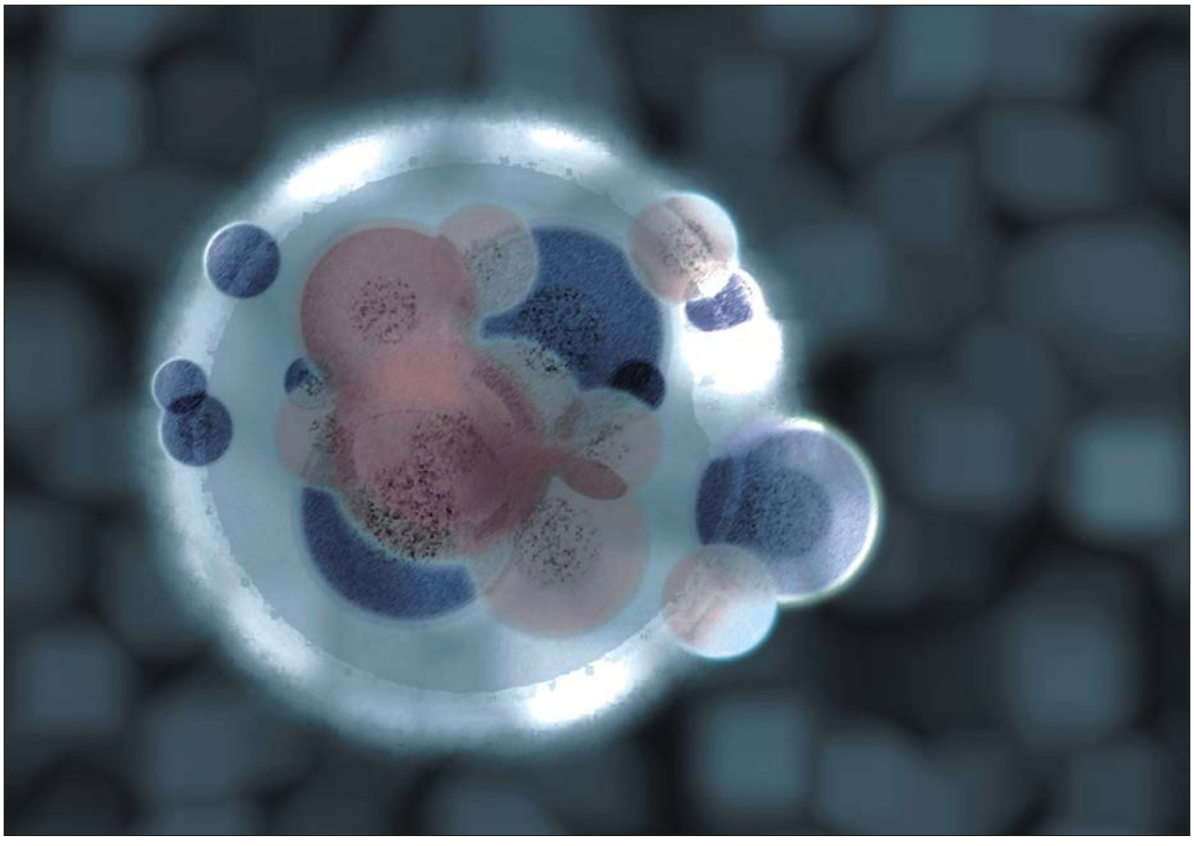

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Genetic Effects of Arsenic and Heavy Metals Pollutants on *Curculigo latifolia* (Lumbah)

