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Applications of inter simple sequence repeat (ISSR) rDNA in detecting genetic variations in *Lymnaea natalensis* snails from certain Egyptian Governorates

Hanaa M. M. El-Khayat*, Kadria M. A. Mahmoud, Hoda Abdel-Hamid and Hanaa M. Abu El Einin

Department of Environmental Research and Medical Malacology, Theodor Bilharz Research Institute, P. O. Box 30, Imbaba, Giza, Egypt.

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Inter-simple sequence repeat (ISSR)-PCR technique was used to assess genetic variation and phylogenetic relationships between *Lymnaea natalensis* collected from Giza, Ismailia, Damietta, and Beheira governorates in Egypt and compared with lab-bred snail in addition to characterization of watercourses from these sites. Five ISSR primers generated 47 amplified bands, of which 63.63% showed high polymorphism. All tested primers detected the common band (approximately 455 bp) in all *L. natalensis* studied. Three bands (318, 782 and 2013 bp for primers HB8, HB12 and HB13, respectively) are characteristic for *L. natalensis* collected from Ismailia, El Behira and Damietta governorates, respectively. These markers were used to estimate genetic similarity among the varieties using Jaccard's similarity coefficient. The similarity matrix was used to construct a dendrogram. The most abundant snails *Physa acuta* (56.0%) and the lowest abundant snails *Planorbis planorbis*, and *Melanoides tuberculata* (0.5) were found associated with *L. natalensis* in many governorates. *Eichhornia crassipes* is the only aquatic plant which grows and is found associated with *L. natalensis* in all canals of governorates. Also, dragon fly is the only macroinvertebrate collected from all canals, then shrimps and true bugs. Heavy metals were detected in water samples of all tested governorates with different significant differences. In conclusion, the present study used for the first time the ISSR PCR technique for studying genetic variations of *L. natalensis* snails in Egypt. *L. natalensis* snails can survive when associated with other snails, plants, and insects and can tolerate the heavy metals in water.

Key words: *Lymnaea natalensis*, inter-simple sequence repeat (ISSR)-PCR, dendrogram, heavy metals, macroinvertebrates.

INTRODUCTION

Fascioliasis is considered to be one of the most important parasitic diseases transmitted by freshwater lymnaeid snails and caused by *Fasciola hepatica* (*Lymnaea columella*) and *F. gigantica (Lymnaea natalensis*) (Mas-Coma et al., 2009). The Distribution of *Lymnaea* species in a given area is concerned with temperature and types of water body (De Kock et al., 2003). In South Africa, De Kock et al. (1989) reported that the most successful colonist of all freshwater snail species is *L. columella*, found associated with *Bulinus tropicus* and *L. natalensis*, and it is considered

the most widely distributed freshwater snail species in this region. In Egypt, Ibrahim et al. (1999) stated that *Lymnaea natalensis* is the most common species prevalent in Egypt compared to other species such as *L. columella, L. truncatula* and *L. stagnalis*. *L. natalensis* is found to live together with *Biomphalaria alexandrina* in most irrigation regions. Snail vectors were distributed according to different degrees of aquatic plants reflecting the degree of species preference plants for snails' life (Kader, 2001). In addition, Ashour et al. (2008) recorded that, morphometric analysis of *Lymnaea* spp. snails collected from different gover-norates revealed that the height, width, aperture and number of whorls of snails' shells belong to *L. natalensis* in all the surveyed Egyptian Governorates. The highest number of *Lymnaea* snails/site was collected from Giza (38 snails) while Kafr El-Sheikh has low number (8.8 snails). It was reported that Fayoum, Gharbiya, Damietta and Qaluobiya Governorates had the highest percentage of snail infection with *Fasciola.* They also found that *Lymnaea* snails were correlated with some factors such as prevalence of other snails and aquatic plants.

Molecular techniques such as random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR) analysis and DNA sequencing (Puslednik et al., 2009) have been extensively used as diagnosis tools as well as for the study of genetic structure, variations or relationships between populations of *Lymnaea* snails. Restriction fragment length polymorphism (PCR-RFLP) techniques were targeted to the first and second internal transcribed spacers (ITS1 and ITS2) rDNA and to the mitochondrial 16S ribosomal gene (16S rDNAmt) (Carvalho et al., 2004).

Inter-simple sequence repeat polymerase chain reaction (ISSR)-PCR technique was chosen for assessing genetic variation in the populations of *L. natalensis* snails. ISSRs are used as simple sequence repeats anchored at the 5'- or 3'-end by a short arbitrary sequence as PCR primers (Zietkiewicz et al., 1994). They are considered as powerful tool for genetic mapping and assessment of genetic diversity between closely related species and also to detect similarities between and within species as well (Moreno et al., 1998; Ghariani et al., 2003).

