

* The Chongpyung and Kunsan strains of *P. manchouricus* and the Gongju strain of *B. (G.) misella* had 10 males and 10 females in each aquarium.

** Fifteen (five males and 10 females) German snails of *B. tentaculata* were cultured in an aquarium.

The egg-laying of the first generation of *P. manchouricus* was from April through June, 1984, and then stopped. The second generation was cultured in the same manner as the first. The mortality of the young snails was much lower than the field-collected parent snails. During 10-months of cultivation, only one out of 23 young snails had died (4.3% mortality).

B. Comparative Morphology

1. **Shells**: All adult shells of all the specimens of *B. tentaculata* have about five whorls. These are sculptured with fine transverse growth lines. The apical whorls of the shells are usually present even in eroded shells. The aperture lip of the shell has a dark edge. The color of the rest of the adult shell is normally yellowish green. The maximum length of the shells of this species is about 11 mm (Table 6).

The shells of *P. manchouricus* are all characterized by having spiral keels and heavily eroded apexes, especially in old shells. The lip of the aperture is thickened and, in some individuals, dark brown. The color of adult shells is horn or amber. The maximum length of the shells was 10.8 to 11.7 mm (Table 6).

The shells of *B. (G.) misella* are much smaller than those of the other two species. None of the shells were over 7.12 mm in maximum length. The shells of this species are yellowish green (as in *B. tentaculata)*, have about 5 whorls, which are sculptured with fine transverse growth lines and sometimes with very fine spiral lines. The apex of the shell is usually not eroded. The aperture lips are not thickened (Table 6).

A three-day old shell of *P. manchouricus* studied with the scanning electron microscope exhibited sculpture of fine growth lines and spiral lines (Fig. 3). No specific sculpture in the embryonic whorl of *P. manchouricus* was ob-

Table 5. Growth of *Parafossarulus manchouricus* (Kunsan Strain) in the conventional aquaria system

Days after Hatching		Length in mm		Day after Hatching		Length in mm	
		Width ($\bar{x}$±S.D.)	Height ($\bar{x}$±S.D.)			Width ($\bar{x}$±S.D.)	Height ($\bar{x}$±S.D.)
Day	0	0.70±0.07	0.90±0.01	Day	69	5.12±0.25	9.14±0.56
Day	1	0.77±0.13	0.94±0.10	Day	72	5.14±0.23	9.10±0.59
Day	3	0.72±0.07	1.04±0.09	Day	75	5.16±0.21	9.11±0.67
Day	6	0.95±0.08	1.18±0.16	Day	78	5.17±0.25	9.10±0.59
Day	9	1.09±0.14	1.68±0.14	Day	81	5.19±0.26	9.13±0.58
Day	12	1.15±0.11	1.74±0.22	Day	84	5.18±0.22	9.13±0.56
Day	15	1.30±0.16	1.78±0.32	Day	87	5.18±0.26	9.10±0.59
Day	18	1.58±0.27	2.19±0.46	Day	90	5.16±0.25	9.11±0.58
Day	21	1.75±0.33	2.67±0.40	Day	93	5.21±0.23	9.10±0.58
Day**	24	2.19±0.24	3.18±0.42	Day	96	5.19±0.20	0.09±0.58
Day	27	2.39 ±0.28	3.53±0.40	Day	99	5.15±0.28	9.12±0.57
Day	30	2.77±0.33	4.25±0.48	Day	102	5.17±0.28	9.10±0.57
Day	33	3.27±0.40	5.00±0.55	Day	105	5.26±0.24	9.10±0.59
Day	36	3.87±0.45	5.90±0.66	Day	108	5.22±0.18	9.10±0.60
Day	39	4.29±0.33	6.73±0.56	Day	114	5.26±0.24	9.07±0.58
Day	42	4.57±0.39	7.28±0.78	Day	120	5.25±0.17	9.08±0.58
Day	45	4.82±0.27	8.10±0.67	Day	126	5.23±0.22	9.08±0.59
Day	48	5.04±0.37	9.67±0.74	Day	132	5.30±0.18	9.11±0.58
Day	51	5.18±0.35	8.99±0.62	Day	138	5.24±0.17	9.15±0.57
Day	54	5.18±0.24	9.10±0.57	Day	144	5.26±0.20	9.10±0.58
Day	57	5.06±0.27	9.15±0.59	Day	150	5.25±0.25	9.15±0.59
Day	60	5.12±0.32	9.18±0.58	Day***	156	5.24±0.20	9.18±0.57
Day	63	5.11±0.26	9.17±0.58	Day	162	5.26±0.26	9.17±0.56
Day	66	5.15±0.25	9.17±0.57	Day	192	5.32±0.20	9.18±0.60

* The shell length of 23 baby snails were measured every three days up to Day 108, and then measured once a week.

** Baby snails were transfered from the finger bowls to green plastic trays (7.5 x 10 x 30 cm).

*** Between Day 150 and Day 156 the snails laid nine egg masses (F_2).

served, except for slight spiral wrinkles on the surface. This scanning electron microscopic pattern of the embryonic shell of *P. manchouricus* is distinctly different from that of hydrobiid snails.

Table 6 presents data on relative spire height (RSH), and sculptural rank of the species of *Bithyniidae studied here.* All pairs of species and populations (see Table 6) were significantly different ($P<0.05$) with respect to relative spire height except for Korean versus Japanese population of *P. manchouricus* (although the sculptural rank of the Japanese population was higher than that of the Korean bithyniids).

On the other hand, the RSH values between the Korean (Chongpyung strain) and the Taiwanese populations of *P. manchouricus* were quite significantly different ($P<0.001$). Additionally, the Taiwanese population had the highest sculptural rank (4). It is obvious that there are morphological differences among the geographic populations of *P. manchouricus* in terms of relative spire height of the adult shells.