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Abstract: Understanding plant response to chemical pollution is important for the management of a healthy ecosystem. Levels of arsenic and heavy metals and their genetic effects on *Curculigo latifolia* (Lumbah) growing in the Mamut River riparian zone were evaluated. *Curculigo latifolia* from Mamut demonstrated significantly higher levels of As, Cd, Cu and Zn compared to the same plant specimens from control site at Kipungit ($p<0.05$). Inverse Sequence-Tagged Repeat (ISTR) was used for estimating the genetic relatedness between *C. latifolia* accessions in Mamut and Kipungit. Genetic identity values (J) between Kipungit and Mamut accessions was 0.638 indicated that Mamut and Kipungit accessions were closely related. Shannon's diversity index for Mamut was 0.166 and for Kipungit it was 0.202, respectively indicating that Kipungit accessions were significantly more diverse than Mamut accessions ($p<0.05$). Partial MT gene was sequenced for *C. latifolia* from Mamut and Kipungit accessions. Average number of synonymous (d_S) and non-synonymous (d_N) substitutions per site in Mamut accessions was 0.31 and 0.22, respectively. These values are greater than the average number of d_S (0.11) and d_N (0.14) in the Kipungit accessions. The ratio of d_S/d_N in Mamut accessions was >1 indicating selection against most of amino acid changes and the high number of d_N in Mamut accessions may indicate a specific role of MT gene in adaptation to the environmental stress caused by arsenic and heavy metals.

Key words: Heavy metal pollution, genetic diversity, metallothioneins gene polymorphism, metal bioaccumulation

INTRODUCTION

Study of plant response to chemical pollution is important for the management of a healthy ecosystem. Heavy metals have been found to accumulate in surface organic layers and consequently affect the biological activity in forest soils because of their strong affinity to organic matter (Hernandez *et al*., 2003). The effects of metal contamination on living organisms have been documented, including the decrease of density, reduction in the number of sensitive species and change of species distribution pattern.

Understanding the effects of pollutants on the genome is of crucial importance to preserve the evolutionary potential of the endangered natural populations (Maes *et al*., 2005). There are few analyses of the relationship between the bioaccumulation of contaminants and the genetic diversity in natural populations (Van Straalen, 2000).

The ability of species to respond to novel environment and to disturbances caused by human activities depends on the extent of diversity and the kind of diversity that is available. Disruption of genetic equilibria has a direct bearing on the decline of diversity, subsequent increase of vulnerability to environmental stress and extinction of species (Bickham *et al*., 2000). Detection of changes in genetic diversity is a useful tool for monitoring the impact of human activities on natural populations, as they can detect impacts even in the absence of population extinction or after recolonisation (Carvajal *et al*., 2000).

Commonly used indicators of pollution effects, such as species diversity and population densities, will often return to normal shortly after removal of the pollutant(s), but do not reflect altered population gene pools (Bickham *et al*., 2000). There is a growing interest among ecotoxicologists in the extent to which contaminant exposure can alter allelic variations in populations (Benton and Guttman, 1992). The assessment the genetic diversity of populations could be a valuable addition to more traditional tools for determining the effects of environmental pollution (Nadig *et al*., 1998).

In short-lived plants there is abundant evidence that pollution changes the genetic constitution of populations specially when herbaceous or grassy plants are exposed to heavy metals (Geburek, 2000). Populations might

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respond with increased genetic variation resulting from new mutations directly induced by a mutagen, or decreased genetic variation resulting from population bottlenecks or selective sweeps that will also affect allele frequencies (up to fixation). In both cases, changes in allele frequencies can result as a consequence of adaptation to the contaminated environments (Bickham *et al*., 2000). There is an urgent need to develop and establish new toxicological approaches to asses the potential cytotoxic and genotoxic effects of heavy metals found in the environment (Parris and Adeli, 2002).

Curculigo latifolia (Lumbah) belongs to the family hypoxidaceae. It is a wild plant growing in Malaysia and India. It is a perennial herb with tuberous rhizomes. The plants grow about 3 feet high. Their lance-shaped, plicate (crinkled from top to bottom), evergreen leaves grow directly from the rhizome. The leafstalks are one-third the length of the leaves and they overlap one another at their bases to form a thick stem. Small bunches of flowers having six petals are produced in the axils of the leaves. It is usually found in forest near running fresh water. The plant characterized by a unique protein (Curculin) which exhibits sweet-tasting and taste-modifying activities (Yamashita *et al*., 1990). In Malaysia *C. latifolia* fruits are eaten to stimulate appetite and used to give sweet taste to the water in which they are steeped (Wiart, 2000). It is also used as traditional cure for jaundice. *In vitro*, extract of the rhizome inhibited hepatitis B virus conforming thereby a traditional use of the plant (Wiart, 2000).