The main advantage of ISSRs is that no sequence data for primer construction are needed; only low quantities of template DNA are required. Among the techniques that can be used to ascertain population structure, PCR-ISSR has proven to be outstanding in the analysis of natural populations of many plant (Taheri et al., 2012), fungus (Priyanka et al., 2013), insect and vertebrate species (Wolfe, 2005).

The main objectives of the present study were to assess genetic variation and genetic pattern of *Lymnaea* snails collected from four different Governorates and to compare them with laboratory breeding snails using ISSR markers, with the characterization of environmental parameters on their collecting sites.

MATERIALS AND METHODS

Study area and sampling

The freshwater canals in the geographical areas; Giza (Talkhaneya canal), Ismailia (Bort-Said canal), Damietta (Al-Sinania canal) and El- Beheira (El-Kandak El Sharky canal) governorates in Egypt were investigated for their biological and ecological parameters. Biological samples included macroinvertebrates and aquatic vegetation were collected from water canals starting from April to November (2011).

Ecological survey

An ecological survey was carried out in a total of 14 sites representing the four examined canals. Aquatic plants and macroinvertebrate samples including *L. natalensis* snails were collected from the examined sites by using a net, 15 cm deep and a mesh size of 3 mm. For each examined site five consecutive dips were taken according to Takougang et al. (2008). At each site during collection, water temperature, conductivity and pH were recorded and the aquatic plants were gathered. The collected macroinvertebrates and water samples were transferred to the Environmental Research and Medical Malacology Department at Theodor Bilharz Research Institute (TBRI). The medically important snails were examined immediately for any evidence of infection and the water samples were filtered and prepared for chemical analysis.

Biological assessment

In the laboratory, the macroinvertebrate organisms were sorted and taxonomically identified to the lowest possible taxonomic level according to Bouchard (2004) and Leska (1998). The water quality of the canals was biologically assessed using macroinvertebrates' information according to Sullivan (2007). Six comparable matrices were adopted, species richness (equals the total number of individuals represented within the sample), *Ephemeroptera, Plecoptera*, and *Trichoptera* (EPT) index (equal to the total number of individuals represented within these three pollution sensitive insect orders in the sample), ratio of EPT to Chironomidae (is calculated by dividing EPT index by the total number of individuals classified as Chironomidae the more tolerant, ratio of the scraper and filtering collector index) (this index is independent of taxonomy and is calculated by dividing the total number of individuals classified as scrapers by the total number of individuals classified as filtering collectors within the sample), contribution percent of major macroinvertebrate group (equals the abundance of the numerically dominant major group relative to the total number of

*Corresponding author: E-mail: fab201355@yahoo.com

organisms in the sample) and the sixth one was Hilsenhoff Biotic Index (HBI). Hilsenhoff Biotic Index (HBI) developed by Hilsenhoff (1982) and modified by Hilsenhoff (1987) was used to summarize the organic pollution tolerance of water body critters; values of HBI range of 2-4 indicate slightly enriched, 4-7 enriched, and 7-10 polluted. It is calculated by dividing the sum of multiplies of the number of taxa of the same taxonomical group by their tolerance value (values ranging from 0 to 10 based on tolerance to organic pollution) then dividing by the total number of organisms within the sample. The bio-assessment was compared between macroinvertebrate matrices of each site and the community of the reference site, which was carefully selected. Percent of matrices similarity of each site to the reference one was calculated and awarded 6, 4, 2, 0 points and total points were determined.

Analysis of water samples

The prepared water samples from different sites of the canals were analyzed by graphite furnace and flame of an atomic absorption spectrophotometer (AVANTA, Pal 3000) to determine the concentration of the elements: cadmium (Cd), lead (Pb), copper (Cu), manganese (Mn), iron (Fe), nickel (Ni), sodium (Na), potassium (K) and calcium (Ca).