In the populations of *P. manchouricus*, Taiwanese snails revealed the greatest RSH value (2.09±0.10) and the highest sculptural rank (4) among those populations which had values that were significantly different from the Korean population. And, since the Taiwanese population has a greater spire height,

Table 6. Characteristics of the shells of bithyniid snails used in this study

Species	Localities collected	n	Aperture length range (mm)	Shell length range (mm)	H_{sp} Range	H_{sp} Mean±S.D.	SR
P. manchouricus							
Chongpyung strain	Chongpyung, Korea	20	4.76-5.52	9.39-10.91	1.91-2.07	1.99±0.04	2
Japanese strain	Kurume, Japan	16	4.27-5.25	9.10-10.80	1.92-2.25	2.06±0.08	3
Taiwanese strain	Taipei, Taiwan	20	4.45-5.76	9.61-11.60	1.85-2.23	2.09±0.10	4
B. tentaculata							
Michigan strain	Michigan, U.S.A.	20	4.38-5.14	8.74-11.06	1.96-2.24	2.08±0.08	1
B. (G.) misella							
Gongju strain	Gongju, Korea	17	2.77-3.42	5.41- 7.12	1.95-2.24	2.05±0.07	1

n=Number of specimens

H_{sp}=Relative spire height

SR (Sculpture rank); Rank 1=the smoothest species of the species being compared (with no spiral keels).
Rank 4=Shells with heavy spiral keels.

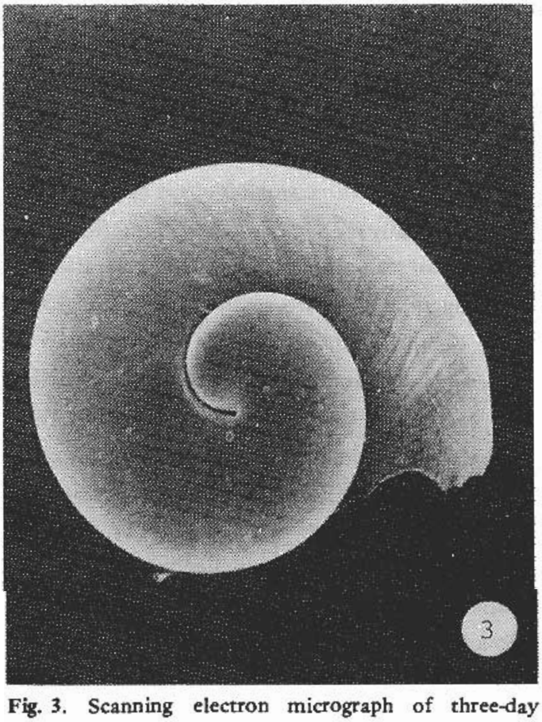

Fig. 3. Scanning electron micrograph of three-day old shell of *P. manchouricus* (Kunsan strain). Transverse lines begin forming near the end of the first whorl, and spiral lines soon after (x 70).

it therefore has a smaller apertural perimeter.

2. Opercula: The opercula in all of the bithyniid species used in this study are calcareous and were concentric with a paucispiral nucleus. The inner sides of the opercula are flat and lack sculpture. The operculum of a newly hatched (3-day old) *P. manchouricus* consists almost entirely of the paucispiral nucleus.

Its margin is thickened with calcareous material. Among individuals of the various species, there are variations in sizes of the opercula. But, in none of the species of bithyniid snails observed here can the opercula be withdrawn inside the shells' boby whorls (a character which differentiates the Bithyniidae from the Hydrobiidae). That is, the various opercula match exactly the sizes of the respective shell apertures (peritremes) into which they fit. *B. (G.) misella*, the smallest species I studied, had the smallest opercula.

3. **Radulae**: Scanning electron microscopy on the radulae of *B. tentaculata* (Michigan and

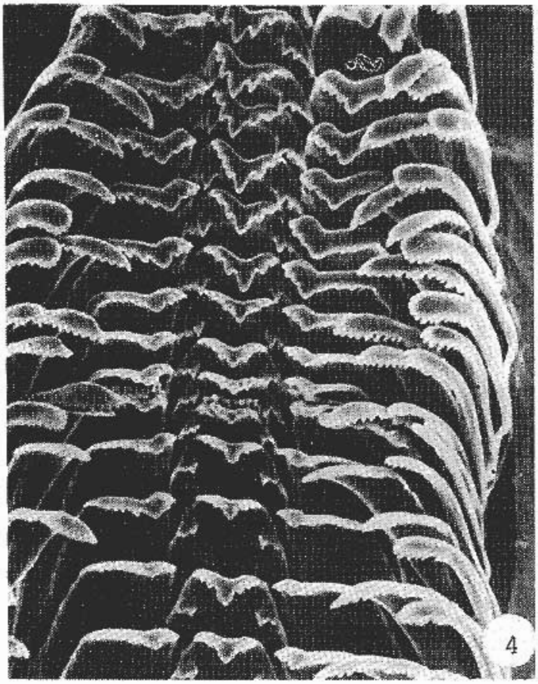

Fig. 4. Scanning electron micrograph of the radula of *P. manchouricus* (Chongpyung, Korea strain). The central teeth are multicuspid and have numerous basal denticles. The laterals are also multicuspid, and are hatchet-shaped. The marginal teeth consist of two teeth on each side, the inner and outer marginals. The marginals are slender and are also multicuspid. Radular formula=2:1:1:1:2. (X 200).