The Inverse Sequence-Tagged Repeat (ISTR) analysis is a PCR-based DNA marker technology. In addition to the repetition of simple sequence motifs like in mini-and microsatellite DNAs, plant and animals genomes contain extended repetitive elements capable of transposition (Finnegan, 1985). The practical applications of ISTR in plants are apparently manifold and would range from general determination of biodiversity, characterization of DNA of wild-growing species, gene bank management, studies on population genetics, finger printing varieties for identification, tracking introgression of genes and systematic studies to possibly marker-assisted selection (Rohde, 1996).

Metallothioneins (MT) are low molecular weight proteins (6000-7000 Da) that have high cysteine content (up to 30%) (Chan *et al*., 2003; Kelly and Martin, 2004). MTs in plants contain a high percentage of cysteine sulfhydryl groups, which bind and sequester heavy metal ions in very stable complexes (Rauser, 1990). MTs are grouped into class I (MT1), II (MT2) and III (MT3). Class I and II are polypeptides of direct gene products, Class III (MT3) are non-translational cysteine-rich molecules named phytochelatins (Ma *et al*., 2003). MTs have been a focus in the plant tolerance research (Hoof *et al*., 2001). Evidence presented in the literature support the idea that MT is involved in detoxification of toxic non-essential metals such as cadmium (Chan *et al*., 2003). Metals ions are sequestered by MTs through complexation with-SH group of the cysteine rich motifs (Ma *et al*., 2003).

The goal of this study was to determine (1) the levels of arsenic and heavy metals and bioaccumulation ability of *C. latifolia* growing in the Mamut River riparian zone, (2) the effects of arsenic and heavy metals on genetic diversity of *C. latifolia* and (3) the effects of arsenic and heavy metals on MT gene polymorphism in *C. latifolia*.

MATERIALS AND METHODS

The study area: The study area was located at a 500 m stretch of the Mamut river at the Poring Park (6° 5' N, 116° 40' E) in Sabah, Malaysia (Fig. 1). The Mamut River has a width of approximately 5 m and with a water depth of 0.5-1 m. The river received effluents, mostly as runoff from the copper mine several kilometers upstream. Studies of the Mamut River by Lee and Stuebing (1990) and Ali *et al*. (2004) on the effect of copper mine runoff indicated that the river itself and soil in the riparian zone were contaminated by high level of several metals, notably copper.

The riparian zone at the study site was about 15-20 m wide and was covered with secondary rainforest. During stormy conditions, the river swells to flood this riparian zone where sediment carried downstream from the mine sites is deposited. To compare the results of the study, a control site, i.e., the Kipungit River was chosen. This control site was located 600 m from the Mamut River. The forest environment and soil conditions are similar to that of the Mamut River except that the Kipungit River is not affected by the copper mine discharge.

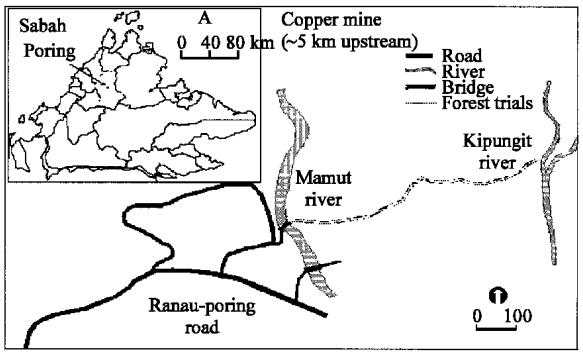

Fig. 1: The location of Mamut and Kipungit Rivers at Poring Hot Spring

Chemical analysis: For chemical analysis a total of 18 samples of *C. latifolia* were collected from the Mamut riverbank and same number was collected from Kipungit riverbank. The above ground parts of the plants (not including the roots) were used for chemical analysis.

