Journal of the Egyptian Society of Parasitology, Vol. 47, No. 3, December 2017
J. Egypt. Soc. Parasitol. (JESP), 47(3), 2017: 575 – 588

SELECTION FOR CADMIUM-TOLERANCE IN *BIOMPHALARIA ALEXANDRINA* SNAILS CHARACTARIZATION BASED ON GENETIC STUDIES, AND SUSCEPTIBILITY TO *SCHISTOSOMA MANSONI* ASSOCIATION

By

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Abstract

Biomphalaria alexandrina snails were examined for their tolerance to cadmium (Cd) toxicity through three generations produced by self-fertilization by exposing snails to serial concentrations of Cd and selection for the most tolerant ones through the successive generations. Results showed a gradual increase in the LC_{50} & LC_{90} values via successive generations indicating tolerance upgrading phenotype. Also, the selected and non-selected snails were examined for susceptibility to *Schistosoma mansoni* and hematological criteria. *B. alexandrina* showed refractory tendency with significant increase in hemocytes; granulocytes; indicating that defense mechanism was stronger than other ones. The dendro-gram based on the similarity matrices of ISSR-PCR banding patterns resulted with ten random primers, showed that the selected groups of the 1st and 2nd generations were the most similar and clustered together forming a distinct group that in turn cluster with non-selected laboratory group that in turn cluster with parent group (field, non-selected), while the 3rd selected generation was the most divergent group, indicating its genetic drift. The mating system and selection succeeded in evolution of Cd tolerance snail isolate; characterized genetically, physiologically, showed refractory tendency to *S. mansoni* infection.

Keywords: *Biomphalaria alexandrina*, toxicity curve, cadmium-tolerance, genetic selection, hemocytes, *Schistosoma mansoni*, infection, ISSR, similarity, dendrogram.

Introduction

Generally, molluscs play a role in the balance of nature, and also act as biological indicator, in determining the degree of pollution of water and terrestrial environment. The genus *Biomphalaria* was intensely studied due to its relation to the schistosomiasis (Barbosa and Coimbra, 1979). Moreover, *Biomphalaria* has been used for more than 30 years for toxicological studies and as a bio-indicator for environmental monitoring studies. Many aspects make this genus a good model for laboratory studies; has a wide geographical distribution, low dispersion and is easily collected, easy to breed, need little space, can reproduce throughout the year under controlled conditions and have a short life-span (Tallarico *et al*, 2012). Heavy metals are common environmental pollutants to the aquatic organisms. Several studies have documental that *Biomphalaria* exposed to Cd continued to be reproductively active and no significant effect of heavy metal exposure was demonstrated (Abd Allah *et al*, 1997). This tolerance to acute metal levels may result from genetic selection (genotypic) which might be expressed phenotypically; or through an adjustment of biochemical or physiological mechanisms (phenotypic) without a genetic basis (Lam, 1996). Irrespective of the basis, mechanisms of metal tolerance in aquatic invertebrates may involve in reducing uptake or increasing excretion, leading to low accumulation (Bryan and Gibbs, 1983) or storage in inert forms, which may be coupled with enhancing uptake (Brown, 1978 and Depledge and Rainbow, 1990).

As a consequence of studies by Richards and Merritt, 1972; Richards 1973, 1977; 1984; El-Khayat *et al.* (2005) and Saber *et al.* (2007) it was concluded that the susceptibility of *Biomphalaria* species to *Schistosoma mansoni* infection is a inheritable trait that genetically controlled. Characterizing of host and parasite population genetic struc-

ture and estimating gene flow among populations depending on allozyme analysis and molecular tools was essential to understand co-evolutionary interactions between host and parasites (Jrane and Theron, 2001; Charbonnel *et al*, 2002; Prugnolle *et al*, 2005; El-Khayat *et al*, 2007; Wethington and Lydeard, 2007; Mohamed *et al*, 2012; Standley *et al*, 2014).

The snail immune-biology is strongly related to the circulating cells in the hemolymph or hemocytes and to humoral factors within the snail plasma (Lie, 1982; Loker and Bayne, 1986; Van der Knaap and Loker, 1990). Two main types of hemocytes are found in the *Biomphalaria* hemolymph: the granulocytes and the hayalinocyte. Some authors suggest that the hayalinocyte and the granulocytes are distinct cell types, while others consider that they represent different developmental phases of the same cell type. Seasonal factors and diverse physiological experimental conditions affect the total number, type and behavior of the hemocytes and the capacity of mollusks to respond strongly to stimuli depends on hemocytes viability and functional capacity (Oliver and Fisher, 1995). In this way, the number of hemocytes may vary according to certain stimuli, such as responses to infection by trematodes (Sullivan *et al*, 1984). Oliveira *et al*. (2010) recorded a differential behavior of defense cells that may be related to the resistant or susceptible phenotype, significant change of the number of granulocytes, while no statistically significant differences for the hayalinocyte. Kambale and Potdar (2010) found that the number of granulocytes was more in freshwater snail *Bellamya bengalensis* than the bivalve *Lamellidens marginalis*, which indicates that their humeral defense mechanism was stronger. Wright *et al*. (2017) succeeded for the first to identify and characterize an H^+ channel in *B. glabrata* hemocytes that may function in immune defense responses against larval *S. mansoni*.

Several studies (Newton, 1963; Sullivan e*t al*, 1984; Jelnes, 1987; Yasuraoka e*t al*, 1987; Saber *et al*, 2005; 2007, El-Khayat and Gawish, 2006; El-Khayat e*t al*, 2005; 2008) isolated different snail lines at laboratory scales by self-fertilization and selections through many generations for certain characters susceptibility to infection, tolerance to chemical or plant molluscicides and different mantle pigmentation.These authors determined genetic alterations in these selected snail isolated by several techniques, enzymatic, protein and molecular. Inter simple sequence repeat polymerase chain reaction (ISSR)-PCR technique was used as simple sequence repeats anchored at the 5`- or 3`-end by a short arbitrary sequence as PCR primers (Zitkiewicz e*t al*, 1994). It was considered as a 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 e*t al*, 1998; Ghariani e*t al*, 2003; Szenejko *et al*, 2016). Also, The ISSR technique has been reported as a good marker to differentiate between geographically different *Lymnaea natalensis* populations and a laboratory isolate (El-Khayat *et al*, 2015).

