

Genetic Differentiation through ISSR Marker in the Common Puddle Frog (*Occidozyga lima*) Affected by Heavy Metals from a Municipal Landfill

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Abstract

The concentrations of arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb) and manganese (Mn) in water, sediment and *Occidozyga lima* tissues were investigated, as well as the genetic differentiation of *O. lima* from a municipal landfill and a reference area. The heavy metal concentrations were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES). Inter simple sequence repeats (ISSR) with dendrogram construction and genomic template stability (GTS) were employed to determine genetic differentiation. The concentrations of As (0.0135 ± 0.01 mg/L), Cr (0.0866 ± 0.08 mg/L), and Pb (0.0808 ± 0.09 mg/L) in the water from the municipal landfill exceeded the standards (0.01, 0.05, and 0.05 mg/L, respectively), and the As concentration (8.9930 ± 1.47 mg/kg) in the sediment from the municipal landfill exceeded the standard (3.9 mg/kg). The heavy metal concentrations in *O. lima* from the municipal landfill and reference area were within the standards. Genetic differentiation of *O. lima* was determined using ISSR fingerprints from 12 successful ISSR primers that generated 535 total bands for dendrogram construction. The dendrogram results separated *O. lima* into two clusters corresponding to the two sample sites. The genetic differentiation ranges of *O. lima* from the municipal landfill and reference area were 40.74 - 58.62% and 80.70 - 93.10%, respectively. The *O. lima* in the municipal landfill had heavy metal accumulation and a low %GTS. These results demonstrated the genotoxicity of heavy metals to frog that live in polluted areas by monitoring contaminant exposure.

Keywords: Frog; Municipal landfill; Heavy metals; Genetic; Genotoxicity

1. Introduction

The human population has increased because of growth in the economy, industry, and agriculture, which has increased the volume of solid waste (Eggen *et al.*, 2010; David *et al.*, 2020). Solid waste can be disposed of using a variety of management practices.

The most common solid waste management approach in developing countries is municipal landfills. Thus, there are an increasing number of municipal landfill sites, and these municipal landfills are not being appropriately managed in accordance with hygienic standards.

The main environmental problem of municipal landfills is the decomposition of organic substances, which results in the production of a strong odor and can result in disease-causing insects. In addition, the leachate from the decomposition of organic substances contains large amounts of toxins such as heavy metals. Therefore, heavy metal contamination of leachates from municipal landfills should be considered (Al-Yaqout et al., 2003; Ihedioha et al., 2017; Phoonaploy et al., 2019). Previous studies reported that water sources and sediments in the Khon Kaen municipality landfill in northeast Thailand were contaminated with As, Cd, Cr, Pb, Cu, Fe, Mn, Ni and Zn, and there was an accumulation of heavy metals in the frogs and snakehead fish in this area (Phoonaploy et al., 2016; Phoonaploy et al., 2019). Heavy metals can have toxic effects on organisms (Maselli et al., 2010; Maselli et al., 2019). The Roi Et municipal landfill is the same designed structure, operation and municipal waste as the Khon Kaen municipal landfill, and the Roi Et municipal landfill is ranked as the 15th dirtiest province in the country. These municipal landfills do not have an appropriate solid waste disposal system according to sanitary principles (Silapaksa et al., 2019). When leachate contaminates water, sediment, and soil, the toxicity of heavy metals may affect living organisms due to ingestion into the body. Heavy metals, such as As, Cd, Cr and Pb, are not necessary for organisms. These heavy metals can accumulate in water, sediment, and soil and are toxic to organisms living in municipal landfills because they are toxic in small quantities (Phoonaploy et al., 2019; Neeratanaphan et al., 2020). As living things in the ecosystem, local people who use the area or water for consumption may experience heavy metal toxicity (Kagalou et al., 2004). In addition, the accumulation of heavy metals in living organisms can affect the functioning of the body. The effects on the cell wall cause the balance of electric charges to be disrupted and cell wall destruction subsequently occurs, causing abnormalities in cell division (Suttichaiya et al., 2016). The chromosomal abnormalities of swamp frogs (*Fejervarya limnocharis*) affected by leachate at the Khon Kaen municipality