The plant samples were carefully washed with deionized water and oven-dried at 60°C for 24 h. Twenty five milliliter of HNO_3 was added to 2 g of leaf tissue sample and heated gently for 30 min at 70-80°C. After cooling, 15 mL of $HClO_4$ (60% w/w) was added and the heating continued until dissolution of the sample occurred and the solution turned colourless. After cooling, it was filtered through a 0.45 μm membrane (Millipore) followed by dilution with distilled water before metal analysis. The concentrations of As and nine heavy metals (Cd, Cu, Pb, Zn, Cr, Ni, Fe, Mn and Co) in plant extracts were determined by using inductively coupled plasma optical emission spectrometer (ICP-OES) (Optima 4300 DV).

Extraction of genomic DNA: Samples of leaf materials of *Curculigo latifolia* (Lumbah) were collected from Poring area in Kinabalu Park, Sabah, Malaysia. The voucher specimen of *C. latifolia* has been deposited in the Herbarium of Kinabalu Park, Sabah. Twenty samples were collected randomly from Mamut riverbank and another twenty from Kipungit riverbank. Samples were kept in separate plastic bags, wrapped with moisturized tissue paper and kept in 4°C fridge. During transportation from Poring to University Kebangsaan Malaysia the samples were stored in Coleman box with ice bags. In the Laboratory, collected samples were kept in the refrigerator at 4°C until DNA extraction was carried out.

Genomic DNAs were isolated and purified using CTAB method (Doyle and Doyle, 1987). The extraction and purification was carried out by RNase hydrolysis, phenol-chloroform extraction and ethanol precipitation.

ISTR analysis: The ISTR primers consisted of 6 forward (F) and 6 reverse (B) primers as shown in Table 1. Initially, 36 combinations of ISTR primers pairs were tested with 20 ng template DNA. Primers (F3*B3) and (F2*B2A) produced good quality products and consistent fragment patterns. Therefore, only these two pairs were applied later in PCR reactions for all samples.

PCR reactions were performed according to Rohde (1996) in a final volume of 25 μL containing 40 ng of genomic DNA, 200 μM dNTPs, 2.5 mM $MgCl_2$, 1X PCR buffer, 2.5 pmole of each primer and 1 unit Taq DNA polymerase. Thermal cycles were consisted of preheating step at 95°C for 3 min, denaturing step at 95°C for 30 sec, annealing step at 45°C for 30 sec and an extension step at 72°C for 2 min for 40 cycles followed by a post extension at 72 for 10 min and then stored at 4°C.

Table 1: ISTR primers

Primer code	Sequence (5'→3')
F1	AGGAGGTGAATACCTTAG
F1A	TTTTCTACTTCATGTCTGAAT
F2	AAAATGGCATAGTCTCTC
F3	GTCGACATGCCATCTTTC
F4	TATAGTACCTATTGGGTG
F5	ATATATGGACTTAAGCAAGC
B1A	TTTTCTACTTCATGTCTGAAT
B2A	AATAAATCGATCATCGACTC
B2B	GGATATCCTATGAATCAAGC
B3	ATTCCCATCTGCACCAAT
B4	ATGTCATCCACGTACAAT
B5	CTTCTGTGAAAGTCCTAG

Three microliter PCR products were mixed with 3 μL loading dye and denatured at 95°C for 1 min. Then the mixture was immediately loaded into 7% polyacrylamide gel. The gels were run at 250V for 4 h. The gels were silver stained and visualized on a light box.

MT gene sequence comparison: Gene sequence of MT for *C. latifolia* was not available in Gene bank. MT sequence was obtained from two monocot species, *Saccharum* sp. (Genbank accession number CF577198) and *Zea mays* (Genbank accession number CD219210). The sequences were aligned in FASTA format using CLUSTALW program (Thompson *et al*., 1994). Alignment was made between *Saccharum* sp. and *Z. mays* sequences to identify the regions of homology. Left and right primers were designed from highly conserved regions using Primer3 program.

Using the designed primers PCR reaction was performed using 3 DNA samples of *C. latifolia* collected from Kipungit riverbank. In a final volume of 25 μL, the PCR reaction was optimized. Amplification was carried out successfully and the products were purified using QIAquick PCR purification kit (QIAGEN). The final products were sequenced by Macrogen Inc. Using BioEdit version 5.0.6 (Hall, 2001) the sequences were edited for end terming and base editing. Based on the sequencing results, new primers were designed. Using the newly designed primers, PCR reactions were re-optimized. The optimum PCR conditions obtained were applied to all samples. The PCR products were purified using QIAquick PCR purification kit (QIAGEN) and the products were sequenced by Macrogen Inc. Using BioEdit version 5.0.6 (Hall, 2001) the sequences were edited.