DNA extraction

Snail feet of the collected *L. natalensis* from each canal in different governorates and lab-bred *L. natalensis* snails were collected from Giza Governnerate and maintained in the Medical Malacology laboratory for several years were dissected, fixed in 70% ethanol and maintained at 4°C till used. Genomic deoxyribonucleic acid (DNA) was extracted from one snail using phenol / chloroform method as described by Vidigal et al. (1994). The foot of lymnaeid snail specimens were suspended in 400 µl of lysis buffer (10 ml Tris-HCl, pH 8.0, 100 ml EDTA, 100 ml NaCl, 1% sodium dodecyl sulfate SDS) containing 500 µg/ml Proteinase K (Promega, Madison, WI, USA) and digested for 1 h at 37°C with alternate shaking each 15 min. The extraction was then performed with phenol-chloroform and DNA was precipitated with ethanol. The pellet was dried and resuspended in 30 µl sterile TE buffer (pH 8.0). This suspension was stored at -20°C until use. DNA integrity and concentrations were estimated by comparison with molecular weight standard on 0.7% agarose gel electrophoresis. Genomic DNA of different groups was subjected to polymerase chain reaction (PCR) using primers (HB8, HB10, HB11, HB12, and HB13). Primers sequencing were: HB8: $(GA)_6$ GG, HB10:$(GA)_6$ CC, HB11:$(GT)_6$ CC, HB12:$(CAC)_3$ GC, HB13:$(GAG)_3$ GC.

PCR conditions

A total PCR reaction volume of 50 µl contained the following: 5 µl DNTPs (2.5 mM), 5 µl $MgCl_2$ (25 mM), 5 µl Buffer (10 x), 4 µl Primer (10 pmol), 0.5 µl Taq DNA polymerase (250 U) (Promega), 5 µl template DNA (250 ng) and 25.5 µl H_2O (d.w). The amplification was carried out in a DNA PTC100TM system using the following cycling conditions: predenaturation of DNA at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for s, annealing at 55°C for 1 min, and extension at 72°C for 1 min and a final extension of 20 min at 72°C. PCR reactions were analyzed through electrophoresis using 1.5% agarose gel electrophoresis.

Data analysis

The data are presented as mean ± standard deviation for the calculation concentration of heavy metals. The heavy metals, temperature, conductivity, HBI and total points parameters were compared statistically with one way ANOVA test by using SPSS computer program, version 20 for Microsoft Windows, 2007.

ISSR-DNA fragments were scored as 1 or 0 for the presence or absence of bands, respectively. The obtained data were subjected to analysis with GelAnalyzer3 (Egygene) software. The level of similarity between species was established as the percentage of polymorphic bands, and a matrix of genetic similarity was compiled using Jaccard's similarity coefficient (Jaccard, 1908). Similarity coefficients were used to construct the dendrogram using the unweighted pair group method with arithmetic average (UPGMA). All calculations were performed with the NTSYS-pc 2.02 software package (Rohlf 2000).

RESULTS

Results of distribution of bio specimens and their association with *L. natalensis* are represented in Table 1. A total of 221 freshwater snail specimens were collected from 14 different sampling sites along four canals, each represents one governorate. Snails were identified as 125 *Physa acuta* (56% of the total snail sample), 28 for each *Lanistes carinatus* and *Cleopatra bulimoides* (12.7%), 17 *L. natalensis* (7.7%), 9 *Theodoxus niloticus* (4.1%), 7 *Bulinus truncatus* (3.2%), 3 *Succinea cleopatra* (1.4%), 2 *Bellamya unicolor* (0.9%) and 1 for each of *Planorbis planorbis*, and *Melanoides tuberculata*, (0.5%). The spatial distribution of snails showed that *L. carinatus* had the higher distribution and was collected from 9 sites (64.0 %), followed by *L. natalensis* that was collected from 6 sites (42.9%), *P. acuta* and *C. bulimoides* were collected from 5 sites (35.7%), *B. truncatus* was collected from three sites (21.0%), *B. unicolor*, *P. planorbis* and *T. niloticus* were collected from 2 sites with 14.3% and *S. cleopatra* was collected from one site with 7.0%. The most abundant snails *L. carinatus* was found in association with *L. natalensis* in many governorates, while in El-Behira Governorate, three species: *Physa acuta,Theodoxus niloticu*s and *Cleopatra bulimoide*s were found associated with *L. natalensis* snails. In relation to aquatic plants, *Eichhornia crassipes* the only aquatic plant which grows and is found in associations with *L. natalensis* in all canals of governorates, followed by *Jussiaea repens* which found in all Governorates except El-Behira governorate (Table 1). Also, Dragon fly is the only macroinveertebrate collected from all canals, followed by shrimps, true bugs and fishing spider was not collected from Damietta governorate (Table 1). In contrast, Water boatman, *Plecoptera Stoneflies* and *Corbicula consobrina* were collected only from Damietta governorate. El-Beheira governorate possessed the highest amount of species of collected macroinvertebrates (15 species) while the lowest were Ismailia and

Table 1. Distribution of biospecimens in the examined canals represented Egyptian Governorates and their association percentage with *Lymnaea natalensis* (frequency of bio-specimen coexistence with *L. natalensis* / No. of sites of *L. natalensis*) x100).