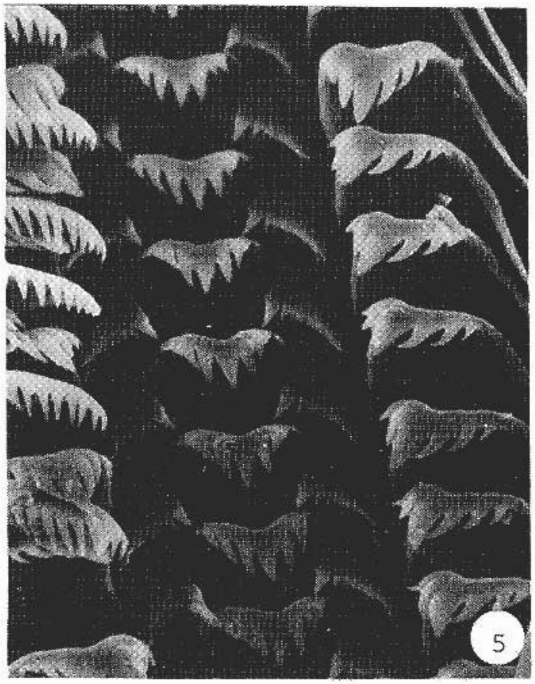

Fig. 5. SEM picture of the radula of *B. (G.) misella* (Gongju, Korea strain) (X 900).

German strains), *P. manchouricus* (Chongpyung, Korea and Taiwan strains) and *B. (G.) misella* (Gongju, Korea strain) was carried out to discern the fine morphology of the radulae to see if they had any taxonomically useful characters.

The radular formula of all the bithyniid snails studied is 2:1:1:1:2 (Figs. 4-5). The central teeth are all multicuspid and have numerous basal denticles. The lateral teeth are also multicuspid and, like the marginal teeth, lack basal denticles. The marginals consist of two teeth on each outer edge of the radula (an inner and an outer tooth). The marginal teeth are slender and are also multicuspid.

The central tooth of *P. manchouricus* (Chongpyung strain) is spade or arrowhead-shaped, with a single larger central cusp and two or (more often) three smaller cusps (progressively smaller distally) on each side. Sometimes one or more of the side cusps exhibits an anomaly by being split into two smaller cusps, and sometimes a small denticle occurs between two of the cusps. There are two to five basal denticles on each side of the central tooth. These denticles occur on a ridge near the lateral margins of the teeth. The more median denticle on each side is largest, with the other denticles becoming increasingly smaller distally. The smaller most distal basal cusps are often fused into a bumpy ridge.

The lateral teeth of *P. manchouricus* (Chongpyung, Korea) have a broad spade-or arrowhead-shaped mesocone, flanked by three or four shorter and narrower ectocones and three shorter and narrower entocones. On each side of the mesocone, the ectocones and entocones decrease progressively in size distally.

The inner marginal teeth of *P. manchouricus* (Chongpyung strain) have about 15 cusps, all nearly of equal size. Some of the more distal cusps have a tendency to fuse. The outer marginals are more slender than the inner marginals, and have fewer cusps (about nine) (Fig. 4).

Other populations of *P. manchouricus* and *B. tentaculata* showed very similar morphologies of Their radulae to the Chongpyung strain of *P. manchouricus*. In relative sizes, the radulae of *P. manchouricus* and *B. tentaculata* (Michigan strain) so not differ greatly from each other.

The central teeth of *B. (G.) misella* from Gongju, Korea differ from those of the above species in several respects, of which the most noticeable are (1) the central cusp (mesocone) is only slightly larger than the flanking cusps (ectocones), (2) there are usually four ectocones on each side of the mesocone, (3) the ridge bearing the basal denticles is more broadly flexed off of the lateral margins of the tooth and (4) the bases of the central teeth are relatively wider.

The lateral teeth of *B. (G.) misella* have three or four ectocones and three entocones. The mesocones in one of the specimens are long, spade-shaped or triangular, and are sharply pointed. In the other specimen the cutting edge of the mesocone is more rounded. The inner marginals of *B. (G.) misella* are also very similar, except that there are several more cusps, and there seems to be less tendency for some of the cusps to fuse (Fig. 5). I did not detect any noticeable differences in the outer marginals between the various species and populations.

4. Egg Masses: The eggs of the three species of bithyniid snails studied here were all laid in elongate masses with two rows per mass. The eggs were not surrounded by a common gelatinous matrix, but simply were struck to each other at their surfaces. Some masses of *P. manchouricus* appeared to have a gelatinous margin, but this did not seem to be a constant characteristic.

The egg masses of *P. manchouricus* contained 4-44 eggs, *B. tentaculata* 6-48 eggs and *B. (G.) misella* 5-23 eggs. The incubation period of the eggs of *B. (G.) misella* was approximately 3 week at room temperature (ca. 25°C), which was the longest of the species studied. The eggs of *B. tentaculata* were the largest in size (mean diameter=1.38mm), followed by *P. manchouricus* (mean diameter=1.29 mm), and those of *B. (G.) misella* were significantly smaller (mean diameter = 1.06 ± 0.04, P<0.001) (Table 7).

Table 7. Comparison of eggs laid in laboratory aquaria by three species of bithyniid snails

	P. manchouricus (Kunsan strain)	*B. (G.) misella* (Gongju strain)	*B. tentaculata* (Michigan strain)
Size (Max. axis in mm)	1.29mm±0.07	1.06mm±0.04*	1.38mm±0.50
Total number of eggs measured for determining size	30 eggs from 10 egg masses	50 eggs from 10 egg masses	20 eggs from 5 egg masses
Range of the number of eggs per egg mass	4-44	5-23	6-48
Incubation period (days) for hatching at room temperature	15-17	20-22	12-14

*Significantly smaller than other eggs (P<0.001).