Data analysis

Genetic diversity parameters: ISTR amplified fragments were scored manually for band presence (1) or absence (0). Genetic diversity was calculated using phenotypic method of Shannon's index of diversity (King and Schaal, 1989). The presence (1) and absence (0) of fragments in

phenotypic method were treated as phenotypic characteristics because ISTR markers are dominant, therefore it was assumed that each band represented the phenotype at a single bi-allelic locus (Williams *et al*., 1990).

The degree of polymorphism for a particular population was quantified with shannon's diversity index:

$$H'_o = -(\sum \pi_i \ln \pi_i)$$

Where, π_i is the frequency of of phenotype I.

The average diversity over (n) populations was calculated as:

$$H_{pop} = (1/n) \sum H'_o$$

The total genetic diversity was calculated as:

$$H_{sp} = \sum(-\pi_i \ln \pi_i)$$

The proportion of diversity present within population was calculated as:

$$H_{pop}/ H_{sp}$$

and among populations as:

$$Gst = (H_{sp}-H_{pop})/H_{sp}$$

Using NTSYS-pc version 2.0 (Rohlf, 1990) genetic identity values were calculated using SIMQUAL (similarity for qualitative data).

Nucleotide sequence analysis: MT sequences were analyzed to obtain nucleotide composition, genetic distances, nucleotide substitution at synonymous and non-synonymous sites and conserved motifs. Nucleotide substitution at synonymous and non-synonymous sites were estimated by SNAP tool from Los Alamos HIV sequence database (http://hiv-web.lanl.gov) using the modified Nei-Gojobori Nei and Gojobori (1986) method. Nucleotide distance values were estimated according to Kimura (1980) two-parameter models among and between Mamut and Kipungit accessions using PAUP program version 3.1 (Swofford and Begle, 1993).

RESULTS AND DISCUSSION

Contamination and bioaccumulation of As and heavy metals in *C. latifolia*: *Curculigo latifolia* (Lumbah) from the Mamut riverbank was clearly contaminated by several heavy metals. The levels of Cd, Cu, Fe and Zn in *C. latifolia* collected from Mamut area were significantly higher when compared to that from the Kipungit riverbank ($p<0.05$) and this was consistent with the higher levels of these metals in the Mamut soil (Table 2). The metals content in *C. latifolia* samples from Mamut area was generally 5-9 times higher than that of Kipungit for Co, Cr, Cu, Fe and Ni. Other metals (As, Cd, Mn, Pb and Zn) were about 2 times or less compared to Kipungit samples.

Table 3 shows a comparison between the data obtained in this study with that of Freitas *et al*. (2004), which involved the analysis of 24 plant species from 13 families found in the Sao Domingos Mine in the south east of Portugal and that of Bech *et al*. (1997) for the analysis of eight plant species in Northern Peru.

Obviously, the levels of As and metals found in various plant species varied widely depending on the species and the location of the mine. Nevertheless, *C. latifolia* from the Mamut riverbank showed the lowest amount of As and metals compared to the plant species investigated in other copper mines. The content of As and metals in *C. latifolia* was also below the safe limit permissible by FAO/WHO for food (FAO, 1992) (Table 3).

Table 2: The average content of As and heavy metal concentrations in *C. latifolia* leaf samples collected from Mamut and Kipungit riverbanks

Metal ($\mu g\ kg^{-1}$)	Mamut	Kipungit
As	045.0±20	016.3±11
Cd	021.6±2	014.3±12
Co	150.0±44	020.0±13
Cr	340.0±300	065.0±30
Cu	3678.0±160	450.0±200
Fe	48320.0±720	9840.0±6300
Mn	06913.0±4600	6200.0±4800
Ni	0710.0±880	100.0±60
Pb	0073.3±46.0	030.0±11
Zn	3773.0±1110	1770.0±640