landfill were investigated by Phoonaploy et al. (2016). They found that the level of Cr exceeded surface water quality standards and that the chromosomal abnormalities of frogs in the municipal landfill area were affected by leachate compared with those of frogs in the reference area. The percentage of breaks per cell in frog chromosomes in both regions was significantly different ($p < 0.05$). A previous study indicated that chromosomal breakage ultimately results in DNA damage (Yadav and Trivedi, 2009).

The common puddle frog (*Occidozyga lima*) can be found in the area of the Roi Et municipality landfill that the local people generally consumed. This species of frog lives both on land and in water and is exposed to toxins from municipal landfills. The life cycle of *O. lima* comprises breeding outside the body and laying of eggs in water, causing the eggs to come into contact with toxins in the water (Shane et al., 2011). After entering the tadpole stage, *O. lima* remain in the water and are exposed to toxins again until they reach the adult stage. In the adult stage, frogs consume small insects as food in the food chain. This causes the accumulation of toxins in the bodies of frogs (Burlibasa and Gavrila, 2011). Heavy metals are absorbed by frog species, and the effects are biomagnified due to their relatively high trophic levels in the food chain (Richter et al., 2007; Burlibasa and Gavrila, 2011). Toxicity can result in acute and long-term effects such as the inhibition of cellular enzyme activities, interference with DNA repair and gene expression, the induction of chromosomal abnormalities, and gene pool mutations (Taiwo et al., 2014; Temwiriyankul et al., 2014). According to reports, heavy metal-induced DNA damage in aquatic organisms can be used as a sensitive bioindicator to test for genotoxicity (Maselli et al., 2010; Salem et al., 2014; Maselli et al., 2019). Currently, genotoxicity tests are acceptable for investigating genotoxicity following metal exposure using molecular biology approaches. DNA fingerprinting techniques include the use of random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) to compare genetic relationships between populations, which is an effective way to show changes in DNA structure. The DNA banding patterns are

used to calculate the percentage of genomic template stability (%GTS). The GTS percentage indicates the ability of a species to maintain genetic consistency (Neeratanaphan *et al.*, 2014; Silprasit *et al.*, 2016; Maselli *et al.*, 2019; Chowrong *et al.*, 2022). A previous study revealed a significant decrease in the GTS percentage of fish, which is one of the first molecular reactions to DNA damage in Nile tilapia. Neeratanaphan *et al.* (2014) and Chowrong *et al.* (2022) reported a decrease in the percentage of GTS in aquatic plants (*Pistia stratiotes*) from Pb induction by ISSR bands. Thus, the purposes of this study were to detect As, Cd, Cr, Pb, and Mn concentrations in the in water, sediment and frog (*O. lima*) samples, including genetic differentiation by ISSR markers, in *O. lima* surrounding area of the Roi Et municipal landfill compared with the reference area.

2. Methodology

2.1 Study area

The study area is located at a Roi Et municipal landfill in the Mueang district of the Roi Et province, Thailand (Figure 1). The geographic coordinates of the study site are latitude 16°05'26.2"N and longitude 103°38'32.3"E. The distance between the duplicate samples in the affected area and the municipal landfill was less than 50 meters. The reference area is located at the Khon Kaen province, Thailand, where there was no heavy metal contamination from leachate. The geographic coordinates of the reference area latitude 16°12'23.8"N and longitude 102°35'49.6"E.

2.2 Water, sediment and *O. lima* samples collection

Five random samples of water, sediment, and *O. lima* were collected in the rainy season near the municipal landfill and in the reference area. The water samples were acidified with nitric acid, and the sediment samples were dried at room temperature. *O. lima* samples were caught randomly with nets. Heavy metal concentrations were measured in the water, sediment, and muscle of *O. lima* samples. The liver of *O. lima* was used for genetic differentiation analyses.