The present study investigated *Biomphalaria alexandrina* for their tolerance to cadmium (Cd) toxicity by exposing snails to serial concentrations of Cd and selection for the most tolerant ones through successive generations in a trial to isolate Cd tolerant snail line. Selected tolerant and non-selected progenies were tested and compared for their susceptibility to *S. mansoni* infection, hematological analysis through total and differential hemocytes count and by molecular analysis through similarity matrices based on PCR-ISSR markers.

Material and Methods

Chemicals: Cadmium chloride: were supplied by Sigma-Aldrich, United Kingdom.

Snail samples: *B. alexandrina*, the interme diate host of *Schistosoma mansoni* were collected from irrigation canals in Giza Governorate, thoroughly washed and maintained

under laboratory conditions in plastic aquaria, fed on green lettuce leaves for 4 weeks before being used as parent. During this period snails were examined weekly for their natural schistosome infection by exposure to a light source for an hour to detect any cercarial shedding.

Maintenance of snails: Adult parental snails were selected as healthy negative for natural infection of uniform size of 10-12 mm. Each 50 snails were maintained in plastic aquaria containing 5 liters of dechlorinated tap water, which was changed weekly and snails were fed on boiled lettuce twice a week.

Estimation of cadmium (Cd) toxicity: Serial concentrations of Cd (0.5, 2, 4, 6, 8, &12 ppm/liter in glass beakers) were done in 3 replicates and 10snails/replicate was added. Another set with three replicates was done using dechlorinated tap-water only as a control. Exposure and recovery periods were 24 hours each and mortality counts were recorded and corrected according to (Abbott, 1925). Mortality regression lines were established by SPSS Computer Program 20.0.

Tolerant snail selection: All survivors in the Cd concentration >4 ppm were maintained individually to produce the next generation by self-fertilization, in plastic aquaria; each containing 5 liters of de-chlorinated tap water. Egg clutches were allowed to be hatched and the newly hatched snails reared in the same aquaria with their parents. Snails were fed on boiled lettuce twice a week, water has been changed monthly but release of departs and complete the water volume was done weekly. Adult 1st generation snails from tolerant parents were tested for their Cd tolerance and selection and isolation for the most tolerant snails was done like their parents to produce the second generation that in turn produced the third generation with the same manner. For each Cd exposure toxicity test starting from the first generation, another test was done using negative mature field *B. alexandrina* of 10-12 mm size at the same time and conditions. Mortality regression lines were established by SPSS Computer Program and used as a control.

Susceptibility to *Schistosoma mansoni* infection: Two groups the selected *B. alexandrina* of the 3rd generation and lab breeding snails (as control) of the same size 5-7mm were examined for susceptibility to *S. mansoni* infection. Each group was exposed individually to 7-10 freshly hatched *S. mansoni* miracidia that supplied by Schistosome Biological Supply Program (SBSP) at Theodor Bilharz Research Institute (TBRI). Snails were put in contact with miracidia overnight and then snails of each group were washed thoroughly and maintained in a separate aquarium under laboratory conditions. Water temperature was maintained between 24-26^{o}C throughout the period of experiment. Snails were tested weekly for shedding cercariae from 25 day post-exposure by exposing them, individually, to fluorescent light in 2ml of water for 2 hours at 25°C. Cercarial suspension of each snail was counted using Bouns' fluid and cercarial production/ infected snail were recorded (Parh and James, 1977).

Hematological analysis: Changes in hemocytes of *B. alexandrina* as to their total and differential hemocytes count (granulocyte and hayalinocyte) were determined in snail samples from parents and their tolerant progenies of 1st, 2nd & 3rd generations. Hemolymph samples were obtained from examined snails by puncturing the previously cleaned shell (with 70% ethanol) between the first and second inner whorls. The number of hemocytes/mm^{3} of hemolymph was counted in a Burker-Turk hemocytometer (Van der Knaap *et al*, 1981) for total count. Monolayers of hemocytes were prepared and stained with Giemsa's stain for 20min according to the method of Abdul-Salam and Michelson (1983). Light microscopy preparation of hemolymph smears were fixed in methanol and stained with Giemsa stain (Cheng and Guida, 1980), then differential hemocytes counts were performed (Brown,

1980). Data were presented as mean±S.E. (M) and n represented the number of snails used in the experiment. Statistical calculations were based on analysis of variance (ANOVA). Differences were considered significant when $P < 0.05$.

Molecular analysis: Extraction: Snail feet from the five parents snail groups, 1st, 2nd & 3rd generations and Lab breeding were dissected free, fixed in 70% ethanol and maintained at 4°C till used. Genomic deoxyribonucleic acid (DNA) was extracted from five snail samples (Junghans *et al*, 1990). From each sample, 0.1g to 0.5g of foot snail tissue was ground in a motor and pestle in liquid nitrogen until a fine powder was obtained. After grinding, thawing of ground tissues was prevented and transferred to 1.5ml Eppendorf tube. Then 700µ of extraction buffer was added and mixed well. Tubes were incubated at 4°C for 10min and then centrifuged at 12000r.p.m for 10min. The supernatant was transferred to a new sterile Eppendorf tube & 500µ of phenol: chloroform: isoamyl at a ratio of 25:24:1was added to the supernatant and mixed. The tubes were centrifuged at 12000r.p.m for 5min, then the aqueous phase was transferred to a new sterile tube and 750µ of cold isopropanol were added and mixed then incubated at 4°C for 20min. Then, tubes were centrifuged for five min. to aggregate the DNA pellets then were washed in 70% ethanol and left to dry for about 30min. The pellets were re-dissolved in 100µ TE buffer then RNAase (5units/µl) for each sample 10µl were added to remove RNA from samples and was incubated at 37°C for two hours to have pure DNA which was kept in refrigerator till use. DNA concentrations were measured by UV-Spectro-Photometer at a wave length of 260-280nm. DNA integrity and concentrations were estimated by comparing with molecular weight standard on 0.7% agarose gel electrophoresis.