Table 3: A comparison of metal contents in various plant species from different mine sites

Metal	Bech *et al*. (1997) ($mg\ kg^{-1}$)	Freitas *et al*. (2004) ($mg\ kg^{-1}$)	Present study ($mg\ kg^{-1}$)	Permissible level by FAO (1992) ($mg\ kg^{-1}$)
As	112-5280	0.3-23.5	0.045±0.020	0.2-1.0
Cu	36-1880	3.6-28.9	3.700±0.160	5-30
Fe	(0.58-7.08)%	-	48.300±0.7	2-15
Mn	40-1490	-	6.900±4.6	-
Ni	-	1.1-7.8	0.700±0.9	-
Pb	-	2.9-84.8	0.070±0.05	0.3-2.0
Zn	61-604	12.2-343.2	3.800±1.1	5.0

Table 4: Genetic diversities of Kipungit and Mamut populations of *C. latifolia* estimated by Shannon's diversity index using ISTR marker

Populations	ISTR marker
Kipungit genetic diversity (M H'_o)	0.202
Mamut genetic diversity (K H'_o)	0.166
Mean genetic diversity of populations (H_{pop})	0.184
Total genetic diversity (H_{sp})	0.423
Genetic diversity within populations (H_{pop}/H_{sp})	0.436
Genetic diversity among populations (H_{sp}-H_{pop}/H_{sp})	0.564

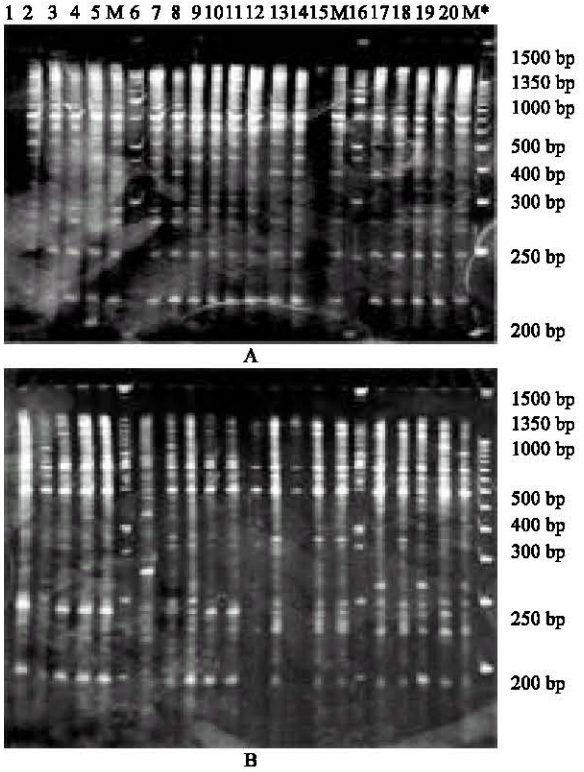

Fig. 2: ISTR analysis of *C. latifolia* using primers (A) F3*B3 and (B) F2*B2A. Lanes 1-10 represent Kipungit samples and 11-20 represent Mamut samples. Lanes M and M* contain 100 and 50 bp DNA ladder, respectively

ISTR analysis: ISTR primers generated a total of 72 reproducible amplification fragments ranging from 1.5 kb to 0.2 kb. A total of 65 loci out of 72 (90%) examined in Kipungit samples were polymorphic, whereas in Mamut samples 63 out of 72 loci (87.5%) showed polymorphism (Fig. 2).

The genetic diversity in Mamut and Kipungit populations was estimated using Shannon's index. Depending on primers used, the numbers of fragments yielded were different, F2*B2A generated a total of 33 bands, while F3*B3 gave 39 bands. The total genetic diversity (H_{sp}) was 0.423 and the mean genetic diversity of populations (H_{pop}) was 0.184. The genetic variability within populations (H_{pop}/H_{sp}) was 0.436 of total genetic diversity. Most of the variability was found among populations (0.564). The genetic diversity (H'_o) in Kipungit (0.202) was significantly higher than that in Mamut (0.166) ($p<0.05$). J-values were 0.720 for Mamut accessions and 0.661 for Kipungit accessions (Table 4).