2.3 Analyses of heavy metal concentrations in water, sediment and frog samples

American Chemical Society (ACS) grade HNO₃ was used to digest a 25 mL water sample on a heated plate (American Public Health Association; APHA, 2012). The 0.5 g dried sediment sample was digested on a hot plate with 5 mL of HNO₃ (ACS grade) and 3 mL of 30% H₂O₂. A 0.5 g sample of dried *O. lima* muscle was digested with 10 mL of 30% H₂O₂ (ACS grade), 5 mL of ACS grade H₂SO₄, and 5 mL of ACS grade HNO₃ on a hot plate (Yang *et al.*, 2013; Hashim *et al.*, 2014). All solution samples were adjusted with deionized water to 25 mL for the water and *O. lima* samples and 50 mL for the sediment samples. The heavy metals (As, Cd, Cr, Pb and Mn) in the final solutions were examined by inductively coupled plasma optical emission spectroscopy (ICP-OES). The limits of detection (LOD) of As, Cd, Cr, Pb and Mn were 0.001, 0.0001, 0.0002, 0.001, and 0.0001 mg/L, respectively (wavelengths of 188.979, 226.502, 267.719, 220.353,

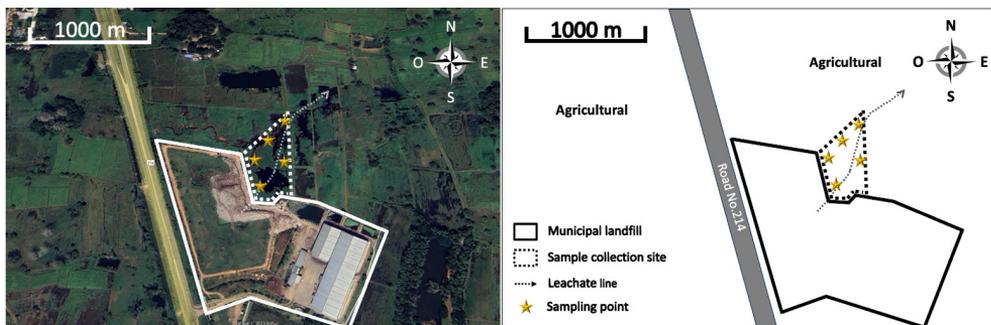


Figure 1. Municipal landfill area and the five sample collection locations.

and 259.372 nm, respectively) (Chand *et al.*, 2013). For quality control and to evaluate the accuracy of the analysis, a laboratory-fortified matrix (LFM), method blanks, and duplicate samples were utilised (APHA, 2012). The measured heavy metals had a recovery value range of 85 - 115%.

2.4 DNA extraction and ISSR analysis

The liver is an organ that has cell division all the time including this organ eliminated the toxins from the body of organism. Therefore, the liver is an appropriate organ to study for the genetic differentiation. The livers of the *O. lima* specimens collected from the municipal landfill and reference area were analyzed. The liver tissues were stored in 100% ethyl alcohol, and the nucleic acids were extracted using a kit (GF-1 tissue extraction: Vivantis, Malaysia) before separation of the samples via 0.8% agarose gel electrophoresis. DNA fingerprinting with H₂O, 2x Taq Master Mix (Vivantis, Malaysia), 50 mM MgCl₂, 50 μM primer and 20 ng/μL DNA template was performed. Twenty-four ISSR primers were screened, and the 12 primers (5'- 3') that amplified marker DNA successfully. The polymerase chain reaction (PCR) products were amplified for 40 thermal cycles, consisting of 1) 1 min at 94 °C for denaturation, 2) 1.30 min at 40 °C for annealing, 3) 2 min at 72 °C for extension, and 4) 7 min at 72 °C for final extension. The PCR products were separated by 1.2% agarose gel electrophoresis (Gel XL-100TM, USA). The successfully amplified ISSR bands were noted as present (1) or absent (0). The DNA profiles, appearance of a new band and disappearance of a normal band in the samples from the municipal landfill and reference area were investigated (Sliprasit *et al.*, 2016). The results of all evaluated bands were analyzed for %GTS determination and dendrogram construction (Bornet and Branchard, 2001; Neeratanaphan *et al.*, 2014; Chowrong *et al.*, 2022).