PCR: Genomic DNA of different groups was subjected to polymerase chain reaction (PCR) using primers. ISSR-PCR reactions were conducted using randomly selected ten ISSR primers, for the genotypes of snails (Tab. 1). Amplification was carried out in stratgene PCR 96 which was programmed as follows: Denaturation (one cycle) 94°C for 2min; followed by 30 cycles: as follows 94°C for 30 second, 44°C for 45 second, 72°C for 1 min; and finally one cycle extension at 72°C for 20 min; and 4°C (infinitive). Gel Electrophoresis: 15µl of PCR-product were resolved in 1.5% GTG agarose gel electrophoresis with 1x TAE running buffer. The run was performed at 80 V for 180 min, and the gel was stained with ethidium bromide. A marker of 1 Kb plus DNA ladder 1µg/µl (Invitrogen) with a total of twenty bands ranging from 12000 to 100bp was used. Bands were detected on UV- Tran illuminator and photographed by gel documentation system UVP2000.

Table 1: ISSR primers names and their sequences.

Primer name	Sequence	Primer name	Sequence
197887A	$(CA)_6AG$	HB11	$(GT)_6CC$
844A	$(CT)_8AC$	HB12	$(CAC)_3GC$
844B	$(CT)_8GC$	HB13	$(GAG)_3GC$
HB8	$(GA)_6GG$	HB14	$(CTC)_3GC$
HB10	$(GA)_6CC$	HB15	$(GTG)_3GC$

Results

Toxicity to cadmium (Cd) against parent *B. alexandrina* and their progenies: Toxicity of Cd was examined in adult *B. alexandrina* snails as parents and their selected Cd tolerant progenies through three generations. The presented results showed values of LC_{50} & LC_{90}, 95% confidence lower and upper limits and slopes of mortality regression lines obtained by probit analysis using SPSS program. It has been shown that there is a gradual increase from survivor tolerant Cd parents to the selected tolerant progenies till the third generation, LC_{50} of parents was 2.452 ppm increased to 4.861, 8.466 and 11.5 ppm in the first, second and third generations,

respectively. Consequently, LC_{90} of parents was 4.2, increased to 8.4, 16.2 & 17.5 ppm in the three successive generations, respectively. On the other hand, LC_{50} & LC_{90} values of control snails of the first, second and third generations were fluctuated in narrow range (LC_{50} ranged from 2.548 to 4.6 & LC_{90} ranged from 4.667 to 8.2ppm) suggesting that control snails showed approximately the same Cd tolerance in the three experiments.

Hematological analysis: Two types of blood cells were distinguished under light mic-roscopy; granulocyte and hayalinocyte. Granulocytes are granular (spreading) hemocytes, measuring 20-25µm in diameter, had plentiful cytoplasm with numerous pseudopodia, irregular nucleus and adhere to glass. Hayalinocyte was morphologically round cell with lower nuclear-cytoplasmic ratio, measuring 12-15µm in diameter and markedly basophilic cytoplasm with abundant dense granules. Comparing the total cells count of parent, 1st, 2nd & 3rd snail generation groups after exposing to Cd, showed a high significant increase in the 3rd generation than the parent one with significant alteration in differential count from parent snails started from 1st generation through the three generations. Details were given in tables (1 & 2) and figures (1, 2, 3 & 4).

Table 2: Probit analysis of mortality of parent field *B. alexandrina*, their selected Cd tolerant progenies through three generations and corresponding field controls in ppm).

Snail groups	Tested					Controls				
	LC_{50} (ppm)	upper limit	lower limit (ppm)	LC_{90} (ppm)	Slope	LC_{50} (ppm)	upper limit	lower limit	LC_{90} (ppm)	Slope
Parents	2.452	3.26	1.9	4.2	1.7					
1st generation	4.861	5.55	4.17	8.4	1.5	4.132	5.33	3	6.2	1.39
2nd generation	8.466	10.88	6.5	16.2	1.67	4.6	5.99	3.5	8.2	1.69
3rd generation	11.5	14.26	9.27	17.5	1.54	2.548	3.625	1.72	4.667	1.84

Table 3: Comparison of hematologic parameters in parents and their progenies through successive generations, G1: 1st generation, G2: 2nd generation and G3: 3rd generation.

Types of cells M±SD	Parents	G1	G2	G3
Total cells count	1250 ±141.4	1550 ± 282.8	2300 ±1060.6	2375**±106.1
Granulocytes	57.5 ±0.71	79*** ±0	68.5** ±0.71	64.5** ±0.7
Hyalinocytes	42.5 ±0.71	21*** ±0	31.5** ±0.71	35.5** ±0.7

*significance between parent and each generation 1st, 2nd & 3rd (*significant, **high significant ***more highly significant)

