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Chromosome Aberrations of East Asian Bullfrog *(Hoplobatrachus rugulosus)* around a Gold Mine Area with Arsenic Contamination

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Abstract

The objectives of this study are to investigate the chromosome aberrations of the East Asian Bullfrog (*Hoplobatrachus rugulosus*) in the gold mine area compared to an unaffected area. Three *H. rugulosus* were collected, and chromosome aberrations were studied using bone marrow. The level of arsenic was measured in water, sediment and *H. rugulosus* samples. The average concentrations of arsenic in the water and sediment samples from the gold mine and unaffected areas were 0.03±0.003 mg/l and not detected in water as well as 351.59±5.73 and 1.37±1.07 mg/kg in sediment, respectively. The gold mine values were higher than the permissible limit of the water and soil quality standards, but the arsenic concentrations in the samples from the unaffected area were within prescribed limit. The average concentrations of arsenic in *H. rugulosus* samples from the gold mine and unaffected areas were 0.39±0.30 and 0.07±0.01 mg/kg, respectively, which were both lower than the standard of arsenic contamination in food. The diploid chromosome number of *H. rugulosus* in both areas was 2*n*=26, and the percentage of chromosome breakages of *H. rugulosus* in the gold mine area were higher than the unaffected area. There were eight types of chromosome aberrations, including a single chromatid gap, isochromatid gap, single chromatid break, isochromatid break, centric fragmentation, deletion, fragmentation and translocation. The most common chromosome aberration in the samples from the affected area was deletion. The difference in the percentage of chromosome breakages in *H. rugulosus* from both areas was statistically significant ($p<0.05$).

Keywords: chromosome aberration; arsenic; gold mine; frog; *Hoplobatrachus rugulosus*

1. Introduction

Gold mines contribute to major arsenic contamination in the environment. Arsenic is a toxic heavy metal that bioaccumulate throughout the food chain to organisms and increased in trophic level and deleterious effects on vertebrates, ranging from fish and amphibians to humans. The high toxicity of arsenic poses a serious threat to both human health and ecological systems (Rahman *et al*., 2014). Despite the increasing awareness of arsenic contamination in surface water worldwide, there is a dearth of information regarding its effects on aquatic life. Compounds with arsenic can have toxic effects on aquatic animals at concentrations ranging from a few micrograms to a milligram per liter (Gomez-Caminero *et al*., 2001). Historically active mines that are abandoned with little rehabilitation are particularly notorious for polluting the surrounding soil and groundwater with toxic elements (Haffert and Craw, 2008; Pauwels *et al*., 2010; Liu *et al*., 2010). Arsenic, with a naturally low average crustal abundance (1.7 mg/kg), is substantially concentrated in certain sediments adsorbed to hydrous iron oxides or in the form of sulfide-bearing minerals (Nordstrom, 2002). The natural dispersion of arsenic in the environment is governed by a combination of region and site-specific biogeochemical and hydrological factors (Ravenscroft *et al*., 2009; Smedley and Kinniburgh, 2002). The mines for gold and base metals, which are frequently associated with sulfide mineralization, have, in several settings, induced or exacerbated localized arsenic contamination (Williams, 2001; Eisler, 2004; Garelick *et al*., 2008). The problem of arsenic contamination from the gold mine activities also affects Thailand. Increased levels of arsenic (up to 277 mg/kg) have been observed in the toxic soil where the gold mine is situated, and there are many small waterways running from high elevation at the top

of the plateau to lower areas (Weerasiri *et al*., 2013). Arsenic accumulates in organisms and may become biomagnified in food chains (Singh *et al*., 2010). Because many aquatic organisms directly take up metals directly from the water, the tissue concentrations reflect the metal concentrations in the water. However, carnivores that are at the top of the food chain, such as aquatic birds and mammals (including humans), obtain most of their pollutant burden from aquatic ecosystems by ingestion, especially frogs, which have considerable potential for biomagnification (Richter and Nagel, 2007). Chronic arsenic exposure through ingestion can have severe adverse effects on frogs, especially via the contaminated aquatic ecosystem.