Bio-specimen		Governorates (no. of sites)	% of Distribution					% of Association
			Giza (4)	Damietta (3)	Beheira (4)	Ismailia (3)	Total (14)	
Biospecimens	Aquatic plants	*Eichhornia crassipes*	50	33	50	100	57	83 (5/6)
		Jussiaea repens	50	100	0	100	57	67 (4/6)
		Ceratophyllum demersum	0	67	0	33	21.4	33 (2/6)
		Bermuda grass	0	0	0	0	7.1	17 (1/6)
		Myriophyllum spicatum	0	0	50	0	14.3	0/6
		Typha angustata	0	0	75	0	21.4	17 (1/6)
		Cyperus alopecuroides	25	0	0	0	7.1	17 (1/6)
		Potamogeton sp.	25	0	0	33.3	7.1	0/6
	Snails (total number)	*Lymnaea natalensis*	75(4)	33 (1)	25(11)	33(1)	43(17)	
		Cleopatra bulimoides	0	100 (20)	50(8)	0	36(28)	17 (1/6)
		Lanistes carinatus	25(2)	100 (17)	50(2)	100(7)	64(28)	67 (4/6)
		Physa acuta	50(112)	67(11)	25(2)	0	21(125)	67 (4/6)
		Bulinus truncatus	0	67(4)	0	33(3)	21(7)	33 (2/6)
		Bellamya unicolor	25(1)	33(1)	0	0	14(2)	33 (2/6)
		Planorbis planorbis	0	33(1)	0	0	7(1)	0/6
		Succinea cleopatra	25(3)	0	0	0	7(3)	17 (1/6)
		Theodoxus niloticus	0	0	25(2)	33 (7)	14 (9)	17 (1/6)
		Melanoides tuberculata	0	33(1)	0	0	7 (1)	17 (1/6)
	Macroinvertebrates (total number)	Snails(Scraper, 7)	67(122)	100(56)	67(25)	100(18)	86(221)	
		Stoneflies (predator, 1)	0	33.3{1}	0	0	7.1 (1)	17 (1/6)
		Dameslfly (predator, 5)	0	0	50(6)	0	78.6 (6)	0/6
		Dragon fly (predator, 5)	25(1)	100 (5)	0	33 (1)	35.7(7)	50 (3/6)
		Riffle beetle (scraper, 4)	0	0	25(4)	100 (7)	28.6(11)	17 (1/6)
		Water boatman (predator, 6)	0	33.3(1)	0	0	7.1(1)	17 (1/6)
		Water strider (predator, 6)	50(5)	0	0	0	14.3 (5)	33 (2/6)
		Water scorpion (predator, 7)	25 (1)	0	0	33(2)	14.3(3)	33 (2/6)
		Back swimmer (predator,9)	0	0	25(1)	0	7.1 (1)	33 (2/6)
		True bugs (predator,8)	75(12)	0	100(14)	100 (14)	71.4 (40)	67 (4/6)
		Corbicula consobrina (collector filtering, 5)	0	33.3(2)	50(9)	33(1)	21.4 (12)	0/6
		Shrimps (collector filtering, 4)	25(6)	0	50 (2)	100 (20)	35.7 (28)	17 (1/6)
		Cray fish (predator,5)	25(3)	0	25 (2)	0	14.3 (5)	0/6
		Fishing spider (predator, 4)	25(5)	0	50(3)	33.3(1)	28.6 (9)	17 (1/6)
		Oligochaeta (collector gathering, 8)	25(1)	0	50 (22)	0	21.4 (23)	33 (2/6)
		Chironomidae (collector gathering, 6)	0	0	0	33.3(1)	7.1 (1)	0/6
		Leeches (Predator,9)	0	0	25 (2)	0	7.1 (2)	17 (1/6)
		Maggot larvae (shredder, 10)	25(2)	0	0	0	7.1 (2)	17 (1/6)

Table 2. Physico-chemical parameters and biological assessment of the examined canals represented Egyptian Governorates.