$$\text{GTS (\%)} = (1-a/n) \times 100$$

where *a* is the number of polymorphic bands detected in the samples from the

municipal landfill, which is equal to the sum of the disappearance of a normal band and the appearance of a new band, and *n* is the total number of bands in the samples from the reference area.

2.6 Statistical analyses and data calculations

The analysis of heavy metal concentrations in water, sediment, and *O. lima* samples from the municipal landfill and reference areas was performed using the Mann-Whitney U test (test of two-group differences). The genetic differentiation of frogs was evaluated by %GTS and dendrogram construction. The data were analyzed using R version 3.4.3 at the 95% confidence level.

3. Results and Discussion

3.1 Heavy metal concentrations in water and sediment

The average concentrations of As, Cd, Cr, Pb, and Mn in the water samples from the municipal landfill were 0.0135 ± 0.01, 0.0049 ± 0.01, 0.0866 ± 0.08, 0.0808 ± 0.09, and 1.1218 ± 1.19 while those of the reference area were 0.0016 ± 0.00, 0.0001 ± 0.00, 0.0209 ± 0.02, 0.0031 ± 0.00, and 0.0558 ± 0.07 mg/L, respectively; the concentrations in the sediment samples from the municipal landfill were 10.2059 ± 2.80, 2.6220 ± 0.44, 25.4945 ± 7.65, 25.9513 ± 8.34, and 196.8589 ± 118.08 while those from the reference area were 1.1046 ± 0.15, 0.4841 ± 0.07, 10.4201 ± 1.28, 7.7208 ± 2.11, and 88.3811 ± 27.16 mg/kg, respectively (Table 1). The average concentrations of As, Cr, Pb, and Mn in water from the municipal landfill area were higher than the concentrations allowed for by the Thailand Pollution Control Department (2001) (As: 0.01, Cr: 0.05, Pb: 0.05, and Mn: 1.0 mg/L), while the concentration of Cd did not exceed the standards. The average As, Cd, Cr, Pb, and Mn concentrations in water from the nonaffected area did not exceed the standards. The concentrations of As in sediment from the municipal landfill area were higher than that allowed for by the Thailand Pollution Control Department (2004) (As: 3.9 mg/kg); however, the Cd, Cr, Pb, and Mn

concentrations did not exceed the standards. The average concentrations of As, Cd, Cr, Pb, and Mn in soil from the reference area did not exceed the standards.

Statistical analysis revealed significant differences in the As, Pb, Mn, As, Cd, Cr, Pb, and Mn concentrations between the water from the municipal landfill and the reference area ($p < 0.05$). This result is consistent with previous research where high concentrations of heavy metals were found in water sources near landfill sites (Phoonaploy et al. 2016; Chowrong et al. 2022). In the landfill area, there are batteries (lithium-ion battery and lead acid battery), flashlight batteries, pesticide cans, spray paint cans, engine oil containers and fluorescent lamps; these solid wastes contain heavy metal components. When solid waste is disposed of improperly according to sanitary principles, heavy metals can spread into the environment in water, sediment and soil. Heavy metals, such as As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn, contaminate the municipal landfill (Phoonaploy et al., 2016). The heavy metal concentrations in water samples were lower than those in sediment samples, and might be removed from water by sorption on sediment particles and complexation by organic materials in sediment (Nannoni et al., 2015; Neeratanaphan et al., 2017).

Heavy metal contamination in aquatic ecosystems, which are plant and animal habitats, can cause bioaccumulation in the food chain and food web (Neeratanaphan et al., 2014).