Based on ISTR markers all sampled individuals had a unique genotype. This study showed higher levels of genetic diversity among populations compared to levels obtained within populations. The genetic diversity parameters obtained in this study indicated clearly that the reference site population was more diverse than polluted site population ($p<0.05$). Variation in genetic diversity among populations most likely is due two reasons. Firstly, to self pollination as self-pollinated species usually show much greater differences between populations (Tashida and Yoshimaru, 1996), secondly, due to effects of heavy metals.

Decrease in genetic diversity due to heavy metals pollution was previously observed in gastropod *Littorina brevicula* collected from heavy-metal polluted environments along the southeast coast of Korea (Kim *et al*., 2003), *Leander intermedius* and the isopod, *Platynympha longicaudata* in long term metal pollution in the Spencer Gulf, South Australia (Ross *el al*., 2002) and brown bullhead *Ameirus nebulosus* found in eastern North America (Mandrak and Crossman, 1992).

J value between Kipungit and Mamut accessions obtained from ISTR analysis was 0.638 indicating that Mamut and Kipungit accessions were closely related. This suggests the population in the two areas have developed from one population. The higher value of mean J of Mamut populations compared to Kipungit population showed that the individuals in Mamut were more closely related than individuals in Kipungit.

MT analysis: Using primer3 program Forward primer (ACATGGCTGAGCAGGTGAC) and Reverse primer (TGCCATAGCACGCAACTAAC) were obtained from two monocot species, *Sacchram* sp and *Z. mays*. PCR conditions were optimized using the designed primers. The optimum PCR conditions are summarized in Table 5 and 6. Based on sequence obtained from amplified fragments, a new Forward primer (GCGCTACAATGATGTGCCTA) and Reverse primer (GCCATAGCCACGCAACTAAC) were designed and applied to all DNA samples using the same optimum conditions used before with exception of annealing temperature which was changed to 48°C.

The partial MT gene of *C. latifolia* was 543 bp. The average of nucleotide composition of Kipungit sequences were 31.69% (A), 16.69% (C), 22.69% (G) and 28.93% (T)

Table 5: Optimum PCR reaction conditions for *C. latifolia* samples using Metallothionein designed primer

Reagents	Concentration
$MgCl_2$	2 mM
10X PCR buffer	1X
DNTPS (25 mM)	0.2 mM
Primer MT F (2.5 uM)	3 µM
Primer MT R (2.5 uM)	3 µM
TAQ (0.5 U)	1 U
DNA	20 ng

Table 6: Optimum PCR thermocycles conditions for *C. latifolia* samples using Metallothionein designed primer

Step	Cycles
Preheating	94°C (2 min)
Denaturing	94°C (1 min)
Aunealing	43°C (1 min, 35 cycles)
Extension	72°C (2 min)
Post extension	72°C (10 min)
Hold	4°C

while in Mamut were 30.47% (A), 17.87% (C), 23.32% (G) and 28.34% (T). Sequence similarity analysis with monocotyledons species showed that MT of C. *latifolia* shared 46.6, 42.3, 45.3, 41.2 and 44.8% similarity with MT genes of *Allium cepa*, *Alstroemeria peruviana*, *Crocus sativus*, *Sacchram* sp. and *Zea mays*, respectively.

CLUSTALW (Thompson *et al*., 1994), multiple alignments of sequences of Kipungit and Mamut accessions revealed SNPs. 13.3% of Kipungit and 38.1% of Mamut MT nucleotide sequences were polymorphic. The results showed that the average distance values among Mamut accessions was very high (0.128) compared to that of Kipungit accessions (0.032) ($p<0.001$).

Average of the number of d_S and d_N substitutions per site in Mamut accessions were 0.31 and 0.22 and in Kipungit accessions were 0.11 and 0.14, respectively (Table 6). Statistically the nonsynonymous substitution in Mamut accessions was significantly higher compared to that in Kipungit accessions ($p<0.05$). The selective pressure on MT gene can be determined from estimates of d_N/d_S (Yang and Nielsen, 2000). The ratio of synonymous substitutions to non-synonymous (d_S/d_N) of Kipungit was 0.80 and of Mamut was 1.44.