Heavy metal	Giza	Ismailia	Damietta	El Behira
Cd (ppp)	0.52±0.11	0.22±0	1.31±1.04	0.68±0.33
Pb (ppp)	14.66±2.29	6.10±1.02	152.95±242.36	35.37±37.03
Cu (ppp)	22.39±14.27	133.56±142.48	26.81±8.34	36.32±22.06
Na (ppm)	21.61±6.69^{C}	108.51±21.83^{B}	38.29±20.26^{C}	27.47±6.08^{C}
K (ppm)	6.29±0.24^{C}	3.84±0.15^{C}	9.89±3.42^{B}	6.55±0.43^{C}
Ca (ppm)	12.43±0.53^{C}	9.36±0.44^{C}	12.86±0.33^{C}	17.41±3.74^{B}
Mn (ppp)	10.16±6.63	21.55±18.93	6.51±1.71	14.94±13.91
Fe (ppp)	16.63±17.30	23.90±14.29	11.25±5.47	73.05±67.97
Ni (ppp)	9.73±2.16^{C}	25.44±7.24^{B}	6.07±0.33^{C}	6.53±1.73^{C}
Temperature	32.50±1.29^{A}	23.00±0.00^{C}	31.33±1.53^{A}	25.33±0.58^{B}
Conductivity	309.33±38.40 ^{C}	433.33±2.89^{C}	630.00±160.93^{B}	320.00±9.85^{C}
HBI	6.80±0.32 Enriched	5.90±0.82 Enriched	6.70±0.26 Enriched	6.60±0.88 Enriched
T points	9.50±4.43 moderately impaired	12.67±6.43 moderately impaired	13.33±8.08 moderately impaired	17.50±11.12 Slightly impaired

B, C= highly significant at p<0.01 (ANOVA), ppp= part per billion, ppm= part per million.

Damietta governorates (12 species).

Levels of the examined heavy metals (mean ± SD), the recorded physico-chemical parameters and biological evaluation in all water courses sites are displayed in Table 2. The analysis of variance recorded high significance differences (p<0.01) in the mean concentrations of sodium (Na), potassium (K), calcium (Ca) and nickel (Ni) among the tested governorate samples (Table 2). On the other hand, the heavy metals cadmium (Cd), lead (Pb), copper (Cu), manganese (Mn) and Fe (Ferrous) with no significant changes at p>0.05, Damietta Governorates showed the highest level of Cd and Pb (1.31±1.04 and 152.95±242.36, respectively) while Ismailia showed the highest level in Cu and Mn (133.56±142.48 and 21.55±18.93, respectively). Also, the temperature and conductivity recorded highly significant variation between the four mentioned governorates.

According to the present macroinvertebrate information, El-Tawfikia site in El-Khandak El-Sharky canal, Beheira governorate was determined as reference site and awarded 34 points. So, values of total points of other sites were calculated relative to reference site as values greater than 28 were evaluated as non-impaired, between18-28 slightly impaired, 7-17 moderately impaired and less than 7 points were evaluated as severely impaired. HBI and total points showed no significant changes at p>0.05 (Table 2).

The levels of different physicochemical parameters were compared in sites of *L. natalensis* and those free of them (Table 3). The present results show that there was no relation between the level of heavy metals, temperature and HBI and the distribution of *L. natalensis* as its sites were characterized in some cases by the higher levels: Giza sites. Results of conductivity reveled that all the free sites were characterized by the higher levels. Also, the biological evaluation using macroinvertebrate information showed that *L. natalensis* tolerated moderately impaired water quality till total points equal to 8.3 and did not tolerate severely impaired habitats (<7 points).

Genetic diversity, a similarity coefficients and dendrogram tree among the *L. natalensis* snails collected from 4 governorates (Giza, Ismailia, Damietta and El Behira) and compared with Laboratory snails, were investigated by ISSR-PCR technique. All of the tested primers successfully amplified products from genomic DNA. The number of amplified, monomorphic and polymorphic bands generated by each primer is shown in Table 4.

A total of 47 amplification fragments, ranging from 188 to 2103 bp in size was detected from five ISSR primers depending on the origin of the collected snails and the primer tested (Figure 1). The highest total number of bands (11) was obtained using primers HB10 and HB13, while the lowest number (7) was obtained using primer HB12. By using all primers and according to the total number of amplified bands in each snail group, Ismailia and Damietta snails showed the highest number of bands

Table 3. Physico-chemical parameters and biological assessment (expressed by HBI, organic pollution (OP), and water quality evaluated by macroinvertebrate information (WQ) of sites that included *Lymnaea natalensis* and those free from them in the examined canals represented Egyptian Governorates.