3.2 Heavy metal concentrations in *O. lima*

The average concentrations of As, Cd, Cr, Pb, and Mn in the *O. lima* samples obtained from the municipal landfill were 0.1864 ± 0.09 , 0.0213 ± 0.02 , 0.4855 ± 0.17 , ND, and 13.0491 ± 11.04 mg/kg, respectively, and those in the reference area were 0.0434 ± 0.05 , 0.0247 ± 0.02 , 0.4664 ± 0.08 , ND, and 12.1958 ± 8.39 mg/kg, respectively (Table 2). These heavy metal concentrations were lower than the standards (As = 2, Cd = 0.05, Cr = 2 and Pb = 1 mg/kg) (Ministry of Public Health, 2003).

The statistical analysis revealed a significant difference between the As concentration in *O. lima* from the municipal landfill area and that in frogs from the reference area ($p < 0.05$). Generally, frogs, including *O. lima*, can obtain heavy metals from contaminated environments via ingestion, respiration and direct contact, and these metals can accumulate at each trophic level via biomagnification in contaminated aquatic ecosystems (Phoonaploy et al., 2016, 2019,

Table 1. Heavy metal concentrations in the water and sediment samples from the municipal landfill and reference area.

	As	Cd	Cr	Pb	Mn
Water (mg/L)					
Municipal landfill	0.0135 ± 0.01^a	0.0049 ± 0.01^a	0.0866 ± 0.08^a	0.0808 ± 0.09^a	1.1218 ± 1.19^a
Reference area	0.0016 ± 0.00^b	0.0001 ± 0.00^a	0.0209 ± 0.02^a	0.0031 ± 0.00^b	0.0558 ± 0.07^b
Standard*	0.01	0.005	0.05	0.05	1.0
Sediment (mg/kg)					
Municipal landfill	10.2059 ± 2.80^a	2.6220 ± 0.44^a	25.4945 ± 7.65^a	25.9513 ± 8.34^a	196.8589 ± 118.08^a
Reference area	1.1046 ± 0.15^b	0.4841 ± 0.07^b	10.4201 ± 1.28^b	7.7208 ± 2.11^b	88.3811 ± 27.16^b
Standard**	3.9	37	300	400	1,800

Notes: a,b Indicates a significant difference between values in the same column ($p < 0.05$)

* Water quality standard for surface water sources, Pollution Control Department, Ministry of Natural Resources and Environment, Thailand. (Thailand Pollution Control Department, 2001)

**Soil quality standard, Pollution Control Department, Ministry of Natural Resources and Environment, Thailand (Thailand Pollution Control Department, 2021)

2020; Neeratanaphan *et al.*, 2020; Chowrong *et al.* 2022). Although this study revealed heavy metals in *O. lima* in both areas, they did not greatly exceed the Ministry of Public Health (2003) standard of contaminants in food quality standards. However, this study revealed that the heavy metal concentrations in *O. lima* from the municipal landfill were greater than those in the reference area. Heavy metals, aromatic compounds, halogens, phenols, and pesticides are toxic substances in leachate and may pollute both surface water and groundwater (Fernandes *et al.*, 2015). Excessive amounts of heavy metals in leachate can accumulate in plants and animals (Oman and Junestedt, 2008; Foo and Hameed, 2009; Nannoni *et al.*, 2015), and these toxins may enter the body through the human food chain and pose a threat to human health (Wang *et al.*, 2012), especially local communities that consume plants and animals surrounding municipal landfills. Thus, excessive amounts of heavy metals create a toxic environment for plants, animals, and humans. The potential impacts of heavy metals on this municipal landfill area are a concern. The leachate needs to be treated before being released into

the environment (He *et al.*, 2006). Leachate management is the most important part of solid waste management (Vrhovac *et al.*, 2013). The level and distribution of heavy metal contaminants should be assessed, studied, and monitored due to the impacts to the environment from human activities related to solid waste management in landfills (Biswas *et al.*, 2010).