Aquatic organisms accumulate, retain, and transform arsenic species inside their bodies when exposed to arsenic through their diet and other routes/ sources, such as water, soil, particles, etc. (Edmonds and Francesconi, 1987; Hasegawa *et al*., 2001; Suhendrayatna and Maeda, 2001). However, arsenic biomagnification, a process whereby chemical concentrations increase in the aquatic organisms of each successive trophic level due to increasing dietary exposure (e.g., increasing concentrations from algae to zooplankton to forage fish to predator fish), is not consistent (Maher *et al*., 2011). Amphibians, which are experiencing a rapid population decline on a global scale (Stuart *et al*., 2004), are excellent bioindicators of environmental contamination because of their high sensitivity to contamination and environmental change related stress (Hopkins, 2007). The aquatic stages of amphibians are particularly important because they are vulnerable to exposure from contaminants in the aquatic environment through their permeable skin and gills. The aquatic stage is a phase of intense growth, development and differentiation; hence, contaminants are likely to interfere with the natural process of growth and differentiation. Amphibians exhibit a range of phenotypic and developmental plasticity in response to different types of stress, which include the body size and weight at metamorphosis and/or timing of metamorphosis, etc. (Relyea, 2007; Johansson *et al*., 2010). Therefore, amphibians are often considered sentinels of environmental pollution as well as an excellent model organism for contaminant-induced DNA damage (Maselli *et al*., 2010; Giri *et al*., 2012; Yadav *et al*., 2013). The cytogenetic abnormalities and DNA damage that are induced by gold mine activities indicate that the consumption of arsenic contaminated water could increase the risk of developing adverse health consequences. As a result, it is important to monitor the potential arsenic toxicity from gold mine activities. The measurement of the genotoxicity caused by heavy metals in living things, including aquatic animals, is mainly related to the sensitivity and a short response time (Gupta and Sarin, 2009). Frogs are sensitive indicators because of their genotoxic and mutagenic effects that arsenic at environmentally relevant concentrations has significant sub-lethal effects on frog, which may have long-term fitness consequence to the species and may have similar implications in other aquatic life (Singha *et al*., 2014). Chromosome aberration tests provide a quick method for screening the genotoxic effects of chemical substances that are present in the environment (Leme and Marin-Morales, 2008; Leme *et al*., 2008; Hoshina *et al*., 2008; Hoshina and Marin-Morales, 2009). Investigations of the toxic effects of arsenic, at the cellular level, demonstrate cytogenetic aberrations, which warrant environmental monitoring and risk assessment.

East Asian Bullfrog (*Hoplobatrachus rugulosus*) is a frog species that is important in the food chain within the aquatic ecosystem. Aquatic ecosystem contamination by heavy metals from gold mine activities has been gaining increasing attention. Chronic exposure and accumulation of arsenic by amphibians may result in tissue damage that produces adverse effects in both the directly exposed organisms and indirectly exposed organisms, such as human beings. This study aims to determine the concentration of arsenic in the water, sediment and *H. rugulosus* samples from the gold mine and unaffected areas and including investigate the chromosomal aberration of arsenic contaminated in *H. rugulosus* that affected by gold mine compared with *H. rugulosus* from unaffected area. The latter area was assumed to have no arsenic contamination has a higher economic value, for instance it is used as a corrosion inhibitor because the peels of cacao containing metabolites secondary sizable. Among phenolics, flavonoids, terpenoids, steroids and alkaloids, the peel contains more lone electro pairs (Azizah *et al*., 1999; Osman *et al*., 2004; Okuda and Ito, 2011). In order to accommodate this need, the writer feels interested to conduct a study to determine the inhibitory power of cacao peels extract to the reaction rate of corrosion of steel in sodium chloride solution.

2. Materials and Methods

2.1. Sampling sites

The sampling sites are located at a stream near the gold mine in the Wangsaphung district of the Loei province of Thailand (Fig. 1). The reference site was defined as the reservoir in the Khon Kaen province where there was no gold mine activity. Most of the land near the gold mine was engaged in farming and crop plants, such as rice, bananas, cassavas, soybeans and rubber trees. Within the catchment area where the gold mine is situated, there are many small waterways running from a high elevation at the top of the plateau to lower areas. These waterways combine and eventually join the Loei River (Weerasiri *et al*., 2013).

2.2. Sample collections

The samples of water, sediment and *H. rugulosus* were collected from the sampling area at the affected stream in the gold mine which has a distance from tailing pond about 50 meters (Fig. 1). The unaffected area was randomly selected. Each sample was analyzed for arsenic concentrations and chromosome aberrations. The water samples were fixed by nitric acid and the sediment samples were dried by air before analysis for the arsenic concentrations.