Site		Cd (ppp)	Pb (ppp)	Cu (ppp)	Na (ppm)	K (ppm)	Ca (ppm)	Mn (ppp)	Fe (ppp)	Ni (ppp)	Temp.	Cond.	HBI	OP	T. points M	T. points SD	WQ
Giza	*L. natalensis*	1.7	44.6	53.4	74.7	19.2	37.7	32.0	62.5	27.1	33.0	309.3	6.9	E	10.7	2.4	M
	Free	0.4	14.0	36.2	11.7	6.0	12.0	8.7	4.1	11.8	31.0		6.5	E	6***	2.4	S
Damietta	*L. natalensis*	0.9	15.3	26.1	19.2	10.0	12.5	7.5	13.6	6.0	31.0	450.0	6.8	E	12	3.1	M
	Free	1.5	221.8	27.2	47.8	9.8	13.0	6.0	10.1	6.1	31.5	720.0	6.7	E	9.0*	2.7	M
Beheira	*L. natalensis*	0.5	90.8	21.4	26.7	6.7	18.6	22.7	25.0	4.3	25.0	331.0	7.7	P	10	2.7	M
	Free	2.2	50.6	1.5	83.2	19.5	51.0	37.1	267.2	21.8	25.0	629.0	7.1	P	10.3ns	2.7	M
Ismailia	*L. natalensis*	0.0	7.1	298.1	84.5	3.7	8.9	15.2	32.3	33.2	23.0	430.0	7.1	P	9.8	3.0	M
	Free	0.1	5.6	51.3	120.5	3.9	9.6	24.7	19.7	21.6	23.0	435.0	7.1	P	10.1ns	2.3	M
Total	*L. natalensis*	0.8	39.5	99.8	51.3	9.9	19.4	19.4	33.4	17.7	28.0	380.1	7.1	P	10.6ns	4.8	M
	Free	1.1	73.0	29.1	65.8	9.8	21.4	19.1	75.3	15.3	34.1	594.7	6.9	E	8.9	9.6	M

E = Enriched, P = polluted, M = moderately impaired, S = severely impaired. T= temperature. *,***Significant difference between sites with *L. natalensis* and those free of them, at $p<0.05$ and 0.001, respectively. ppp= part per billion, ppm= part per million

(11), while the lowest number (seven bands) was detected in El Behira groups. All tested primers revealed the percentage level of polymorphisms between *L. natalensis* snails collected from four governorates and laboratory snails ranging from 0.1% for primer HB11 to 63.63% for primer HB13. Three monomorphic bands were detected (318, 782 and 2013 bp) for primers HB8, HB12, HB13 respectively. These bands are characteristic for *L. natalensis* collected from Ismailia, El Behira and Damietta governorates respectively. All tested primers (HB8, HB10, HB11, HB12, and HB13) detected approximately the common band found in all groups of snails (452, 455, 463, 495 and 466 bp, respectively). This band was considered to be characteristic bands for *L. natalensis* snails.

The similarity coefficients estimated among the *L. natalensis* snails groups ranged from 0. 6-0.95. The highest value (0.95) was recorded between the Ismailia group and the highest value between Ismailia and Giza (0.95) while the lowest (0. 6) was between Giza and laboratory snails groups (Table 5). The UPGMA dendrogram (Figure 2) showed three main clusters. The first one including two groups Damietta and El Behira, the second one included two groups Giza and Ismailia and the third major group included only the laboratory snails group.

DISCUSSION

The evaluation of the freshwater surface using biological assessment depending on macro-invertebrate information was more efficient in defi-ning the polluted sites (El- Khayat et al., 2011a).

Table 4. Total number of amplicons and the level of polymorphism among the five snail populations as revealed by ISSRs.

Primer	Total number of amplified bands	Polymorphic amplified bands	Percentage of polymorphism bands
HB8	8	3	37.5
HB10	11	2	18.18
HB11	10	1	0.1
HB12	7	3	42.85
HB13	11	7	63.63