3.3 DNA fingerprinting of *O. lima*

Table 3 shows that the 12 successful ISSR primers generated 535 total bands containing 83 characteristic and 61 polymorphic bands. Figure 2 shows the ISSR fingerprint profiles of *O. lima* from the municipal landfill and the reference area. The dendrogram results categorized the *O. lima* samples into two groups, representing the two areas. The first group corresponds to the municipal landfill, and the second group corresponds to the reference area (Figure 3). According to the dendrogram of *O. lima* in the study area, individual 4 was separated from individual samples 1, 2, 3, and 5, which had lower As and Cr concentrations (0.1864 ± 0.09 and

Table 2. Heavy metal concentrations in *O. lima* samples from the municipal landfill and reference area.

	Heavy metal concentrations (mg/kg)				
	As	Cd	Cr	Pb	Mn
Municipal landfill					
Individual 1	0.3451	0.0526	0.7542	ND	31.5737
Individual 2	0.1749	0.0096	0.3824	ND	6.3525
Individual 3	0.1523	0.0271	0.5483	ND	15.0466
Individual 4	0.1193	0.0108	0.3688	ND	6.0696
Individual 5	0.1405	0.0062	0.3722	ND	6.2030
Average \pm SD	$0.1864 \pm 0.09^*$	0.0213 ± 0.02	0.4855 ± 0.17	-	13.0491 ± 11.04
Reference area					
Individual 1	0.0058	0.0138	0.5477	ND	7.8891
Individual 2	0.0060	0.0344	0.4524	ND	13.0675
Individual 3	0.0136	0.0277	0.4023	ND	7.4659
Individual 4	0.1009	0.0421	0.5584	ND	26.4370
Individual 5	0.0907	0.0053	0.3712	ND	6.1196
Average \pm SD	$0.0434 \pm 0.05^*$	0.0247 ± 0.02	0.4664 ± 0.08	-	12.1958 ± 8.39
Standard**	2	0.05	2	1	-

Notes: ND = Not detected,

**Standard for contaminants in food according to the Notification of the Ministry of Public Health of Thailand No. 273/2, Ministry of Public Health, (2003),

* Indicates a significant difference between values in the same column ($p < 0.05$)

0.4855 ± 0.17, respectively) than the Cd, Pb and Mn concentrations, and the genetic similarity values of *O. lima* in the municipal landfill were lower than those in the reference area. The results of this study show that low

As and Cr concentrations in *O. lima* resulted in genetic differentiation. Maselli *et al.* (2019) and Guezgouz *et al.*, (2021) reported that heavy metals affect genetic changes in frogs.

Table 3. Twelve successful primer sequences for ISSR-PC fingerprinting.

No.	Primer	Nucleotide sequences	Total bands	Monomorphic bands	Polymorphic bands
1	P1	AGAGAGAGAGAGAGAGG	34	3	2
2	P3	CTCTCTCTCTCTCTGC	62	3	8
3	P4	CACACACACAAC	43	1	7
4	P5	CACACACACAGT	67	1	8
5	P6	CACACACACAAG	55	1	8
6	P7	CACACACACAGG	76	2	10
7	P11	GTGTGTGTGTGTCC	60	1	6
8	P12	CACCACCACGC	10	2	0
9	P13	GAGGAGGAGGC	33	2	1
10	P14	CTCCTCCTCGC	42	3	4
11	P17	GACAGACAGACAGACA	26	1	2
12	P22	CCCCGTGTGTGTGTGT	27	2	4
Total			535	22	61