C. Chromosome Cytology

1. **Chromosome Cycle** : The chromosome cycle was studied mainly from spermatogonial divisions of germ cells in *P. manchouricus* (Kunsan, Korea strain). Some observations were made as well of chromosomes during female gametogenesis. These same gonadal chromosome stages were observed also in the other bithyniid populations. No specific difference was observed in details of the chromosome cycle in the various species and populations.

The chromosomes of early mitotic prophase appear as rather fuzzy and diffuse stands with extremely irregular margins. The individual chromosomes could not be seen as separate distinct entities in this early mitotic stage (Fig. 6).

The mid-prophase mitotic chromosomes are shorter, more deeply stained, and have smoother margins than those of early prophase cells as a result of increased chromosome contraction. The area of the centromere appears as a lightly stained or non-stained portion of each strand and this area produces the primary construction of the chromosome. The chromosome numbers can easily be counted in this stage. Spermatogonial mid- or late prophase chromosomes are shown in Fig. 7.

Mitotic metaphase chromosomes are greatly contracted, deeply stained and have smooth margins. The centromeric regions, where the bi-polar spindle apparatus attaches, are well defined in this stage, and the chromatids of each chromosome are clearly joined together at the centromeric region. The secondary constructions were usually observed on the chromatids of a pair of the large chromosomes. The 34 spermatogonial metaphase chromosomes of this species range in length from 2.88 μm for the largest pair to 0.79 μm for the smallest pair (Fig. 8).

After the pre-meiotic division, the nucleus enlarges to form the first meiotic prophase nucleus. In the leptotene stage the chromosomes appear as maximally extended single strands, which are lightly stained with more darkly stained bead-like chromomeres along their length. The synapsis of homologous chromosomes begins in zygonema. The chromosomes begin to pair at the polarized ends, while the remainder of the strands are yet unpaired. Fig. 9 shows a late leptotene or zygotene nucleus in spermatogonial division.

After completing the synapsis of homologous strands, the pachytene chromosomes are easily distinguishable by their double thickness and reduced number (haploid). Pairing is chromomere-by-chromomere along the length of the homologous strands. Late pachytene chromosomes (bivalents) are much shorter than leptotene or zygotene chromosomes, due to the increased contraction of the strands. Two darkly stained nucleolar organizers can be seen during pachynema (Fig. 10). Mid to late diakinetic chromosomes are smaller, more deeply stained and have somewhat smoother margins (Fig. 11) due to the increased contraction, although the chromosomes in early diakinesis are still diffuse and have irregular margins. The chromosomes form ring, cross or multiple loop-shaped figures depending on number and position of chiasmata. Ring-shape bivalents with two chiasmata, cross-shaped bivalents with one non-terminal chiasma and multi-looped bivalents with four chiasmata can be seen in Fig. 11.

Metaphase II and Anaphase II dyads were not seen in this study. But, Telophase II monads were found (Fig. 12). Finally, following Telophase II and nuclear condensation, mature sperm cells with long tails are formed (Fig. 13).

(2) Chromosome Numbers: The chromosomes of 10 males and females of *P. manchouricus* (Chongpyung, Chinju and Kunsan strains)