2.3. Analysis of arsenic concentrations in water, sediment and East Asian Bullfrog

A total of 2.5 g of each sample was predigested with 3 mg/l of concentrated nitric acid overnight at 40°C; after cooling, 2 mg/l of 30% hydrogen peroxide was added. The container was covered, placed in a high-pressure stainless steel bomb and then placed in an oven at 160°C for 4 h. After cooling, the solution was diluted with Milli-Q water and transferred into a PET bottle to 50 g. The arsenic concentrations in each sample were determined using inductive coupled plasma-optical emission spectrometry (ICP-OES; model Optima 8300) (Chand and Prasad, 2013). The wavelength analyses of ICP-OES for arsenic were set to 188.979 nm. The accuracy of the metal concentrations results was evaluated with certified reference material (CRM) via the 3111C method (APHA, 2005). Two aliquots of the CRM were spiked with a known level of metal spike standard. One spike was analyzed according to the 3111C method, and the other was analyzed with the 3111B method (APHA, 2005). The metal recoveries were in the 96-100% range, which is considered acceptable (USEPA, 1994).

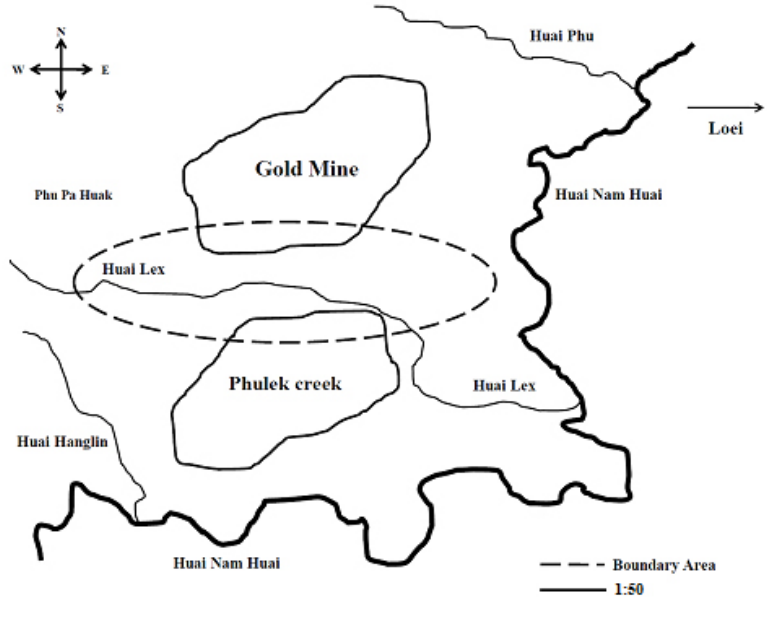

Figure 1. Overview of the gold mine area, the location of the sampling collection site is the boundary area

2.4. Chromosome preparation and conventional staining

The *H. rugulosus* samples were transferred to the laboratory. Chromosomes were directly prepared in vivo (Chen and Ebeling, 1968; Nanda *et al*., 1995) as follows. Colchicine was injected into the *H. rugulosus*'s abdominal cavity and left for 8 h. The bone marrow was cut into small pieces and then mixed with 0.075 M KCl. After discarding all large pieces of tissue, 7 mg/l of cell sediments were transferred to a centrifuge tube and incubated for 30 min. KCl was discarded from the supernatant after centrifugation at 2,000-2,500 rpm for 10 min. The cells were fixed in a fresh, cool fixative (3 methanol: 1 glacial acetic acid) that was gradually increased to 7 mg/l before centrifugation at 2,000-2,500 rpm for 10 min; then, the supernatant was discarded. The fixation was repeated until the supernatant was clear; then, the pellet was mixed with 1 mg/l fixative. The mixture was dropped onto a clean and cold slide using a micropipette, which was followed by an air-dry technique.

Conventional staining was prepared using 20% Giemsa's solution for 30 min (Rooney, 2001). Ag-NOR banding (Howell and Black, 1980) was performed by adding four drops of 50% silver nitrate and 2% gelatin on the slides. The slides were sealed with cover glasses and incubated at 60°C for 5 min. Afterwards, the slides were soaked in distilled water until the cover glasses were separated.

2.5. Chromosome counting and recording of abnormal chromosomes

Chromosome counting and recording of the abnormal chromosomes was performed on mitotic metaphase cells under a light microscope. Two hundred clearly observable and healthy cells that were spread chromosome plates were selected and photographed. The fundamental number (NF, number of chromosome arms) was obtained by assigning a value of two arms to metacentric, submetacentric and acrocentric chromosomes and one to a telocentric chromosome. All parameters were used in karyotyping. The cytotoxicity was evaluated from chromosome aberrations by studying the percentage of chromosome breakages on 100 metaphase cells per individual sample under a light microscope.