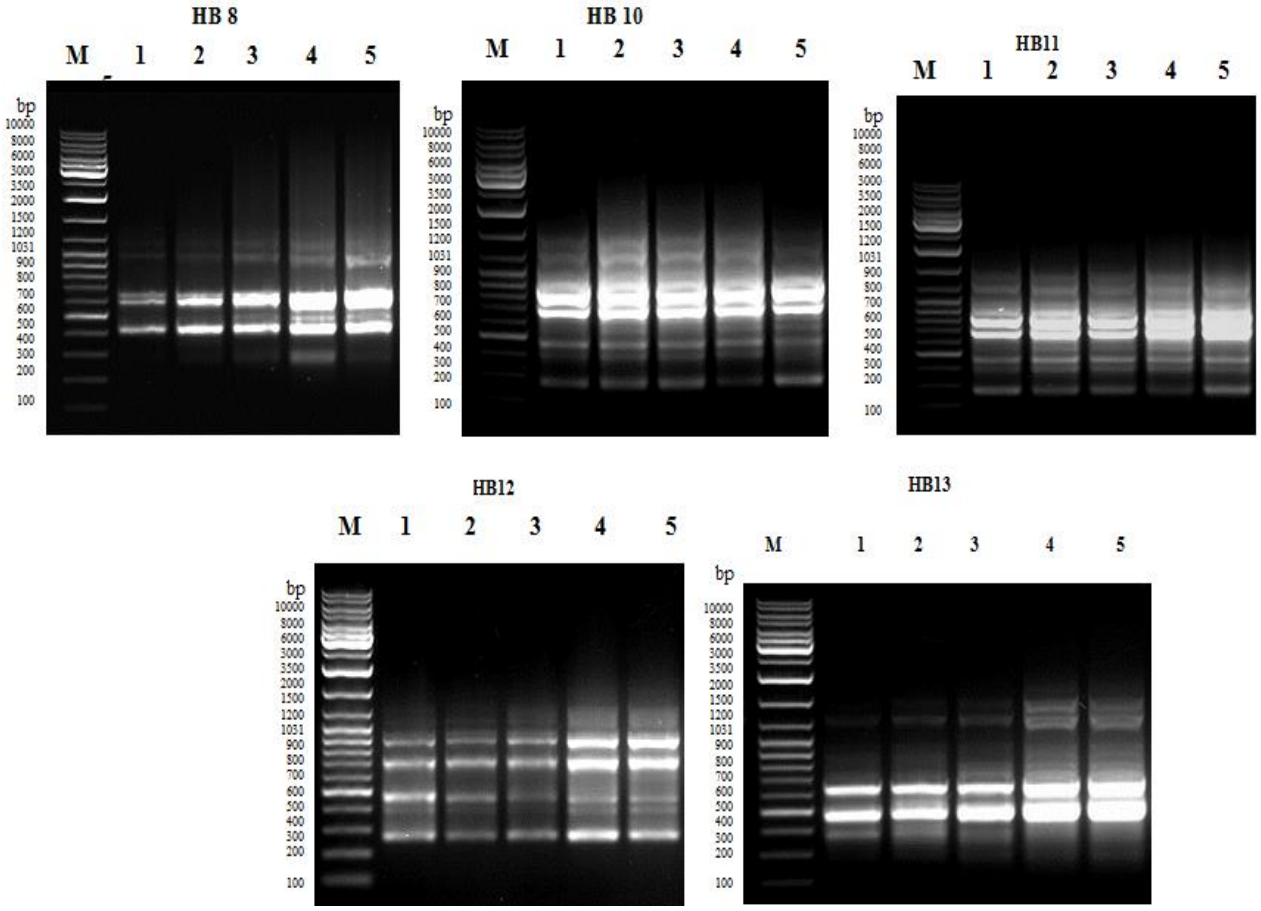

Figure 1. PCR products of genomic DNA from *Lymnaea natalensis* snails collected from 4 Governorates with ISSR primers. Lane M, DNA marker; lane 1, laboratory snails; lane 2, Giza; lane 3, Ismailia; lane 4, Damietta; lane 5, El Behira Governorates.

This was in accordance with EPA (2011), Mandaville (2002), Karr and Chu (2000) and Bartram and Balance (1996) who indicated that the biological assessments provide direct measures of the cumulative response of the biological community to all sources of stress to which the organisms were exposed over a period while chemical assessments are designed to protect the biological community of a water body from different toxic levels of pollutants, is valid only for the instance in time when the sample was collected.

In this study, the toxigenic cadmium (Cd), lead (Pb), copper (Cu), cobalt (Co), sodium (Na), potassium (Ka)

Table 5. Genetic similarity indices between *Lymnaea natalensis* snails collected from four Governorates and Laboratory snails based on ISSR fragment analysis.

Collection	Laboratory snails	Giza	Ismailia	Damietta	El Behira
Laboratory snails	--	0. 6	0.75	0.82	0.77
Giza		--	0.95	0.91	0.83
Ismailia			--	0.84	0.86
Damietta				--	0.91
El Behira					--