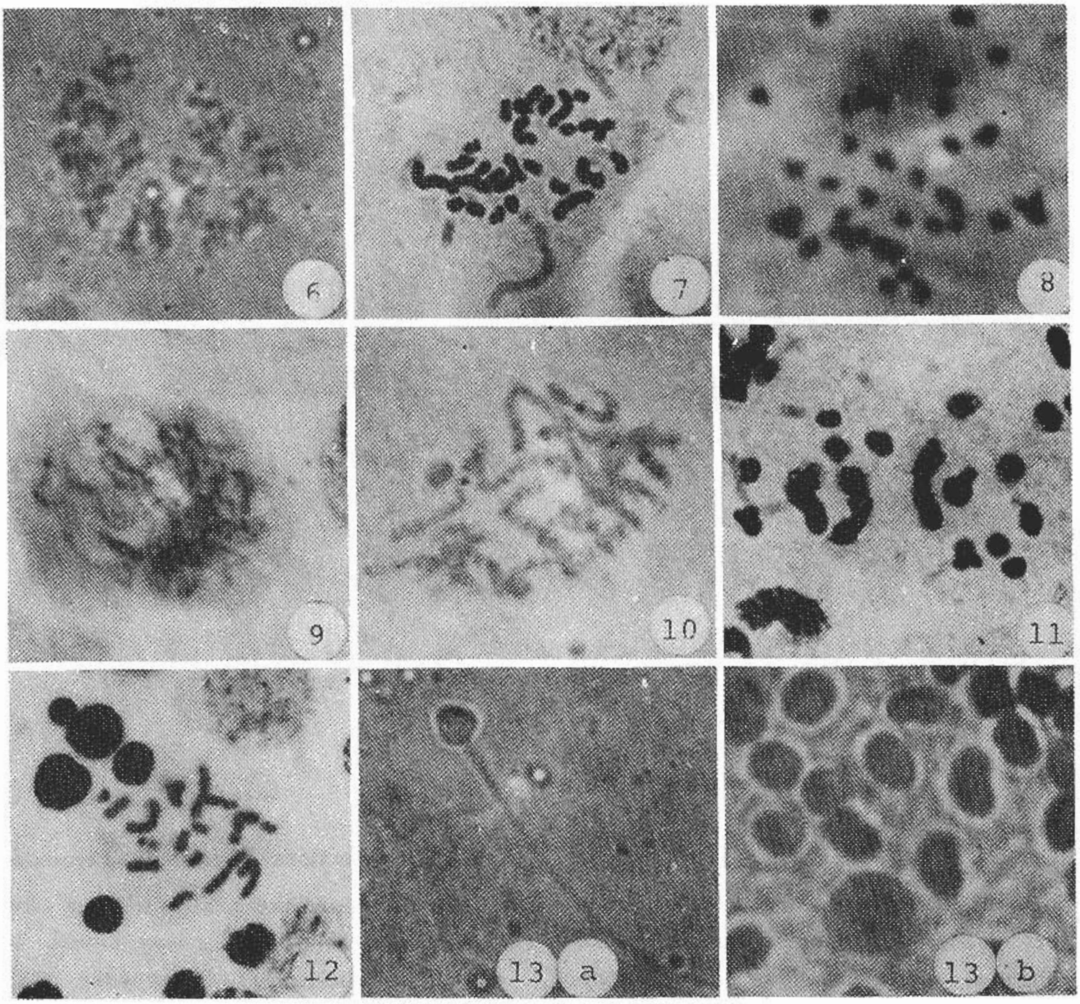

Fig. 6. A spermatogonial early prophase cell. The nucleic membrane disappears, fuzzy chromosomes can be seen. (3mm=1μm).

Fig. 7. Spermatogonial mitotic mid- or late prophase chromosomes. (3mm=1μm).

Fig. 8. Spermatogonial mitotic metaphase chromosomes (2n=34). (3mm=1μm)

Fig. 9. Late leptotene or zygotene nucleus with the beginning of synapsis of the homologous chromosomes. (3mm=1μm)

Fig. 10. Late pachytene chromosomes (bivalents) with two dark-staining nucleolar organizers. (3mm=1μm)

Fig. 11. Late diakinesis bivalents with chiasmata (n=17). (3mm=1μm).

Fig. 12. The haploid monads in telophase II (n=17).

Fig. 13. Mature sperm. (a) Single sperm; the tail can be seen clearly. (b) Numerous sperm heads. (3mm=1μm)

were observed in this investigation. Those snails had all 17 bivalents during the first meiotic division, and 34 chromosomes were observed during both spermatogonial and oōgonial divisions.

(3) Karyology: The chromosomes in male and female mitotic metaphase cells were karyotyped for analysis of chromosome morphologies in all

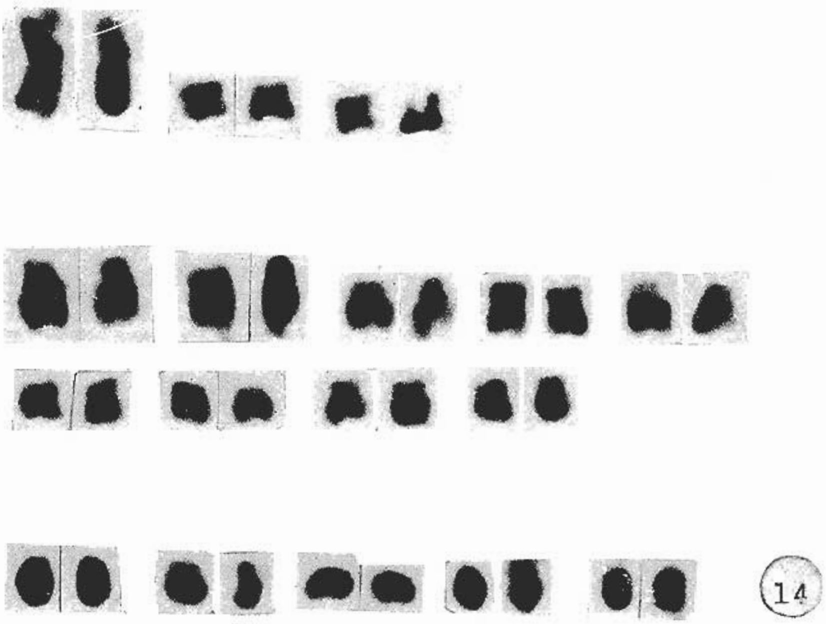

Fig. 14. Karyotype of *P. manchouricus* (male, Chongpyung strain).

the Korea strains of *P. manchouricus*. The chromosome configurations were made directly from photographs of single cells. Korean strains of *P. manchouricus* have a mitotic chromosome number of 34. Karyotypes of spermatogonial and oögonial chromosomes show three metacentric, 9 submetacentric and five acrocentric pairs (Fig. 14). The mitotic metaphase chromosome sizes ranged from 2.9 μm to 0.8 μm, and the ratio of length between the longest and shortest chromosomes was 3.6.

Chromosomal homologies between local strains of *P. manchouricus* cannot be established with certainty without banding studies. So, all the chromosome preparations were stained with quinacrine for the detection of so-called Q-banding patterns on the chromosomes. However, no chromosome was successfully stained in this study. It was difficult to identify the banding pairs becouse of extemely small sizes of chromosomes. Also, no sex chromosomes were found in this study.

D) Natural Infections of Trematode Cercariae

The freshwater intermediate snails of *Clonorchis sinensis, Parafossarulus manchouricus were* randomly collected at Chongpyung, Chinju and Kunsan areas, and *Bithynia (Gabbia) misella* at Gongju area in September 1983 through July 1984 in order to observe shedding trematode cercariae.

The infection rates of cercariae of C. *sinensis,* furcocercus cercariae, *Loxogenes liberum* type I and type II and unknown tail-less cercariae were 0.14%, 0.29%, 5.97%, 0.58% and 0%, respectively, in Chinju strain; and 1.25%, 0%, 6.24% 1.25% and 0.62%, respectively, in Kunsan strain. Cercariae of *L. liberum* type I were found in Chongpyung strain, and no other cercariae were shed in these snails. *B.(G.) misella* shed only microcercous xipidiocercariae with the infection rate of 1.82% (Table 8).