2.6. Statistical analysis

The concentrations of arsenic in the environment, including *H. rugulosus*, and the percentage of chromosome aberrations in *H. rugulosus* from the gold mine and unaffected areas were analyzed using the *t*-test. All statistical tests were conducted at a 95% confidence level.

3. Results and Discussion

3.1. Arsenic concentrations in water, sediment and East Asian Bullfrogs

The arsenic concentrations in water and sediment and *H. rugulosus* samples of the gold mine and unaffected areas are shown in Table 1. The arsenic concentrations in the water and sediment samples from the gold mine and unaffected areas were 0.03±0.003 mg/l and not detected in water as well as 351.59±5.73 and 1.37±1.07 mg/kg in sediment, respectively. The average concentrations of arsenic in the water from the gold mine area were still higher than the levels the Pollution Control Department of Thailand (2001) allowed for the water quality standards (0.01 mg/l). Arsenic was not detected in water from the unaffected area. The concentration of arsenic in the sediment samples of the gold mine area exceeded the standards for soil quality, while the arsenic concentrations of the unaffected area did not exceed the standards for soil quality. Statistical analysis indicated that there were significant differences between the arsenic concentrations in the sediment samples from the gold mine and unaffected areas ($p=0.02$).

The average arsenic concentrations in *H. rugulosus* samples from the gold mine and unaffected areas were 0.39±0.30 and 0.07±0.01 mg/kg, respectively, which were both lower than the standards for arsenic contamination in food (2 mg/kg) according to the Pollution Control Department of Thailand (2001). Statistical analysis indicated that there were no significant differences between the arsenic concentrations in *H. rugulosus* samples from the gold mine and unaffected areas ($p=0.23$).

This study revealed that the arsenic concentrations in the water, sediment and *H. rugulosus* samples from the gold mine area correlated with chromosome aberrations. The arsenic concentrations in the sediment are higher than those in the water from both areas; after being deposited into the sediment, arsenic accumulates in *H. rugulosus*. The arsenic levels met the standards for water and soil quality from the unaffected area, except for the arsenic levels in the water and sediment from the gold mine area. The arsenic concentration was observed in *H. rugulosus* from both areas, and, fortunately, the arsenic levels met Thailand's food quality standard level. This comparative study showed that the accumulation of arsenic in *H. rugulosus* was lower than in the water and sediment because it accumulates in organisms through the consumption hierarchy. In addition, *H. rugulosus* can be found in the water and sediment. It lays large clutches of pigmented eggs in standing bodies of water. The tadpoles are mottled with brown and grow to approximately 2.5 cm. They live at the bottom of shallow puddles and ditches (Lim and Lim, 2002; Das, 2007; Baker and

Table 1. The arsenic concentrations in the water, sediment and *H. rugulosus* samples from the gold mine and unaffected areas

Samples		Water (mg/l)	Sediment (mg/kg)	*H. rugulosus* (mg/kg)
Samples from the gold mine area	Sample 1	0.03	355.43	0.708
	Sample 2	0.03	354.33	0.349
	Sample 3	0.03	345.00	0.121
	Mean±SD	0.03±0.003	351.59±5.73 [a]	0.39±0.30 [ns]
Samples from the unaffected area	Sample 1	Not detected	2.59	0.30
	Sample 2	Not detected	0.93	0.03
	Sample 3	Not detected	0.59	0.03
	Mean±SD	-	1.37±1.07 [a]	0.07±0.01 [ns]
P-value		-	0.02	0.23
Thailand standard		0.01	3.90	2.00

Remarks: Limit of Detection (LOD) for arsenic = 0.006 mg/l; mg/kg; ns= no significant, a=significant

Lim, 2008). The *H. rugulosus* is an amphibian with a lower accumulation of arsenic in the water and sediment and a life cycle that consists of living in both the water and sediment. Therefore, *H. rugulosus* is a creature that is expected to adapt well to the environment. However, the average concentration of arsenic in the water and sediment samples from the gold mine area was higher than in the unaffected area. Statistical analysis indicated that there were significant differences between the concentrations of arsenic in the samples from affected and unaffected areas. These data indicated that the gold mine area has running water and arsenic from the gold mine activity accumulates in the affected area, which is then diluted in water, deposited into sediment, and accumulated in *H. rugulosus*. These process likely accounts for the increased metal concentration during the rainy season. Because this study is a field investigation, there are other possible environmental measures of toxicity in addition to heavy metal contamination (Ansari *et al*., 2004).

