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Cytogenetic Characterization and Genetic Variations Between Freshwater Crayfish *Procambarus clarkii* in Egypt.

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ABSTRACT

Within the last few years, freshwater Crayfish *Procambarus clarkii* (*P. clarkii*) has been successfully established in various sites of the river Nile and its branches. The main aim of this study was to describe improved techniques for obtaining good quality metaphase from a variety of tissues, the mitotic chromosome number and to evaluate the genetic variation among three geographic populations of freshwater Crayfish *P. clarkii,* which inhabits the Nile River in Egypt using the Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) technique. Our results provided that, good metaphase chromosomes were obtained from the regenerated blastema treated with 0.025% for 1 h and hepatopancreas tissues treated with 0.01% colchicine in vivo for 5 – 6 h, and then treated with the hypotonic solution for 1h. From 100 mitotic metaphases, the number of chromosome ranged from 184 to 208 per metaphase with a mode of 190 for 38 % of metaphases. The genetic diversity among three crayfish populations from three different regions (Giza, Qaluabia and Sharkia) generates 159 loci with 10 oligodecamers primers and RAPD profiles exhibited bands between 200 to 2778 bp in length. Average genetic distances among populations ranged from 0.0076 to 0.1735. The estimated average of gene differentiation (*G*ST) value across all loci was 0.2884, suggesting, very low gene flow among the different localities. The phylogenetic tree constructed by unweighted pair-group method of analysis (UPGMA) shows that, Giza, Qaluabia and Sharkia populations, respectively, seems to be approximately as closely linked to each other from the dendrogram. However it can be seemed that, Qaluabia population is more related to Giza populations. We conclude that, the number of mitotic chromosome ranged from 184 to 208 and there is a high level of genetic variation and population differentiation indicated dynamic evolution in these populations as revealed by variation at RAPD loci. This information will help in the selection of high-quality individuals for artificial reproduction.

Keywords: *Procambarus clarkii*, Chromosomes, RAPD-PCR. Genetic variation, UPGMA

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INTRODUCTION

The area of recent distribution of the crayfish covers many regions of the Earth as represented in northern Greece, the Balkans, central and Western Europe, Scandinavia as well as major parts of Eastern Europe to the White Sea in the north (Albrecht, 1983; Holdich, 2002). Members of the genus *Procambarus* are located in North America. *Procambarus clarkii* are mostly found in south-central United States and northeastern Mexico (areas to which this species is native). The red swamp crayfish has also been transplanted to Hawaii, Japan, and even the River Nile (Safra, et al., 1999; Jarmon, 1999).

Procambarus clarkii seems to be introduced latterly in Egypt. Within the last few years, it has been successfully established in various sites of the river Nile and its branches. Although *P. clarkii* has been recorded in Sudan (Huner, 1977), there is no clear evidence that it has migrated from the Sudan northward. There are no records of this species from the most southern parts of Nile or Lake Nasser. The only available explanation is that the initial access and colonization of *P. clarkii* started a commercial aquaculture in Giza (Manial-Sheiha), in the early 1980's, when the first immigrants of this species were introduced from USA. This project was shortly terminated due to administrative failure, meanwhile, *P. clarkii* proved to have definitely been transported in Egyptian waters (Hamdi, 1994).

In general, cytogenetics studies of crustaceans are relatively few and very difficult to perform because their chromosomes are large in numbers (Niiyama, 1962: Roberts, 1969: Mittal and Dhall, 1971;Campos-Ramos, 1997; Dumas and Campos-Ramos, 1999; Zhang et al., 2003; Lee et al., 2004), small in sizes and their shapes are very variable including metacentric, submetacentric, and acrocentric chromosomes (Salema and Heino, 1990; Damrongphol et al., 1991;Tan et al. 2004). Such distinctiveness makes them difficult to karyotype in comparison with the chromosomes of insects and some vertebrate species (White, 1973).

Early karyological studies have provided basic information on the number, size, and morphology of chromosomes which is an important prerequisite to the use of techniques for set chromosome manipulations in fishes (Arai, 2001; Hulata 2001), marine crustaceans, such as the white shrimp *Litopenaeus vannammei* (Dumas and Campos-Ramos, 1999), the Chinese shrimp *Fenneropenaeus chinensis* (Zhang et al., 2003) and *Procambarus* (*Austrocambarus*) *llamasi* (Indy et al., 2010). However, several studies have been carried out on specimens of crayfish from different locations around the world (existing geographical differences in chromosome numbers) as in Japan (Niiyama 1934 and 1959; Murofushi et al., 1984), in Australia (Tan et al., 2004), In Mexico (Indy et al., 2010) and in Europe (Mlinarec et al., 2011).