Table 8. Natural infection rates (%) of bithyniid snails with trematode cercariae

Trematode cercariae	*P. manchouricus*						*B.(G.) misella*	
	Chongpyung St.		*Chinju St.*		*Kunsan St.*			
	No. Posit.	%	No. Posit.	%	No. Posit.	%	No. Posit.	%
Cercariae of *Clonorchis sinensis*	0	0	1	0.14	8	1.25	0	0
Furcocercus cercariae	0	0	2	0.29	0	0	0	0
Cercariae of *Loxogenes liberum (I)*	1	6.7	41	5.97	40	6.24	0	0
Cercariae of *Loxogenes liberum (II)*	0	0	4	0.58	8	1.25	0	0
Tail-less unknown cercariae	0	0	0	0	4	0.62	0	0
Microcercous xipidiocercariae	0	0	0	0	0	0	1	1.82
Total No. of snails examined	15		686		641		55	

Table 9. D.O. and B.O.D.$_5$ values of waters at the collecting areas around Jinyang Lake in 1983

No. of sampling areas	D.O.				B.O.D.
	1	2	3	$\bar{M}$±S.D.	
1	8.7	8.9	8.4	8.7±0.25	1.6
2	7.8	7.8	8.0	7.8±0.11	0.4
3	10.0	8.4	10.4	9.6±1.05	0.8
4	7.8	8.2	8.0	8.0±0.19	0.8
5	5.8	6.0	6.2	6.0±0.19	0.7
Middle of lake	7.1	6.9	–	7.0±0.14	1.2

Remark: Unit=mg/*l* (ppm)

E) Water Chemistry in the Habitats of the Snails

Water samples were collected for physical and chemical-analyses from the snail habitats around the Jinyang Lake of Chinju City. No other localities were targeted for water chemistries because of the expectation of similar results, and of shortage of research fund.

The levels of dissolved oxygen (D.O.) and biochemical oxygen demand (B.O.D.) of the water specimens sampled from the study areas ranged from 6.0 to 9.6 ppm and from 0.4 to 1.6 ppm, respectively (Table 9). As a result it is considered that water system around the

Table 10. Chemical analysis of waters in which the mollusks were collected at the Jinyang Lake areas in October 1983

No. of sampling areas	Zn	Pb	Cd	Cu	Mn	Ca	Mg	Hg
1	0.03	0.005	ND	0.002	0.13	9.48	2.48	0.0005
2	0.03	ND	ND	ND	0.10	15.31	3.97	0.0033
4	0.02	0.008	ND	ND	0.38	7.18	1.80	0.0006
5	ND	ND	ND	ND	1.11	8.32	1.89	ND
Middle of lake	ND	ND	ND	ND	0.15	7.46	1.91	ND

Remarks; Unit=mg/*l* (ppm)
ND=Not detected

Jingang Lake might be relatively clean without any heavy pollution of aquatic microorganisms and organic materials during the period of this study.

On the other hand, eight metalic constituents from the water samples were also assayed, and all metalic ions detected were remarkably low below the legal criteria (Table 10). However, calcium ion in the water samples from the habitats of *P. manchouricus* was considerably higher than others.

DISCUSSION

A) Culture Methods

Successful laboratory culture of any freshwater gastropod is an important first step in pursuing research on such animals, and much effort has been expanded on learning how to successfully maintain and culture various freshwater snails, especially those of medical or veterinary importance (e.g., Bruce and Radke, 1971; Liang, 1974; Kitikoon, 1981). Bithyniids were, in fact, considered to be too difficult to raise outside of their natural environment. However, I was successful in the laboratory culture of the species of bithyniid snails studied, and also during the period of my research. *P. Manchouricus* is an especially important snail to be able to culture in the laboratory because it is the first intermediate host of *Clonorchis sinensis* and *Echinochasmus perfoliatus* for men and other mammals. To sustain a viable research program on this snail, it is necessary to have sufficient research material. Experimental work with *P. manchouricus* in the past, for example, has often been hampered by the unavailability of material due to inadequate methods of snail culture. Previously, no one had successfully cultured snails of *P. manchouricus, B. tentaculata* and *B. (G.) misella* in the laboratory.

B) Egg-laying Characteristics and Snail Growth

Kruatrachue *et al.* (1982) reported that approximately two to three weeks after the introduction of adult snails of *Bithynia siamensis and B. funiculata* to the breeding cultures, the first eggs were laid on the mud surface or on the sides of the containers. However, they did not mentioned whether the snails laid their eggs all year round.

The bithyniid snails employed in this study showed a probable trend of biological rhythm in their reproduction. The snails of all three species of bithyniids seem to prefer to lay their eggs once a year, March through June (although there were a few sporadic egg depositions at other times). However, a two or three-year period of observation is necessary to confirm this seasonal rhythm of egg laying.

Vincent *et al.* (1981) studied the life cycle of *B. tentaculata* over a period of two years in five localities of a freshwater estuary of the St. Lawrence River, Canada. The eggs were laid once a year and growth stopped during winter for about 6 months. The main phases of individual growth were related to age and thermal variations of the water, and the snails occurred during the months of May, June and July. This field study on *B. tentaculata* by Vincent *et al.* (1981) in part supports the present study in terms of egg-laying seasonality of the bithyniids.