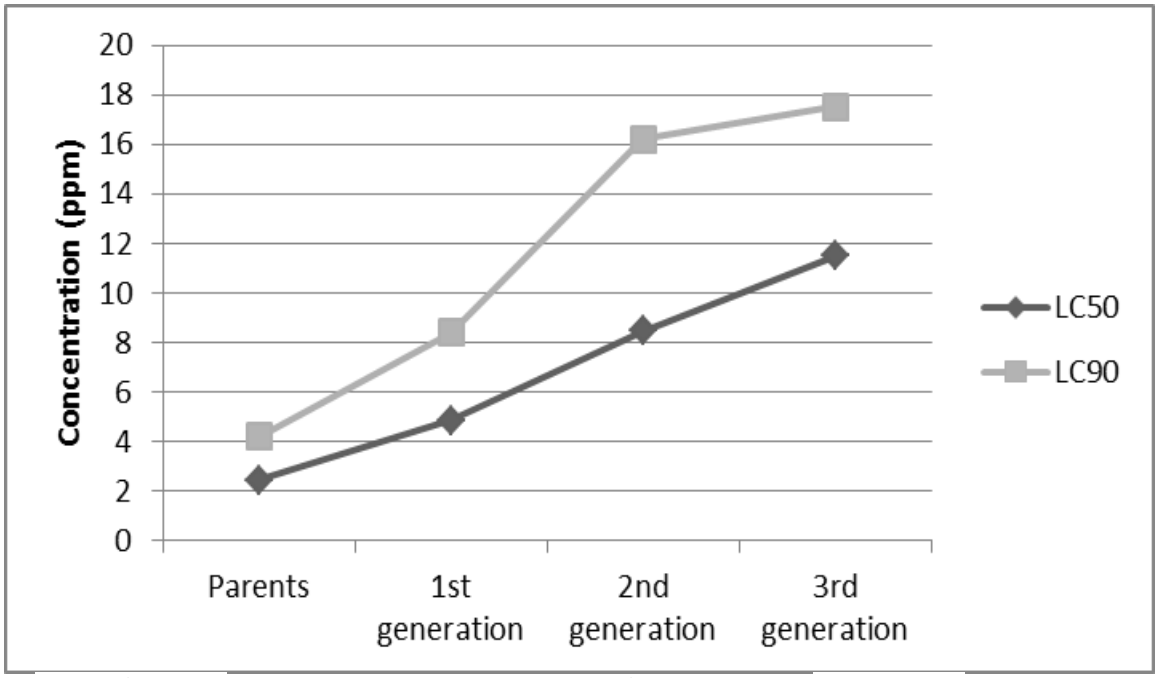

Fig. 1: Toxicity of Cd against *B. alexandrina* snails and selected progenies after 24 h exposure LC_{50} and LC_{90} lethal concentrations.

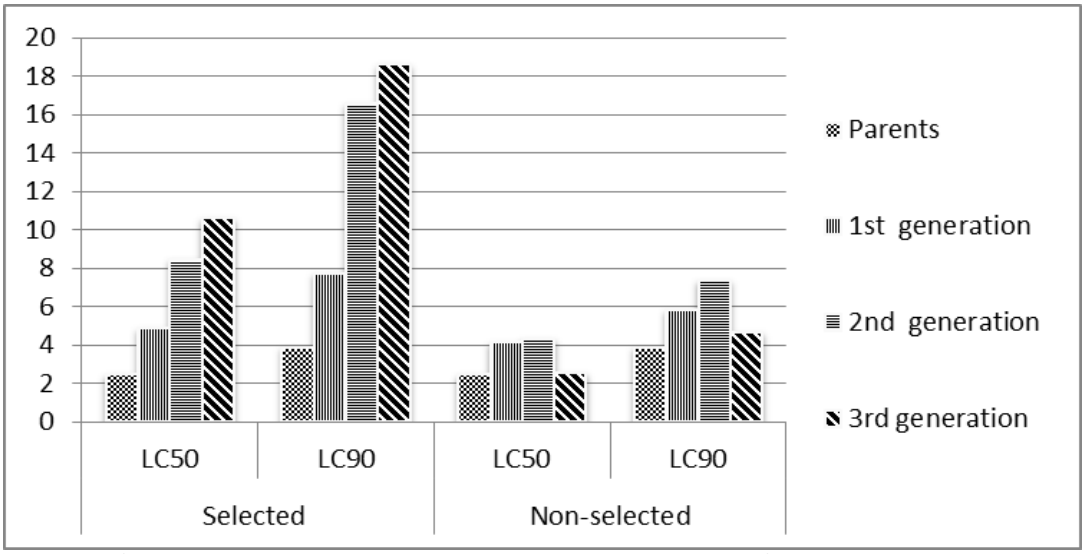

Fig. 2: Comparison of LC_{50} & LC_{90} between parents' *B. alexandrina* and selected progenies and field *B. alexandrina* as a control when exposed to Cd**.**

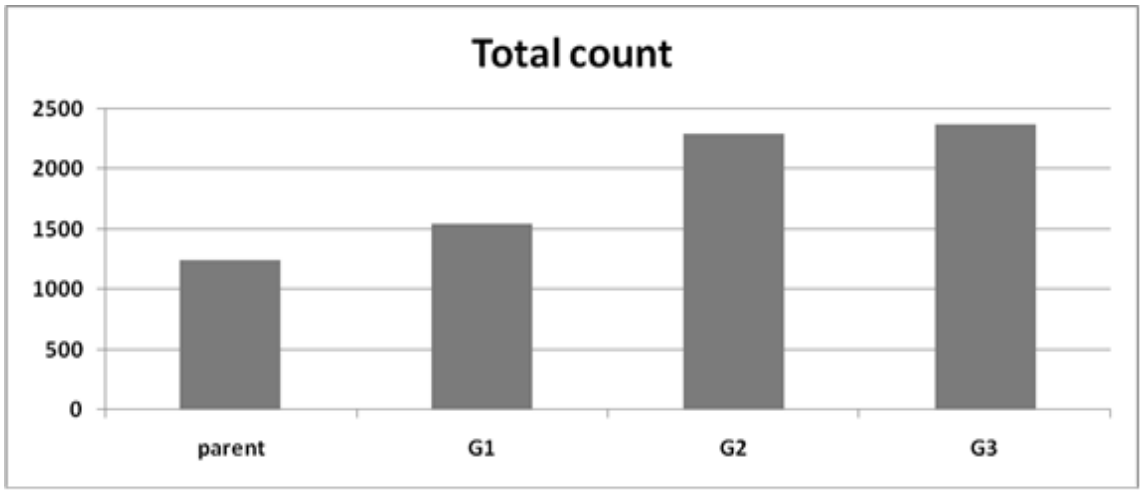

Fig 3: Total blood cells count in hemolymph samples collected from *B. alexandrina* parent snails and their progenies by successive generations, G1: First generation, G2: Second generation and G3: Third generation**.**