3.2. Chromosome evaluation of East Asian Bullfrogs (H. rugulosus)

The diploid chromosome number (*2n*) of *H. rugulosus* from affected and unaffected areas was *2n*=26. Joshy and Kuramoto (2008) reported that the diploid chromosome number (*2n*) of the five species of the genus *Fejervaya* (Anura: Ranidae) from South India were *2n*=26. The karyotype of *H. rugulosus* from both areas consisted of 8 metacentric and 18 submetacentric regions (Figs. 2 and 3). The karyotype of *H. rugulosus* from the affected area showed chromosome aberrations. The *H. rugulosus* from both areas displayed terminal Ag-NOR on chromosome pair 9 (Fig. 4). The *2n* of the mitotic metaphase cells and karyotypes of *H. rugulosus*, conventionally stained with Ag-NOR, are not different in the two areas.

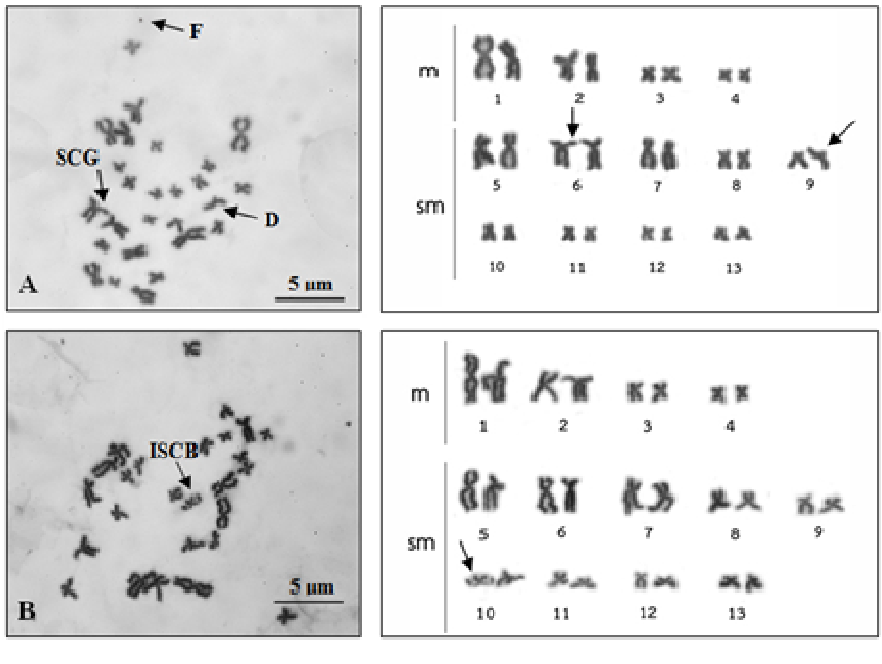

Figure 2. Metaphase chromosome plates and karyotypes of individual (A, B) *H. rugulosus* (*2n*=26) from the gold mine area using a conventional staining technique, the arrows indicate the chromosome aberration