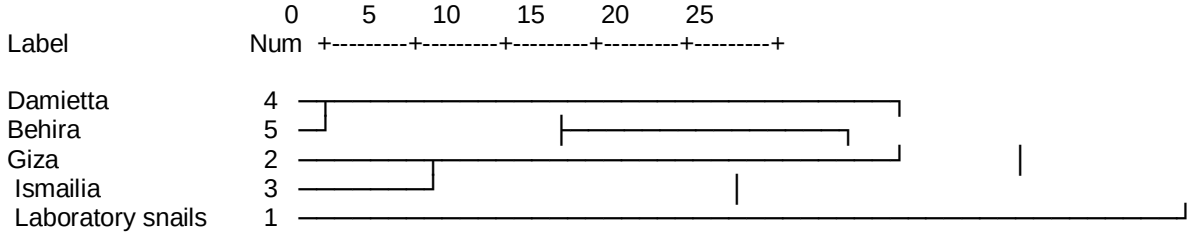

Figure 2. Dendrogram demonstrating the diversity and relationships among *Lymnaea natalensis* snails collected from 4 Governorates and Laboratory snails based on ISSRs

manganese (Mn), ferrous (Fe) and nickel (Ni) were found in all tested canals, Giza (Upper Egypt), Ismailia (Canal Cities), Damietta and Beheira governorates (Lower Egypt). Witt (1982) assumed that in the developing countries 80% of all human illness is associated with polluted water. The pH values play an important role in the interaction between heavy metals and many parameters. Toxicity with heavy metals can increase with basic pH values (Mandour and Azab, 2011). Cadmium is found in surface water as a pollutant from industries (Mandour and Azab 2011). This agreed with the same results found in Damietta governorate which is famous for wood industries and recorded high levels of Cd. Also, Damietta branches receive the water from a number of agricultural drains, which are heavily polluted by industrial and domestic sewage (Abdel Wahaab and Badawy, 2004).

Recently, freshwater snails and bivalves have been used frequently as bioindicator organisms and in several studies of chemical contaminants (Mostafa et al., 2013). In this study, in spite of the accumulations of heavy metals in canals, *L. natalensis* snails could survive. El-Khayat et al. (2011a) found that *L. natalensis* were more tolerant to Cd, Fe, Ni and Mn and more sensitive to Cu than other snails while all snails could live at approximately the same concentrations of Na, K and Ca. Siwela et al. (2010) reported that at elevated levels of heavy metal accumulation, *L. natalensis* snails could survive through some physiological modifications. They found that *L. natalensis* responded to pollution by increasing the activity of catalase and selenium-dependent glutathione peroxidase specific activity in an effort to detoxify peroxides produced as a result of metal-induced oxidative stress.

In this study, the conductivity was measured in all sites and all the free sites were characterized by the higher levels indicating that it may be a limiting factor in *Lymnaea* distribution. Also, the biological evaluation using macroinvertebrate information showed that *L. natalensis* tolerated moderately impaired water quality till the total points equal to 8.3 and did not tolerate severely impaired habitats (<7 points). El-Khayat et al. (2011b) determined by macroinvertebrate matrices that *L. natalenthsis* showed intermediate pollution tolerance between *Biomphalaria alexanrina* and *Bulinus truncates*; severely impaired sites constituted 23% of *B. alexandrina* habitats, 14% of *L. natalensis* and 9.4% of *B.truncatus* sites. Hilsenhoff Biotic Index (HBI) showed the same tolerance to organic pollution.

To analyze various species of *Lymnaea* snails, there are many published reports on the use of molecular marker techniques such as RAPD (Puslednik et al., 2009; Bin Dajem, 2012; Koneva, 2013), RFLP (Carvalho et al., 2004) and SSU rDNA (Bargues et al., 1997). Moreover, Yang et al. (1994) reported that ISSR assay can provide more informative data than other techniques. The present

investigation clearly demonstrated that *L. natalensis* snails collected from different governorates could be distinguished by ISSR primers for the first time in Egypt. Segments of DNA from *L. natalensis* can be reproducibly amplified by short primer range of 10-20 bp nucleotides with the ISSR technique, showing a high level of polymorphism (63.63% for primer HB13). Seven polymorphic bands and the total number of amplified bands were 11; obtained with primer HB13. Thus, this primer may be useful in differentiating between different *L. natalensis* snails throughout the governorates.

In another related study on *B. glabrata* snails, Larson et al. (1996) reported that the identification of polymorphic bands was based on a comparison of banding patterns on the same gel for each of the six snail groups investigated. This suggests that ISSR markers were polymorphic markers suitable to detect the genetic diversity of snails at the DNA level. All tested primers (HB8, HB10, HB11, HB12, and HB13) detect approximately the common band found in all groups of snails (452, 455, 463, 495 and 466 bp). The apparent of this band was considered to be genetic characteristics bands for *L. natalensis* snails and lymnaeid snails from only one source.

In conclusion, the present study considered for the first time the use of ISSR PCR technique in studying genetic variations of *L. natalensis* snails in Egypt. ISSR primers showed a high level of polymorphism between collected snails.

Conflict of interests

The authors did not declare any conflict of interest.

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