Since the mid-1980s; scientists have tried to describe the genetic structure of freshwater crayfish species. A variety of methods have been applied. Early studies based on protein electrophoresis have shown only low levels of variation between populations of European freshwater crayfish (Attard and Vianet, 1985; Fevolden and Hessen, 1989; Agerberg, 1990; Fevolden et al., 1994). During the last decade molecular techniques revealing a higher degree of variability have been developed and applied successfully in population studies of freshwater crayfish. Genetic differentiation of populations had been assessed using mitochondrial DNA (Souty-Grosset et al., 1997; Grandjean and Souty-

Grosset, 2000; Largiader et al., 2000), RAPD-PCR (Macaranas et al., 1995; Schulz, 2000; Gouin et al., 2001), AFLP (Fetzner and Crandall, 1999) and microsatellites (Gouin et al., 2000; Gouin et al., 2002). Phylogenetic studies (Grandjean et al., 2000; Munasinghe et al., 2003), and reconstruction of the phylogeography (Grandjean et al., 2001) were possible using mitochondrial DNA. Information on genetic structure of *P. clarkii* is still scarce. Using RAPD-markers; Schulz (2000) compared two stocks in northeastern Germany (Brandenburg), one in northwestern Germany and two stocks in southern Germany (Bavaria). All five stocks could be clearly distinguished, indicating a high geographic structuring of genetic diversity. Gouin et al., (2002) analysed microsatellite length variations in the ITS1 region of the rDNA and detected highly significant differences between most of the sampled Swedish populations.

Consequently, information on the basic and molecular genetics of the tropical crayfish *P. clarkii* is necessary not only to reinforce its potential for aquaculture, but also for genetic improvements and conservation. The main aim of this study was to describe improved techniques for obtaining good quality metaphase from a variety of tissues, the mitotic chromosome number and to evaluate RAPD-PCR markers for population-level studies in freshwater crayfish to assess the genetic diversity of the tropical freshwater P. clarkii that inhabits the Nile River in Egypt. To our knowledge, the present study is the first one to show the chromosomes and the genetic diversity among freshwater crayfish in Egypt.

MATERIALS AND METHODS

Sampling site and crayfish maintenance

The red swamp crayfish *Procambarus clarkii* were collected from three different regions in Egypt. Thirty adult of *P. clarkii* obtained from Abbasa fish farm (Sharkia governorate), El-Riah El-Tawfiki tributaries (Qaluabia governorate) and River Nile (Giza governorate). The crayfish were transmitted alive and stocked in glass aquaria supplied with continues aerated declorinated water at the faculty of Veterinary Medicine, Benha University. They were fed daily with shrimp pellets throughout this study.

Cytological techniques and preparation of chromosome slides

Metaphase spreads were obtained from regenerated blastema technique

In this technique, the first pair of periopeds was cut and the animals kept in a well aerated tank with feeding. After 8-10 days, regenerated blastema had grown to 2-4 mm in size. It was then removed and treated with 0.025 colchicine solution for 1 h. Minced blastema tissue was kept for hypotonic treatment in 0.9% sodium citrate or potassium chloride (KCl) 0.75 % for 1 h at room temperature. It was then transferred to Carnoy's fixative with three changes at interval of 15 minutes. Metaphase chromosome slides were prepared from the fixed tissue following Kligerman and Bloom (1977). The slides were stained in 10% Giemsa in Sorensons phosphate buffer for 25 minutes and differentiated in distilled water.

Metaphase spreads obtained from adult specimens technique

The adult specimens of *P. clarkii* were injected intraperitoneally with 0.01- 0.1 % colchicine at a rate of 1 ml / 100 gm body weight. The injected specimens allowed to swim in a well aerated aquaria for 4-7 h, after which they were sacrificed and their hepatopancreas, testes and gills tissues dissected out. The rest of protocol was the same as previously described.

Metaphase spreads obtained from tissue culture technique

Small piece of hepatopancreas was minced in 5 ml of RPMI 1640 medium into sterile tubes, then 0.2 ml of 0.05 colchicine was added in each tube in vitro (Williams et al., 1984). Cultures were incubated at room temperature for 1 h. The cells were centrifuged at 1000 rpm for 10 min and re-suspended in hypotonic solution potassium chloride (KCl) 0.75 % for 1 h at room temperature. The rest of protocol was the same as described earlier.

Chromosomes examinations

Mitotic metaphases were examined under microscope with an oil immersion lens at 1000 magnification. We were counting about 100 metaphases to detect the number of chromosomes. The chromosomes at the metaphase were photographed with digital microscope eyepiece camera (Item No. MA88).

Molecular Analysis

Samples collection

To obtain high quality of DNA from *P. clarkii*, it was first necessary to procure several specimens. Approximately thirty individuals were collected; from three different governorates in Egypt (Sharkia, Qaluabia and Giza) only those at least 2.5 – 3.5 cm long were deemed acceptable for this experiment, as the larger the specimen, the more easily tissue could be extracted. In the lab, the *P. clarkii* were placed into plastic bags and placed alive into a -80 ºC freezer. The freezing both killed the specimens and preserved them. It was imperative that they be kept at -80 ºC to arrest the degeneration of DNA due to the decomposition of the cells upon the death of the organism. The specimens were stored in the -80 ºC freezer until their dissection. Leg muscle tissue samples were taken from the specimens per stock.