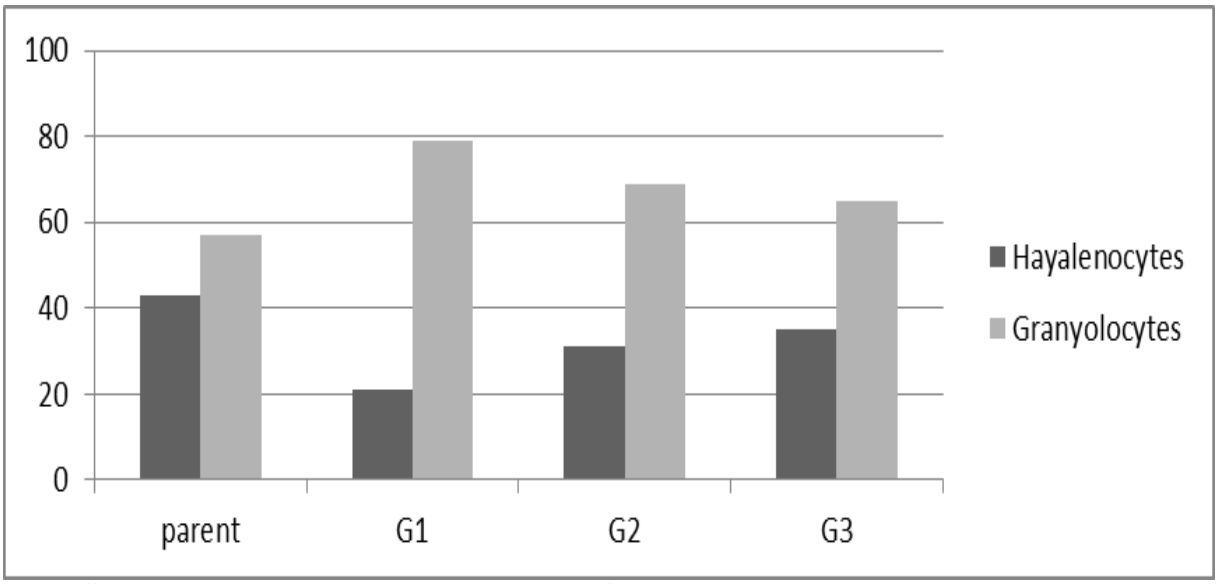

Fig 4: Differential blood cell count in hemolymph samples collected from in *B. alexandrina* parent snails and progenies by successive generations, G1: First generation, G2: Second generation and G3: Third generation.

Susceptibility to *S. mansoni* showed approximately moderate susceptibility of 33.3% infection, pre-patent period of 26 days, number of cercariae/snail of 1790 &survival rate of 15% while selected snails showed resistance to infection, higher pre-patent period over 72 days and higher survival rate of 35% (Fig. 5).

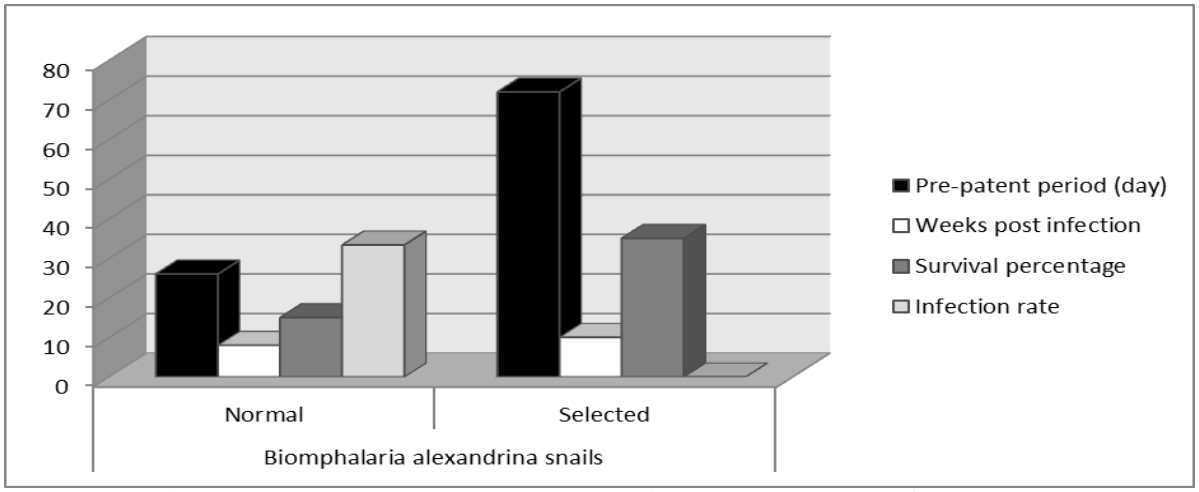

Fig 5: Comparison of susceptibility criteria, pre-patent period, weeks post infection, survival percentage, infection percentage and total number of cercariae/snail between third generation selected for Cd tolerance and laboratory non-selected *B. alexandrina* snails.

ISSR markers in assessing genetic variation: Genetic diversity among parent *B. alexandrina*, selected progenies for Cd tolerance till the third generations and laboratory reared non-selected snails, were investigated by ISSR-PCR technique using ten primers. A total of 88 amplification fragments, ranging from 205 to 2065bp in size were detected, the highest total number of bands (12) was obtained using primers HB08 & HB14, but, lowest number of bands (5) was by using primer HB12 (Tabs, 4, 5 & 6, Fig. 6).

By using all primers and according to the total number of amplified bands in each group, the unselected groups, parents and Laboratory showed the highest number of bands (68 & 67, respectively), while the selected groups under pressure of selection showed less bands 63, 65&57 in G1, G2, & G3, respectively. Genetic analysis showed that the 3rd generation group was the most unique by total 25 polymorphic bands in all primers, 16 absence & 9 excess. It was characterized by absence of 2 bands of 705 & 610bp using the primer 197887A, absence of 2 bands of 980 & 600bp and presence of 2 bands of 915& AF06 using the primer 844A. Absence of 4 bands of 970, 820, 735 & 610bp and the presence of 3 bands of 705, 670 & 31bp using the primer 844B, the absence of 3 bands of 680, 605 & 365bp and presence of 2 bands of 735 and 665bp using the primer HB08. The absence of one band of 540bp and the presence in one band at 670 using the primer HB12; the absence of one band of 520 using the primer HB13, absence of 2 bands of 755 & 420bp and presence of 1 band of 705bp using primer HB14 and at last, the absence of one band of at 890bp using the primer HB15. Also, 1st generation snail was characterized by 3 polymorphic bands; 2 were absent of 730 & 615bp and one excess of 520bp using primer HB11. Also, the 2nd generation snails were characterized by 2 polymorphic bands; one was excess of 640bp using primer 197887A and one was absent of 820bp using primer HB13. Parent group showed characterization on the presence of 3 excess polymorphic bands of 1385, 290 and 840bp by using primers 197887A, HB08 & HB10, respectively. The least characterized snail group was laboratory reared onesthat showed only one excess polymorphic band than the others in all primers and the absence of one band of 505bp by using the primer HB15.