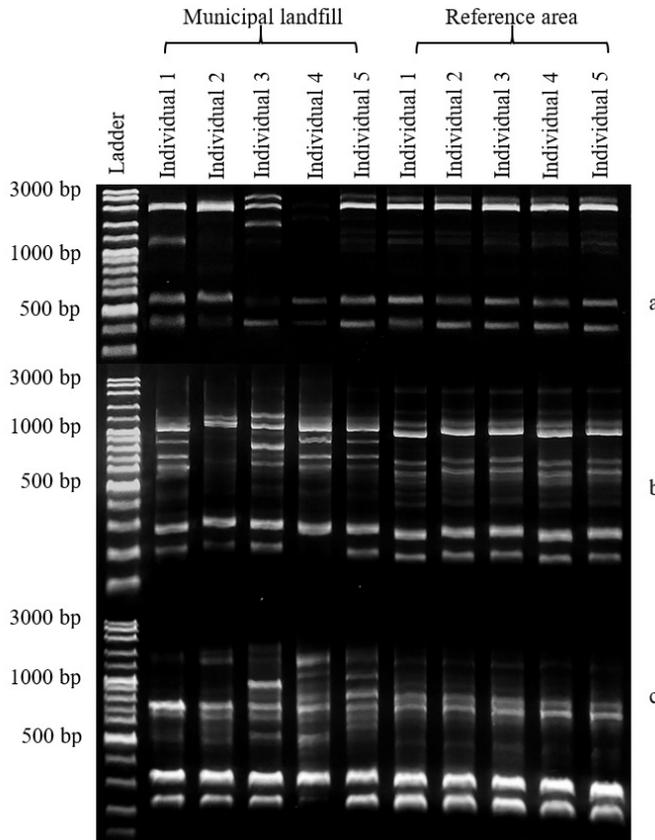


Figure 2. Examples of ISSR fingerprints of *O. lima* samples in the municipal landfill and reference area with the primers CTCCTCCTCGC (a), GTGTGTGTGTGTCC (b) and CACACACACAGG (c).

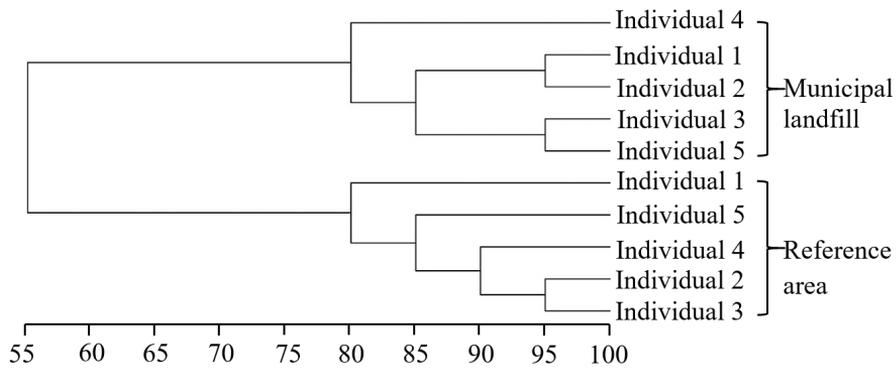


Figure 3. Dendrogram constructed from 12 primers demonstrating the genetic similarity of *O. lima* samples from the municipal landfill and reference area.

Table 4. The percentage of genomic template stability (%GTS) of *O. lima* samples from the municipal landfill and the reference area.

Area	%GTS				
	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5
Reference area					
Individual 1					
Individual 2	84.21				
Individual 3	80.70	93.10			
Individual 4	84.21	89.66	86.21		
Individual 5	84.21	86.21	82.76	86.21	
Municipal landfill					
Individual 1	52.63	53.45	48.28	55.17	48.15
Individual 2	49.12	51.72	48.28	44.83	40.74
Individual 3	52.63	51.72	48.28	48.28	44.44
Individual 4	50.88	50.00	46.55	43.10	42.59
Individual 5	50.88	58.62	53.45	55.17	55.56

3.4 Genomic template stability (GTS) of *O. lima*

Table 4 shows the %GTS of individual *O. lima* in the municipal landfill and the reference area. The %GTS range of *O. lima* from the reference area, 80.70 - 93.10%, was greater than that of *O. lima* from the municipal landfill, 40.74 - 58.62%.