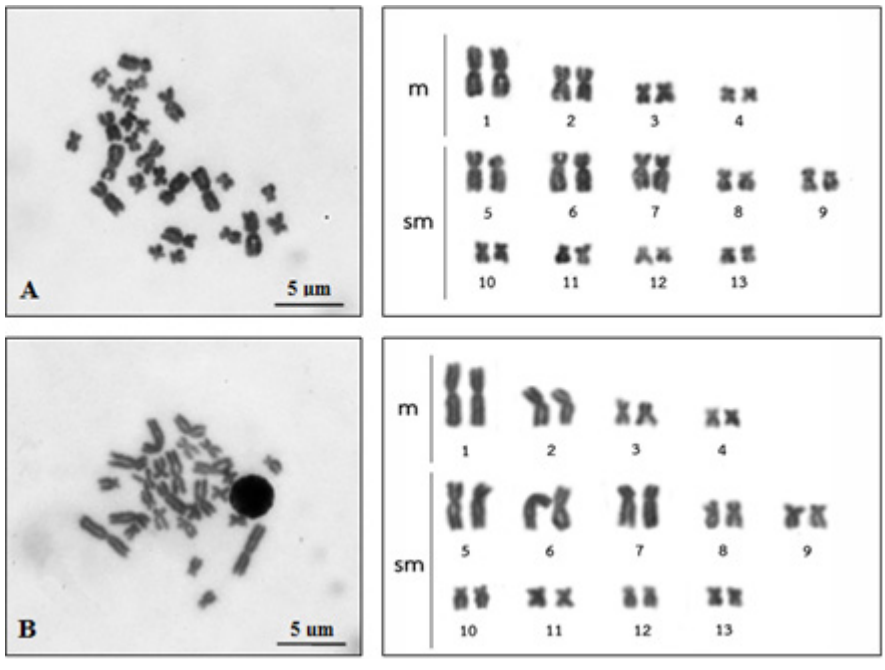

Figure 3. Metaphase chromosome plates and karyotypes of individual (A, B) *H. rugulosus* (*2n*=26) from the unaffected area using a conventional staining technique

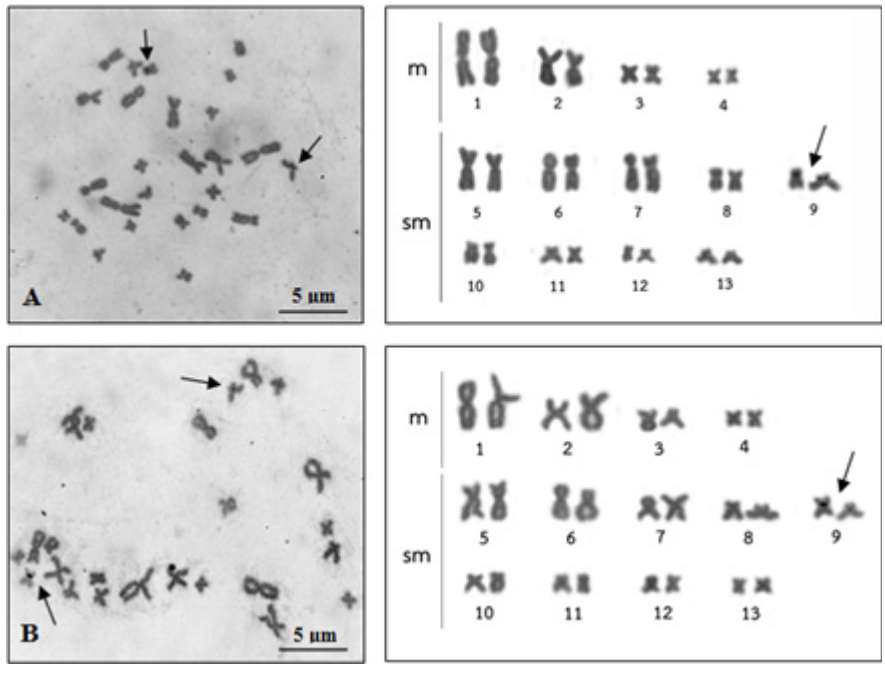

Figure 4. Metaphase chromosome plates of *H. rugulosus* (*2n*=26) of the unaffected area (A) and affected area (B) using an Ag-NOR banding technique, the arrows indicate NOR-bearing chromosomes pair 9