Table 5: Total number of amplicons and level of polymorphism among parent *B. alexandrina*, their selected progenies for Cd tolerance and laboratory breading non-selected snail samples as revealed by ISSRs.

Primers	Total number of amplified bands	Polymorphic amplified bands	Percentage of Polymorphism
197887A	10	9	90
844A	6	4	67
844B	11	7	64
HB08	12	8	67
HB10	6	3	50
HB11	11	5	46
HB12	5	2	40
HB13	8	5	61
HB14	12	6	50
HB15	7	2	29
Total	88	51	58

Table 6: Total number of amplified bands in each snail group; parent *B. alexandrina*, their selected progenies G1: 1st generation, G2: 2nd generation and G3: 3rd generation for Cd tolerance and laboratory breading non-selected (Lab) by using ten ISSR primers.

Primers	Parent	G1	G2	G3	Lab
197887A	6	6	7	3	7
844A	4	4	4	4	4
844B	8	8	8	7	8
HB08	9	7	7	7	9
HB10	6	3	5	3	4
HB11	9	8	9	9	9
HB12	4	4	4	4	4
HB13	7	7	6	5	5
HB14	8	9	8	9	11
HB15	7	7	7	6	6
Total	68	63	65	57	67

Similarity coefficients estimated among all snails groups ranged from 0. 65-0.94. The highest value (0.94) was between 1st & 2nd generations snails, lowest (0. 6) was detected between 2nd generation snail group and 3rd generation group (Tab. 7). UPGMA dendrogram (Fig. 7) showed four main clusters, selected G1& G2 clustered with each other forming first cluster in turn cluster with Lab forming a second one which further cluster with parent forming the third one, that finally cluster with the most divert G3. So, closest relationship was between selected G1& G2, while Lab and parent snail groups showed median distance between all groups and selected G3 was the most divert.

Table 7: Proximity, similarity, matrices among parent *B. alexandrina*, selected progenies for Cd tolerance, G1: 1st generation, G2: 2nd generation and G3: 3rd generation and laboratory breading non-selected (Lab) based on ISSR fragment analysis.

Case	Matrix File Input				
	VAR00001:(parent)	VAR00002 G1	VAR00003G2	VAR00004:G3	VAR00005:Lab
VAR00001	1.00				
VAR00002	0.88	1.00			
VAR00003	0.91	0.94	1.00		
VAR00004	0.70	0.66	0.65	1.00	
VAR00005	0.87	0.90	0.90	0.74	1.00

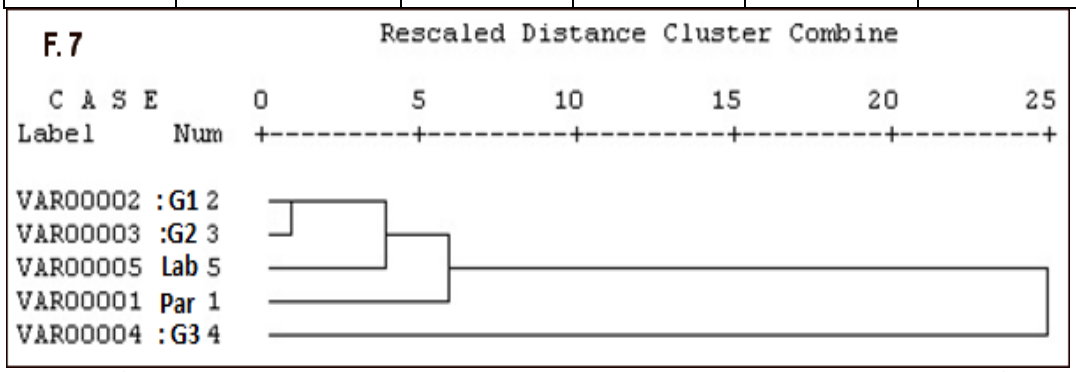

Fig. 7: Dendrogram of diversity and relationships among snail groups; parent *B. alexandrina* (Par), their selected progenies for Cd tolerance, G1: 1st generation, G2: 2nd generation and G3: 3rd generation and laboratory breading non-selected (Lab) based on ISSRs fragments.

Discussion

Environmental parameters influence the organism, producing a non-pathological phenotype, appropriate for that environment causing intraspecific phenotypic variation (Schluter, 2000). Thus, a particular environment can elicit different phenotypes from the same genotype and the ability of organisms to produce different phenotypes under different environmental parameters in natural populations is a critical issue to understand how species might face future changes (Gilbert, 2001). Also, the present work the proposed mating system of self-fertilization and selection for Cd toxicity tolerance in field parent *Biomphalaria alexandrina* snails and their progenies through three generations succeeded to produce snail isolate of higher tolerance than their parents, more than four times. *B. alexandrina* snails showed gradual increase in Cd tolerance through the successive generations produced by self-fertilization. LC_{50} increased from 2.45ppm in parents to 4.86, 8.46 &11.5ppm in 1^{st} 2^{nd} & 3^{rd} generations (G1, G2 & G3), respectively. This result is agreed with El-Khayat and Gawish (2006), on studying the sensitivity of *B. alexandrina* to water extract of the plant molluscicide *Anagallis arvensis* through five generations produced by self-fertilization of the most tolerant snails. The authors showed gradual increase in LC_{50} and LC_{90} values started from the 3^{rd} generation till the 5^{th} generation.