C) Morphological Studies

The differences between the three species of bithyniid snails employed in this study are: (1) the spirally raised ridges on the whorls of *P. manchouricus* against the smooth surface with fine growth lines on the whorls of *B. tentaculata* and *B. (G.) misella,* (2) the thickened lips of the apertures of *P. manchouricus* instead of the sharper lips of *B. tentaculata* and *B. (G.) misella,* (3) the usually eroded apex in *P. manchouricus,* (4) olivebuff color of the shells of *P. manchouricus*, (5) well-rounded whorls in *B. (G.) misella*, and (6) the size of the shell (the shell length of *B. (G.) misella* is not over 7.5 mm, while the shells of the other two species are more than 10 mm in maximum length).

In addition to the above differences, there were some statistical difference among the populations in regard to relative shell heights. Why does morphological variation of the shells take place among the geographical populations? An analysis of shell form in several families of high-intertidal herbivorous gastropods has revealed the existence of morphological gradients (Vermeij, 1973). In Littorinidae, relative spire height and degree of development of external shell sculpture generally increase inter- and intraspecifically, from low to high shore levels. This gradient is also evident from temperate to tropical latitudes. Vermeij (1973) suggested that temperature and desiccation stresses are extremely important physical parameters in the ecological distribution of the species concerned.

Fretter and Graham (1978) are the only other investigators to study the protoconchs of bithyniid snails. They reported that the protoconchs of both *Bithynia tentaculata* and *B. leachi* are "featureless" (although the SEM photographs published by them are not of especially high magnification). In my SEM studies of the protoconch of *P. manchouricus*, I found that the sculpture was basically smooth, but on the protoconch there were small, low spiral wrinkles. But, of special significance in Fretter and Graham's observations and my own is that the bithyniid protoconch sculpture is not like that of the Hydorbiidae. The hydrobiids have a distinctly different protoconch sculpture (see Hadzisce, *et al.*, 1976; Thompson, 1977, 1979). This sculpture consists of closely spaced, shallow polygonal pits. Therefore, the embryonic shell sculpture is another character differentiating the bithyniids from the hydrobiids.

2. Radula.

Scanning electron microscopy (SEM) of the radulae of the three bithyniid species confirmed the radular formula as 2:1:1:1:2. Morphological differences of the radulae between the populations or species of bithyniid snails are described in detail in the Results section. For example, the central teeth of *B. (G.) misella* differ from those of the other species in several respects: 1) the mesocone is larger than the ectocones, 2) there are usually four ectocones on each side of mesocone, 3) the ridge bearing the basal denticles is more broadly flexed off of the lateral margins of the tooth, and 4) the bases of the central teeth are relatively wider.

Walker (1927) reported in his light microscopic study on the radulae of bithyniid snails that radulae of members of the family Bithyniidae are quite similar and do not possess any characters on which generic distinctions can be made. But, as shown in this SEM study, the bithyniid radular morphologies do seem to have some differences even at the species level. But more individuals should be studied, since Itagaki (1965) reported that the denticles of the central tooth and the cusps of the teeth of *P. manchouricus* are variable in their number between individuals.

D) Chromosome Cytology

Von Kemnitz (1914) was the first to produce cytological data on *B. tentaculata*. His chromosomal counts in meiotic divisions of this species, reported as n=24-28, were incorrect. Ankel (1924) found the haploid chromosome number of *B. tentaculata* to be n=17, and later (Ankel, 1933) gave a good photograph of the chromosomes. This chromosome number was confirmed by Keyl (1955) and Butot and Kiauta (1966), and by my observations. I have also found that *P. manchouricus* has the same number (n=17), but the chromosome number of *B. (G.) misella* is n=18. No sex-related, aneuploid or supernumerary chromosomes were found in my studies; all homologous pairs of chromosomes were always well matched. Q-banding technique was applied in this study to confirm sex chromosomes, but the results were unsuccessful. Further studies on banding patterns of snail chromosomes are needed.

The karyotype of animals and plants is a species specific character, and thus it may be helpful to taxonomists studying poorly understood groups. The most extensive comparative karyotype analysis of a snail group has been done by Burch (1967) of the species of the Japanese pleurocerid genus *Semisulcospira*. The systematics of this genus in the Lake Biwa basin was so poorly understood that prior to Burch's study it was not known how many species really existed. Chambers (1982) has recently found karyotypic variation between populations of *Goniobasis floridensis* inhabiting different river basins with indistinguishable shell patterns. His observation suggests the parallel evolution of similar shell patterns in populations which have divergenced chromosomally.

Karyotypic variation between bithyniid species was found (Chung, 1983), and more interesting was the finding of karyotypic variation in the geographic populations of *B. tentaculata*, although no karyotypic variation was observed among the Korean strains of *P. manchouricus* in this study.

The mitotic and meiotic chromosome cycles have been described in detail for the land snail *Catinella vermeta*, which has only 6 pairs of chromosomes (Patterson and Burch, 1966). The chromosome cycle in bithyniid snails did not differ from that found in *Catinella* and other animals.

Non-homologous chromosomes do not normally pair during meiosis and hence are usually observed as univalents in meiotic Metaphase I cells (Burch, 1960,1967). Such univalents may be sex chromosomes, supernumerary chromosomes or duplications of the normal somatic chromosomes. Neither univalent chromosomes in meiotic metaphase nor sex chromosomes were found in this study.

E) Natural Infection of Trematode Cercariae

In the Orient, the family Bithyniidae is represented by the medically important genus *Parafossarulus*, which is the main intermediate host of *Clonorchis sinensis*, and *Echinochasmus perfoliatus*, an occasional echinostome of man (Malek, 1962). The endemic areas of clonorchiasis in man caused by *C. sinensis* infection are closely related to the distribution of the snail intermediate host. The snail host mainly occurs in China, Korea, Japan and Taiwan.