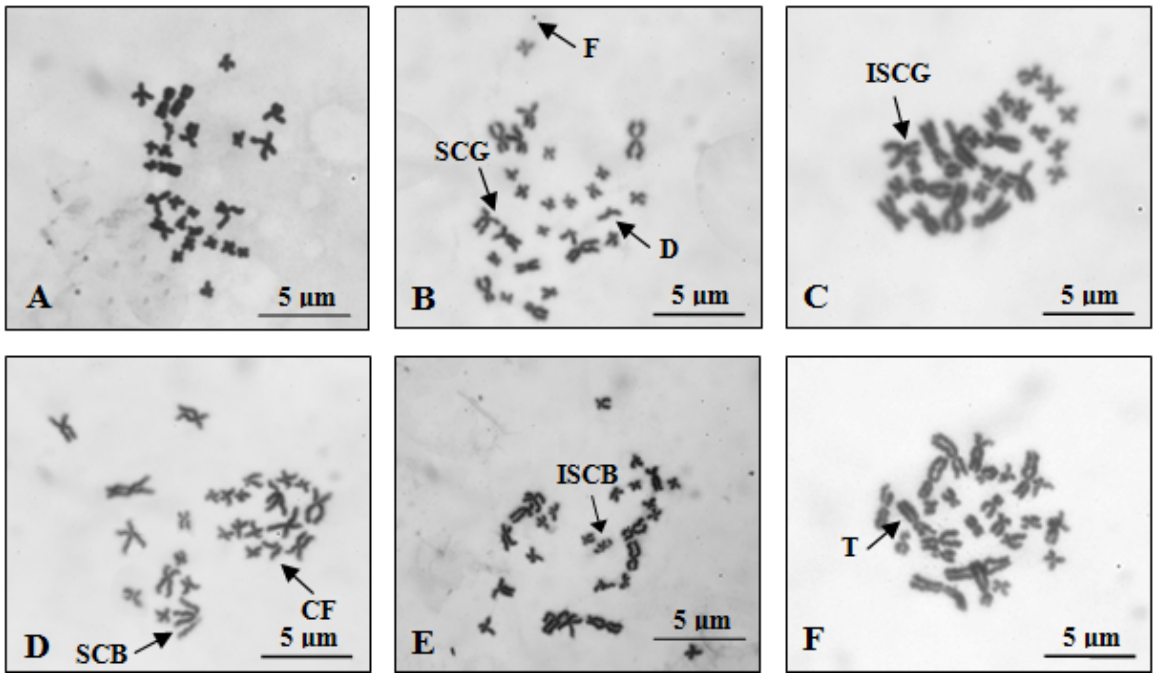

Figure 5. Different types of aberrations in the metaphase spread of *H. rugulosus* (*2n*=26), showing a single chromatid gap (SCG), isochromatid gap (ISCG), single chromatid break (SCB), isochromatid break (ISCB), centric fragmentation (CF), deletion (D), fragmentation (F) and translocation (T) affected by arsenic contamination (A: Unaffected area, B-F: Affected area)

Staining chromosomes with Ag-NOR does not detect the chromosome aberrations of *H. rugulosus*, while conventional staining techniques only detect chromosome aberrations. The different types of aberrations in the metaphase spread cells of *H. rugulosus* samples from the gold mine area are shown in Fig. 5. This study showed that the different types of chromosome aberrations were single chromatid gap (SCG), isochromatid gap (ISCG), single chromatid break (SCB), isochromatid break (ISCB), centric fragmentation (CF), deletion (D), fragmentation (F) and translocation (T). The most common chromosome aberrations in the samples from the affected area were Ds. The numbers and percentages of chromosome aberrations of *H. rugulosus* samples from the affected and unaffected areas are shown in Table 2. The number of chromosome aberrations of the *H. rugulosus* samples in chromosome aberrations of SCG, ISCG, SCB, ISCB, CF, D, F and T were 12, 1, 18, 9, 1, 31, 9 and 1 aberrations from the affected area and 1, 0, 3, 0, 3, 4, 0 and 0 aberrations from the unaffected area, respectively. One hundred clearly observable chromosomes were used for this study; the

Table 2. The number and percentage of chromosome aberrations of *H. rugulosus* samples from affected and unaffected areas

H.rugulosus Samples	Arsenic concentration (mg/kg)	The number of chromosome aberrations								Total number of chromosome aberrations	The cell number of chromosome aberrations	The percentage of chromosome aberrations
		SCG	ISCG	SCB	ISCB	CF	D	F	T			
Affected area												
Individual 1	0.708	3	1	5	4	1	7	6	0	27	14	14
Individual 2	0.349	5	0	5	2	0	13	2	0	27	15	15
Individual 3	0.121	4	0	8	3	0	11	1	1	28	21	21
Average/Total	0.39±0.30^{ns}	12	1	18	9	1	31	9	1	82	50^{a}	50^{a}
Unaffected area												
Individual 1	0.30	0	0	1	0	2	3	0	0	6	6	6
Individual 2	0.03	1	0	0	0	1	0	0	0	2	2	2
Individual 3	0.03	0	0	2	0	0	1	0	0	3	3	3
Average/Total	0.07±0.01^{ns}	1	0	3	0	3	4	0	0	11	11	11
P-value											0.0065^{a}	<0.001^{a}

Remarks : Limit of Detection (LOD) for arsenic = 0.006mg/kg, ns=no significant, a=significant

total number of chromosome aberrations found in the *H. rugulosus* samples of the affected and unaffected areas were 82 and 11 aberrations, respectively. In addition, the cell numbers of chromosome aberration in the *H. rugulosus* samples of the affected and unaffected areas were 50 and 11 cells, respectively. The average arsenic concentrations and average percentages of chromosome aberrations in the *H. rugulosus* samples from the affected and unaffected areas were 0.39±0.30 mg/kg and 50% as well as 0.07±0.01 mg/kg and 11%, respectively. These data indicated that the average concentration values of arsenic and the average percentage of chromosome aberrations of the *H. rugulosus* samples from the affected area are higher than the samples from the unaffected area. Statistical analysis indicated that there are significant differences between the cell number of chromosomal aberrations and percentage of chromosomal aberrations of *H. rugulosus* samples from the affected and unaffected areas with respect to arsenic ($p= 0.0065, 0.001$).