The present results of the total cells count of the selected G1, G2 & G3 snail generation groups after exposing to Cd, it was resulted in high significant increase in the third generation group their parents. This alteration in the hematological content of the selected snails especially in the third generation may be considered as a physiological characteristic that developed under the pressure of selection for Cd tolerance causing stronger immune defense mechanism. Also, in the earlier studies of different snail species, total hemocyte count has been reported to increase by heavy metal pollution (Pipe *et al*, 1995; 1999; Fisher *et al*, 2000) and a significant increase in the percentage of granulocytes (Pickwell and Steinert, 1984). In a trial to cleare how the snail immune-biology is related to the hematological criteria, Malek and Cheng (1974) explained that circulating hemocytes react against foreign bodies, digest and transport nutrients, accumulate various toxic substances, such as heavy metals, pesticides and molluscicides. Helal *et al*. (2003) observed significant increases in *B. alexandrina* hemocytes after exposure to the water extract of the plant *Euphorbia peplus*, after the second week, and after exposure to Praziquantel after 3^{rd} and 4^{th} weeks. They showed behavioral and structural changes in hemocytes, aggregation in clusters, after long exposure to the tested molluscicides. The present study recorded change in susceptibility to *S. mansoni* infection in selected Cd tolerant *B. alexandrina* (G3) from moderatory susceptible as in non-selected ones to refractory tendency suggesting trait association between Cd tolerance and refractory tendenncy. Also, a probable explanation for the decrease in susceptibility that the selection for Cd tolerance evolved snail immunue response; proved by significant alteration in hematological creiteria; to react against forien bodies of the schistosome sporocysts as well as to react against Cd toxicity. Also, Pan (1963), Sullivan and Richards (1981) and Loker *et al*. (1982) proved that hemocytes (phagocytic immune cells) of *B. glabrata*, genetically-selected for susceptibility or resistance to infection by larval *S. mansoni*, have been shown to react differentially to invading miracidia. Circulating hemocytes of susceptible strains do not recognize and kill invading larvae, whereas in resistant snails developing larvae are rapidly encapsulated by hemocytes and killed within 24–48 hours of infection. Also, Zahoor *et al.* (2010) and Negrão-Corrêa *et al.* (2007) suggested that the internal defense system (IDS) is one of the factors that influence the susceptibility pattern of snails. This system was stimulated by the excretory/ se-

cretory products of the penetrating miracidia. Its main action is mediated by the phagocytic hemocytes in cooperation with humoral components. In addition, this change could be explained by conclusion of El-Khayat *et al*. (2008) that there was more expectation of genetic alterations or mutation to occur in snails which practice self-fertilization and snail susceptibility will be affected only if genetic segregation or isolation developed under the effect of mating system leads to certain snail lines which differ in their susceptibly. The abundance of recent advance techniques for screening polymorphic markers has allowed the extension of genetic analysis of *Biomphalaria* snail in aspects regarding identification, susceptibility to schistosome infection and tolerance to molluscicides and environmental pollutants. Mohamed *et al*. (2011) used PCR-RAPD technique to identify various *B. alexandrina* strains collected from five Egyptian governorates. Also, El-Khayat *et al*. (2015) used ISSR-PCR technique for studying genetic variations of *Lymnaea natalensis* collected from four Egyptian Governorates and compared it with laboratory snails. The ISSR-PCR genetic analysis used in the present study among two non-selected snail groups, parent field and Lab *B. alexandrina* and three selected groups for Cd tolerance; 1st, 2nd and 3rd generations revealed that 3rd generation snail group was the most unique by total 25 polymorphic bands produced by ten primers, 16 absence and 9 excess. The similarity coefficient between the five examined groups based on ISSR fragment analysis ranged from 0.65 to 0.94. The highest value was between 1st and 2nd generations and the lowest value was between 2nd and 3rd generation. In addition, the dendograme based on ISSR fragment analysis showed that 3rd generation is the farthest apart other snail groups. This means that 3rd generation of Cd tolerant snails could be genetically changed and became different from other snail groups under pressure of selection for Cd toxicity tolerance.

Conclusion

The outcome results showed that certain *B. alexandrina* snails can tolerate Cd toxicity and this tolerance could be extended and upgraded by selection and self-fertilization mating system to produce genetically different generations for their Cd toxicity tolerance, hematological creiteria and susceptibility to *S. mansoni* infection.

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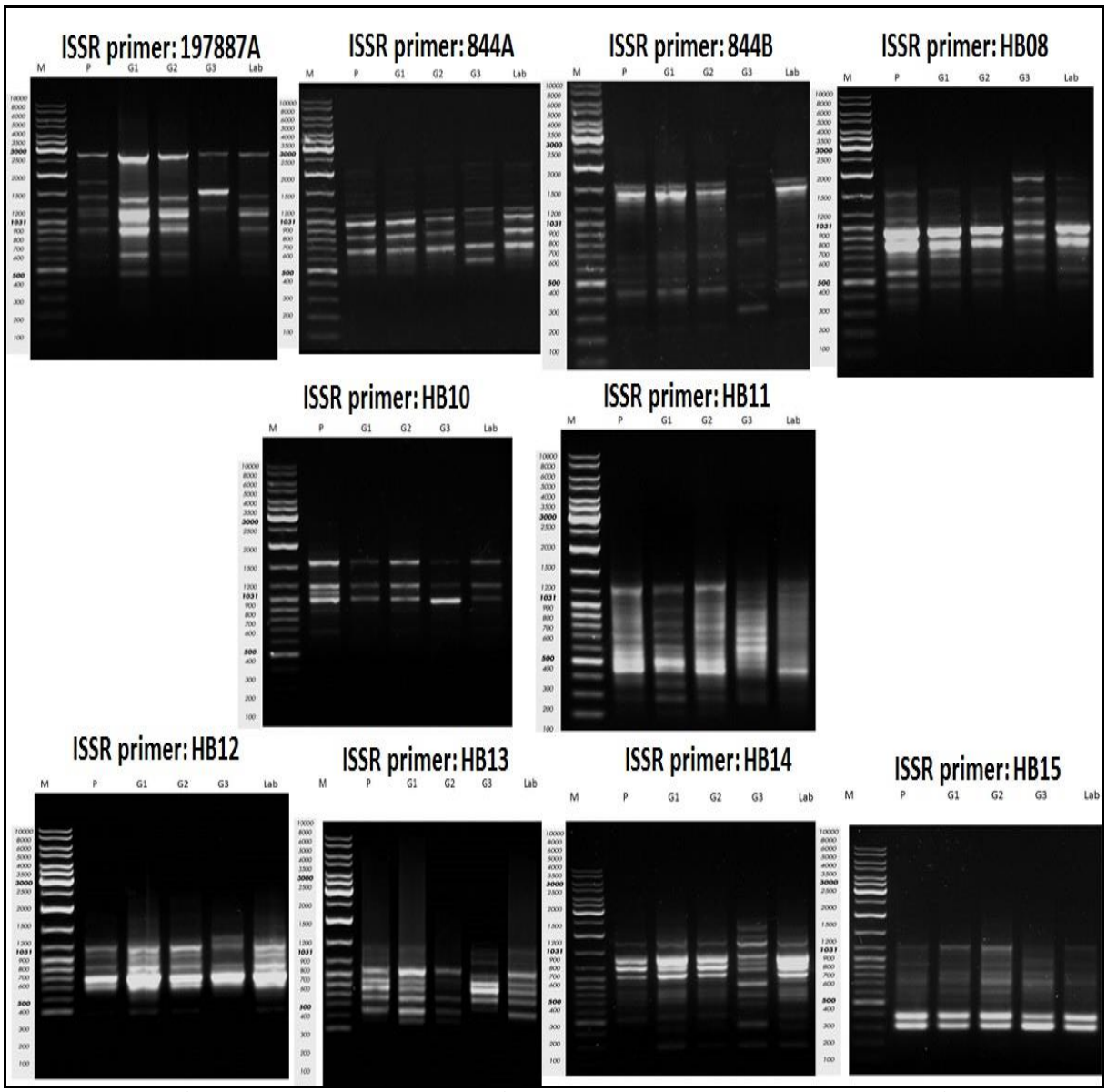