The results of the ISSR fingerprinting of *O. lima* samples affected by municipal landfill leachate differed from those of the reference area samples. These characteristics were affected by heavy metals, consistent with the findings of Maselli *et al.* (2010) and Maselli *et al.* (2019), who reported that *Rana esculenta* was affected by heavy metal contamination in the leachate from municipal landfills. Landfill leachate can affect the DNA fingerprint

patterns of frogs. In addition, laboratory-scale studies by Maselli *et al.* (2019) and Kumer *et al.* (2014) revealed changes in the DNA fingerprints of organisms affected by heavy metal contamination in leachate from landfills; this causes bands of DNA to be missing or new. Similar to Erismis *et al.* (2013), who studied the blood cells of the frog species *Pelophylax ridibundus* affected by heavy metals, the DNA fingerprint characteristics differed from those of the control frog that was not affected by heavy metals. The mechanism of damage to frog DNA comprises heavy metal binding to the sulfhydryl (-SH) group of the receptor enzyme or coenzyme, leading to the inhibition of biochemical reactions and interfering with the biochemical reactions of DNA and RNA (US Department of Health and Human Services, 2007).

However, heavy metal ions can bind to the hydroxyl group of phosphate in nucleic acids, and the electrons are pulled closer to the oxygen and phosphorus atoms. This results in a positive charge, and the ester bond between the phosphate group and the hydroxyl group of the pentose sugar is broken (Guillamet *et al.*, 2004; Chanda *et al.*, 2006; Majumdar *et al.*, 2010). Hydrolysis has a negative effect on transferred RNA (t-RNA), which blocks DNA repair. The DNA cannot be repaired due to DNA strand breaks or abnormal control of the DNA methylation reaction. As a result, protein synthesis is stopped in cells, resulting in genetic abnormalities in the organism (Wilhelm *et al.*, 2010; Intamat *et al.*, 2016; Chowrong *et al.*, 2022). According to the results of the present study, the genetic similarity and %GTS of the frog samples from the municipal landfill were lower than those of the reference area, which might be affected by the presence of heavy metals and DNA damage. DNA damage affects DNA stability (McCulloch and Kunkel, 2008; Huang and Li, 2013).

The genetic stability of the frog *O. lima* cannot be maintained in water sources in the municipal landfill area because of heavy metal contamination. Therefore, *O. lima* from areas contaminated with heavy metal were less able to maintain genetic stability than *O. lima* from the reference area. The heavy metal concentrations in *O. lima* were greater than those in the reference area. If a frog has a high concentration of heavy metals, its genetic stability will be low. In a previous study, Guezgouz *et al.* (2021) and Maselli *et al.* (2019) reported on two species of frogs (*Bufo spinosus* and *Pelophylax klesculentus*). These studies revealed that the genetic stability of frogs decreased with increasing concentrations of heavy metals. The effects of heavy metals, such as oxidative DNA damage, DNA strand breaks, and the inhibition of DNA repair mechanisms, cause genetic damage in *O. lima* (Whiteside *et al.*, 2010, Ventura *et al.*, 2013). DNA damage increases with increasing levels of heavy metal accumulation in frog bodies. In addition, heavy metal toxicity induces a variety of mechanisms that respond to oxidative stress, inducing free radical and

Fenton reactions. The production of free hydroxyl radicals (OH) causes the destruction of macromolecules such as proteins, lipids, and DNA, resulting in cell death (Abarikwu *et al.*, 2017; Barnes *et al.*, 2019).

4. Conclusion

The As, Cr, and Pb concentrations in the water and the As concentration in the sediment from the municipal landfill exceeded the standards. The heavy metal concentrations in *O. lima* samples from the municipal landfill and reference area were within the standards. Low concentrations of As and Cr in *O. lima* resulted in high genetic differentiation. The %GTS in *O. lima* samples from the municipal landfill was lower than that in the reference area. The accumulation of heavy metals in frogs could lead to DNA structure changes and DNA damage. Heavy metal contamination in leachate from the municipal landfill induced genotoxicity in *O. lima*. Heavy metal contamination from this municipal landfill may directly affect the food chain and human health. The leachate needs to be treated before being released into surrounding the environment.

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