Results obtained with the chromosome checks of *H. rugulosus* samples from the arsenic affected and unaffected areas indicate that the diploid chromosome numbers are not different. The NOR location can describe the chromosome evolution. The *H. rugulosus* samples displayed terminal Ag-NOR on chromosome pair 9. In addition, Patawang *et al*. (2014) reported that Ag-NOR was located on the region adjacent to the centromere of chromosome pair 9. The data report basic knowledge about *H. rugulosus*, and its cytogenetics will be applied to studies on breeding, conservation and chromosome evolution in this *H. rugulosus*. The *H. rugulosus* arsenic samples from the affected and unaffected areas were significantly different, and there is a significant difference in the chromosome aberrations between the affected and unaffected samples. The organisms appeared to receive less toxic pollutants and were then able to build up resistance. In addition, the average percentage of chromosome aberrations of the *H. rugulosus* samples from the affected area was higher than the samples from the unaffected area. These data indicate that the chromosome aberrations were more frequent in *H. rugulosus* that lived for a long time in the high arsenic contamination area. However, the chromosome aberrations are not only caused by arsenic; they are also affected by other heavy metals as well as their combination and duration of exposure. The *H. rugulosus* living in the arsenic contaminated area needs to develop some degree of tolerance to arsenic toxicity to survive. The *H. rugulosus* from the affected area adapts to its habitat environment. They can endure arsenic contamination and survive in the ecosystem near the gold mine, which is a highly contaminated area. The *H. rugulosus* are affected by arsenic from gold mine activities, which can also occur in any eukaryotic organism. Humans, for example, can similarly be affected through the food chain or food web. Exposure to high arsenic concentrations is known to cause damage to the nervous system, kidney and liver in human beings. The cytotoxicity confers chromosome abnormalities in developing fetuses and is a major cause of early spontaneous abortions in humans. Aneuploidy accounts for over 90% of fetal loss (Hassold, 1986). In the cytotoxicity evaluation of environmental samples, developments that are similar to classical toxicology have been undertaken. Fortunately, this study only detected a small number of chromosome aberrations in *H. rugulosus* from the unaffected area, which was selected at random. These data indicated that people could consume *H. rugulosus* from this area.

In addition to arsenic, the areas are affected by several other pollutants, such as fertilizers, chemicals and insecticides. These pollutants may contaminate and affect the ecosystem and environment in which *H. rugulosus* resides. This study was performed with a small number of *H. rugulosus*, but the detection of chromosome aberrations suggests that the gold mine areas must be managed better. To assess the real impact of the arsenic from gold mines on the ecosystem, it is necessary to continuously biomonitor this area with more collections and tests.

4. Conclusions

The gold mine activities have high arsenic contamination, which impacts the organisms in ecosystems. Arsenic contamination in the water, sediment and organisms affects animals that are further along in the food chain, causing biomagnification. The arsenic concentrations in the sediment are higher than the water from both areas. Unfortunately, the arsenic concentrations in the water and sediment from the gold mine area were higher than the standard level, while the arsenic levels met the standard of water and soil quality from the unaffected area. Although the concentration of arsenic was lower in *H. rugulosus* from either area, and the average concentrations of arsenic in *H. rugulosus* from both areas met Thailand's food quality standard level, chromosome aberrations were found at extremely low levels in the unaffected area. However, the arsenic concentration in *H. rugulosus* from the affected area was higher than the unaffected area. The average percentage of chromosome aberrations in the *H. rugulosus* samples from the affected area was higher than the samples from the unaffected area. The chromosome aberrations were more frequent in *H. rugulosus* that lived for a long time in the high arsenic contaminated area. Exposure to high concentrations of arsenic causes structural aberrations of chromosomes, without affecting diploid number.

H. rugulosus can endure arsenic contamination and survive in the contaminated ecosystem. Therefore, the accumulation of arsenic in *H. rugulosus* species should be a concern because of its potential effects on human health. The government should be informed of the results of this research so that officials can properly consider of the consumption of *H. rugulosus* that live in contaminated areas.

Acknowledgments

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