Similarity analysis revealed that MT of *C. latifolia* was 41 to 46.6% similar with MT genes of *A. cepa*, *A. peruviana*, *C. sativus*, *Sacchram* sp. and *Z. mays*. Comparison homology of MT genes sequences among these monocotyledons species showed that *A. cepa* shared 45.9, 44.8, 42.9 and 47.2% homology with MT genes of *A. peruviana*, Sacchram sp., *C. sativus* and *Z. mays*, respectively. *Sacchram* sp. showed homology of 45.5%, with *A. peruviana* and 68.9% with *Z. mays*. With exception of homology between *Sacchram* sp and *Z. mays*, homology analysis among MT genes was ranged between 42.2 to 47.2% same as that observed between *C. latifolia* and these species. Low levels of similarity and high genetic variations among MT genes of these different species are more likely due to direct interaction between this gene and the environment.

High level of MT polymorphism was in Kipungit accessions. Nucleotide distances results showed that average of distance among Mamut accessions was highly significant compared to the distance among Kipungit accessions ($p<0.001$). The results indicate that the Mamut accessions MT sequences were more diverse compared to Kipungit accessions. Low level of polymorphism and low genetic variation in Kipungit accession can be explained by the fact that, genes whose expression is under stabilizing selection should exhibit reduced genetic variation within species and reduced divergence within species (Nuzhdin *et al*., 2004).

Table 7: Mean synonymous and nonsynonymous substitution changes in Kipungit and Mamut accessions

Parameters	Kipungit	Mamut
The frequency of synonymous substitution per synonymous site (d_S)	0.11±0.04	0.31±0.3
The frequency of nonsynonymous substitution per nonsynonymous site (d_N)	0.14±0.01	0.22±0.17
d_S/d_N	0.80	1.44

The results of d_S and d_N showed that Mamut accessions have accumulated nonsynonymous substitution at a faster rate than the Kipungit accessions (Table 7). The d_N and d_S values indicating that MT gene was more conserved in Kipungit accessions compared to that in Mamut accessions. Mamut accessions showed a significant higher rate of d_N when compared to Kipungit accessions ($p<0.05$). Nuzhdin *et al*. (2004) reported that a substantial fraction of nonsynonymous substitutions had shown to be adaptive. The d_N value of Mamut may indicate a role of MT gene in helping the plant to adapt to the environmental stress caused by arsenic and heavy metals pollution or may indicate an accumulation of deleterious mutations.

Measure of ds/d_N provides a measure at molecular level of the selection intensity among amino acids sites (Lorenzo *et al*., 2004; Swart *et al*., 2004). A significant excess in the rate of non-synonymous substitution (d_N) of Kipungit compared to the rate of nearly neutral synonymous substitution (d_s) can be used as evidence that a sequence has evolved under positive selective pressure (Yang and Nielsen, 2000; Suzuki, 2004). Mamut accessions results revealed that $d_S>d_N$ indicating that most of the substitutions caused by arsenic and heavy metals were not deleterious.

CONCLUSIONS

In this study ISTR analysis proved to be an effective and a powerful tool for studying genetic variation within populations and among populations. The results showed an obvious link between pollution, bioaccumulation and genetic diversity. ISTR analysis indicated clearly that the control site population was significantly more diverse than polluted site population.

Using two monocot species, *Sacchram* sp. and *Zea mays*, MT gene sequence of *C. latifolia* was obtained. Metallothionein of *C. latifolia* showed higher levels of polymorphism in polluted area compared to the control site.

The results of synonymous (d_S) and non-synonymous (d_N) mutations showed that Mamut accessions have accumulated d_N at a faster rate than Kipungit accessions. The d_N and d_S values indicate that MT gene was more conserved in Kipungit accessions compared to that in Mamut accessions. In Mamut river bank *C. latifolia* accessions revealed that $d_S > d_N$, indicating that most of the substitutions caused by arsenic and heavy metals were not deleterious. The study demonstrated that assessment of genetic diversity parameters and screening candidate gene polymorphism can help in the understanding of the effects of environmental pollution on plant populations and their adaptability.

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