Fig. 6: PCR products of genomic DNA using ten ISSR primers from parent *B. alexandrina* (P); their selected progenies for Cd tolerance, G1: 1st generation, G2: 2nd generation and G3: 3rd generation and laboratory breading non-selected (Lab) snail samples.

Table 4: DNA polymorphism of 5 snail samples; parent *B. alexandrina*, selected progenies for Cd tolerance, G1: 1st generation, G2: 2nd generation and G3: 3rd generation and laboratory breading non-selected (Lab) using ten ISSR primers.

1: ISSR primer 197887A

197887A		Parent	G1	G2	G3	Lab
AF01	2065	1	0	0	1	1
AF02	1385	1	0	0	0	0
AF03	965	1	0	0	1	0
AF04	820	0	1	1	0	1
AF05	735	1	1	1	1	1
AF06	705	1	1	1	0	1
AF07	640	0	0	1	0	0
AF08	610	1	1	1	0	1
AF09	530	0	1	1	0	1
AF10	485	0	1	1	0	1
Total		6	6	7	3	7

3: ISSR primer 844B

844B		Parent	G1	G2	G3	Lab
AF01	1090	1	1	1	1	1
AF02	970	1	1	1	0	1
AF03	860	1	1	1	1	1
AF04	820	1	1	1	0	1
AF05	735	1	1	1	0	1
AF06	705	0	0	0	1	0
AF07	670	0	0	0	1	0
AF08	610	1	1	1	0	1
AF09	490	1	1	1	1	1
AF10	455	1	1	1	1	1
AF11	315	0	0	0	1	0
Total		8	8	8	7	8

5: ISSR primer HB10

HB10		Parent	G1	G2	G3	Lab
AF01	1520	1	1	1	1	1
AF02	965	1	1	1	1	1
AF03	840	1	0	0	0	0
AF04	805	1	1	1	1	1
AF05	710	1	0	1	0	0
AF06	635	1	0	1	0	1
Total		6	3	5	3	4

2: ISSR primer 844A

844A		Parent	G1	G2	G3	Lab
AF01	1020	1	1	1	1	1
AF02	980	1	1	1	0	1
AF03	915	0	0	0	1	0
AF04	600	1	1	1	0	1
AF05	445	1	1	1	1	1
AF06		0	0	0	1	0
Total		4	4	4	4	4

4: ISSR primer HB08.

HB08		Parent	G1	G2	G3	Lab
AF01	1355	0	0	0	1	1
AF02	1105	1	1	1	1	1
AF03	920	0	0	0	1	1
AF04	890	1	1	1	1	1
AF05	735	0	0	0	1	0
AF06	680	1	1	1	0	1
AF07	665	0	0	0	1	0
AF08	605	1	1	1	0	1
AF09	590	1	1	1	1	1
AF10	435	1	1	1	1	1
AF11	365	1	1	1	0	1
AF12	290	1	0	0	0	0
Total		9	7	7	7	9

6: ISSR primer HB11

HB11		Parent	G1	G2	G3	Lab
AF01	1210	1	1	1	1	1
AF02	1020	0	0	0	1	1
AF03	930	1	1	1	1	1
AF04	825	1	1	1	1	1
AF05	730	1	0	1	1	1
AF06	615	1	0	1	1	1
AF07	520	0	1	0	0	0
AF08	485	1	1	1	1	1
AF09	405	1	1	1	1	1
AF10	255	1	1	1	0	0
AF11	205	1	1	1	1	1
Total		9	8	9	9	9

8: ISSR primer HB13

HB13		Parent	G1	G2	G3	Lab
AF01	820	1	1	0	1	1
AF02	610	1	0	0	1	0
AF03	520	1	1	1	0	1
AF04	485	1	1	1	1	1
AF05	410	1	1	1	1	1
AF06	365	1	1	1	1	1
AF07	275	1	1	1	0	1
AF08	210	0	1	1	0	0
Total		7	7	6	5	5

10: ISSR primer HB15

HB15		Parent	G1	G2	G3	Lab
AF01	890	1	1	1	0	1
AF02	680	1	1	1	1	1
AF03	615	1	1	1	1	1
AF04	560	1	1	1	1	1
AF05	505	1	1	1	1	0
AF06	390	1	1	1	1	1
AF07	350	1	1	1	1	1
Total		7	7	7	6	6

7: ISSR primer HB12

HB12		Parent	G1	G2	G3	Lab
AF01	670	0	0	0	1	0
AF02	610	1	1	1	1	1
AF03	540	1	1	1	0	1
AF04	355	1	1	1	1	1
AF05	310	1	1	1	1	1
Total		4	4	4	4	4

9: ISSR primer HB14

HB14		Parent	G1	G2	G3	Lab
AF01	2060	0	0	0	1	1
AF02	1805	0	0	0	1	1
AF03	975	1	1	1	1	1
AF04	905	1	1	1	1	1
AF05	865	1	1	1	1	1
AF06	755	1	1	1	0	1
AF07	705	0	0	0	1	0
AF08	685	1	1	1	1	1
AF09	610	1	1	1	1	1
AF10	520	1	1	1	1	1
AF11	420	1	1	1	0	1
AF12	315	0	1	0	1	1
Total		8	9	8	9	11