Kim (1974) conducted an ecological study on *C. sinensis* in Korea. He concluded that *Clonorchis* infection in fish was closely related to the occurrence and distribution of *P. manchouricus*. Relatively low prevalence of the infection (2.3-3.1%) was found in this snail in both high and low endemic localities, with no significant difference between them. In this study, a total of 1,397 Korean bithyniid snails were crushed to obtain trematode cercariae. A low infection rate of cercariae of *C. sinensis* was also found. The Chongpyung strain of *P. manchouricus* did not shed *Clonorchis* cercariae, but it is obscure wheather sample size (15 snails only) affected this result.

Shiba (1933) found two species of bithyniid snail, *Bulimus striatus* Benson 1842 *(=P. manchouricus)* and *Bulimus kiusiuensis S. Hirase 1927 (=Bithynia (Gabbia) misella)*, in Korea and reported that *Bulimus striatus* would seem to serve as the first intermediate host of *C. sinensis*, but the role of *B. kiusiuensis* was unknown.

However, Soh (1978) suggested the possibility that *B. (G.) misella* might serve as the snail intermediate host of *C. sinensis*. Little has been known about the susceptibility of bithyniid snails so far.

One out of 55 snails, or 1.82%, of *B. (G.) misella* collected in Gongju area released cercariae belonging to microcercous xiphidiocercaria group (Table 8). However, no snails of *B. (G.) misella* were found to be harboring any other trematode cercariae. Although the susceptibility test of this snail to *C. sinensis* is obliged to determine this snail as the first intermediate host of *C. sinensis*, *B. (G.) misella* is seemed not to be related with the transmission of human clonorchiasis.

F) Water Chemistry in the Habitats of the Snails

The habitats of *P. manchouricus* were generally ponds or ditches connected with main river systems, where the depths of water were relatively shallow. Water current was slow or standing, but the levels of dissolved oxygen were considerably high. The habitats were also made up of muddy basin with high levels of calcium ions. The outcomes in this study might be useful for the selection of snail habitats in the future studies.

It would be very important epidemiological aspect to assay the levels of fecal contamination in the snail habitats in terms of the life cycle of trematode parasites. Checking fecal materials in the surveyed areas was not carried out in this study. However, epidemiological studies on the exposure of snails to fecal materials are strongly recommended especially in the endemic areas of clonorchiasis

SUMMARY

Five different populations of *Parafossarulus manchouricus* (Chongpyung, Chinju and Kunsan, Korea; and Japan and Taiwan), a population of *Bithynia (Gabbia) misella* (Gongju, Korea) and two different populations of *Bithynia tentaculata* (Michigan, U.S.A. and Bodensee, Germany) were compared in regard to egg-laying characteristics, morphology, chromosome cytology, natural infections of parasites and ecology of habitats.

A satisfactory culture method was devised for laboratory rearing of the snails. Tropical fish food (Tetra SML) and powdered green leaves (Ceralife) were used as the main food sources for the snails. Benthic diatoms such as *Navicula* and *Gomphonema* from the periphyton were also essential for satisfactory growth, especially for the baby snails. The powdered leaves were ingested by ciliary filter feeding. The aquaria were stabilized with small stones from a local stream. Young *P. manchouricus* snails grew to adult size in about 54 days after hatching. They laid eggs 150-156 days after hatching. The whole cycle (birth to egg-laying) took approximately 5 months. The three species of bithyniid snails are iteroparous and lay eggs once a year.

There were no major morphological differences in the shells of genera or subgenera studied here. They did exhibit the following rather minor differences. The shell of *Parafossarulus* has spirally raised ridges, and its apex is usually eroded; the other two genera lack these characteristics. The shell of *B. (Gabbia) misella* is small, not exceeding 7.5 mm in length, while the shells of the other two species are larger, being more than 10 mm in length.

Scanning electron microscopy (SEM) of the protoconch of *P. manchouricus* reveals nearly smooth sculpture with small, low, spiral wrinkles. This sculpture is quite different from that of the Hydrobiidae, a family to which the bithyniids are frequently assigned.

Scanning electron microscopy of the radulae

of the three bithyniid species showed that their radular morphologies are very similar, but there are some small differences, which may be species-specific.

There were some statistical differences in shell heights between the Korean and the other populations of *P. manchouricus*, and between this species and the other two bithyniids as well. The shell differences between the several populations of Korean *P. manchouricus* may be related to environment.

Details of the chromosome cycle of these bithyniid snails are similar to those reported for other snails. No specific differences were observed in the chromosome cycle between the various species and populations of snails employed in this study. Reported for the first time in molluscs are two darkly stained "nucleolar organizers" during pachytene stages of meiosis. Two different chromosome numbers were observed in the three bithyniid species: n=17 in *B. tentaculata* and *P. manchouricus*, and n=18 in *B.* (G.) *misella*. No sex chromosomes or supernumerary chromosomes were seen. There were no morphological differences in karyotypes of three Korean strains of *P. manchouricus*.

The infection rates of cercariae of *Clonorchis sinensis* in Chinju and Kunsan strains of *P. manchouricus* were 0.14% and 1.25%, respectively. However, *Clonorchis* cercariae were found in Chongpyung strain of *P. manchouricus* and Gongju strain of *B. (G.) misella*.

The habitats of *P. manchouricus* around Jinyang Lake were relatively clean without any heavy pollution of aquatic microorganisms and organic materials during the period of this study. The levels of dissolved oxygen (D.O.) and biochemical oxygen demand (B.O.D.) of the water specimens sampled from the study areas ranged from 6.0 to 9.6 ppm and from 0.4 to 1.6 ppm, respectively.

Eight metalic constituents from the water samples were also assayed, and all metalic ions detected were remarkably low below the legal criteria. However, calcium ion in the water samples from the habitats of *P. manchouricus* was considerably higher than others.

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