Genomic DNA Extraction

Genomic DNA was extracted using the method of Sambrook and Russell (2001). Briefly, muscle tissues were homogenized in 1.5 ml microcentrifuge tube containing 1 ml lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS and 100 µg/ml proteinase K.) pH 8.0. Samples were incubated for 16 h at 37°C. DNase-free RNase was added to a final concentration of 20 µg/ml and the digest incubated for another 60 min. After precipitation of the SDS by adding potassium acetate solution to a final concentration of 0.75 M in respect of potassium and 1.25 M in respect of acetate, the DNA was precipitated with one

volume of isopropanol. The pellet was washed with 70% ethanol, dried and redissolved in 10 mM Tris-HCl (pH 8.0).

Quantity, Purity and Quality of DNA

Quantitative estimation of DNA sample was done by a double beam UV-spectrophotometer by measuring the DNA concentration at 260 nm. Purity of DNA was checked by means of absorbance ratios A260/A280 for protein contamination. Further, the samples were run on 1% agarose electrophoresis to check the quality of DNA (Sambrook et al., 1989) along with one kb plus DNA ladder. Only high quality of DNA was used for RAPD analysis.

RAPD-PCR Amplification

To generate RAPD profiles from *P. clarkii* DNA, a set of 10 oligodecamers primers (Table1) from the Operon Technologies (Operon Technologies Inc., Alameda, Calif.: A, B and C) were used. Primers were designated as useful if they yielded well-amplified, distinguishable polymorphic bands. Finally three primers (OPA20, OPB09 and OPB14) were selected and used to amplify DNA from all individuals. DNA amplification reactions were performed under conditions reported by Williams et al. (1990) and Plotsky et al. (1995).

Table 1: Sequence, operon codes and GC content of random primers used to study variation in freshwater crayfish *P. clarkii*.

Primers	Sequence (5'- 3')	GC%	Primers	Sequence (5'- 3')	GC%
OPA07	5-GAA ACG GGT G-3	60	OPB03	5-CAT CCC CCT G-3	60
OPA04	5-AAT CGG GCT G-3	70	OPB09	5-GGT GAC GCA G-3	60
OPA09	5-GGG TAA CGC C-3	70	OPB12	5-CCT TGA CGC A-3	60
OPA15	5-TTC CGA ACC C-3	60	OPB14	5-TCC GCT CTG G-3	70
OPA20	5-GTT GCG ATC C-3	60	OPB20	5-GGA CCC TTA C-3	60

PCR amplification was conducted in 50 µl reaction volume containing 100 ng genomic DNA, 100 mM dNTPs, 40 nM primer, 2.5 units of Taq DNA polymerase buffer. The PCR reactions were carried out in thermocycler (Perkine Elmer 9700) programmed with a first denaturation of 5 min at 94 °C, followed by 45 cycles of 1 min denaturation at 95 °C, 1 min annealing at 36 °C and 2 min extension at 72 °C. Final extension at 72 °C for 5 min was allowed before holding the reaction at 4 °C for 10 min. Reaction products were stored at 4 °C prior to electrophoresis.

A volume of 5 µl of each sample was mixed with 2 µL 6× gel loading buffer and used for electrophoresis on 1.2% agarose gel. RAPD patterns were visualized and documented using the Gel Documentation system, Gel-Pro Analyzer (Media Cybernetics). A Φx174 DNA digested with HaeIII were used as known molecular size DNA markers.

Recording of data and Statistical analysis

The RAPD patterns were scored for the presence and absence of amplicons. In a binary matrix the presence of a band was recorded as one and the absence as zero. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating polymorphic loci, Nei's (1973) gene diversity (h), allele frequencies, genetic distance (D) and genetic identity (I). All calculations were

carried out using the population genetic analysis software, PopGene version 1.31 (Yeh et al., 1999).

RESULTS

Cytological Data Analysis

In this study a good quality metaphase plates were not obtained from neither gill nor testes tissues, Fig 1(a&b). However, good metaphase chromosomes were obtained from the blastema and hepatopancreas tissues in vivo, as shown in Fig 1(c&d). The tissue culture technique also gives metaphases but they were not good quality, represented in Fig 1(e). Longer incubation led to a greater chromosome condensation. In this study, the most condensed chromosomes were obtained by the 7hs treatment, Fig 1(f). The most effective colchicine and hypotonic treatment for suitable metaphase plate were for 5-6 hr and 1hour, respectively.

The existence of micro-chromosomes, the large number and small size of chromosomes with progressively decreasing length in *P. clarkii* make it difficult to identify them individually. A *total* of 100 mitotic metaphases from hepatopancreas were examined to determine the number of diploid chromosome. The counts of chromosome ranged from 184 to 208 per metaphase with a mode of 190 for 38 % of metaphases Table 2.

Table 2: Illustrated the numbers of diploid chromosome in a total of 100 metaphases spread from hepatopancreas of the *P. clarkii.*

Total Number of Chromosomes	184	189	190 *	192	196	198	200	202	208
Number of Metaphases	6	5	38	14	8	6	11	9	3

Mode of diploid chromosome number (2n = 190)

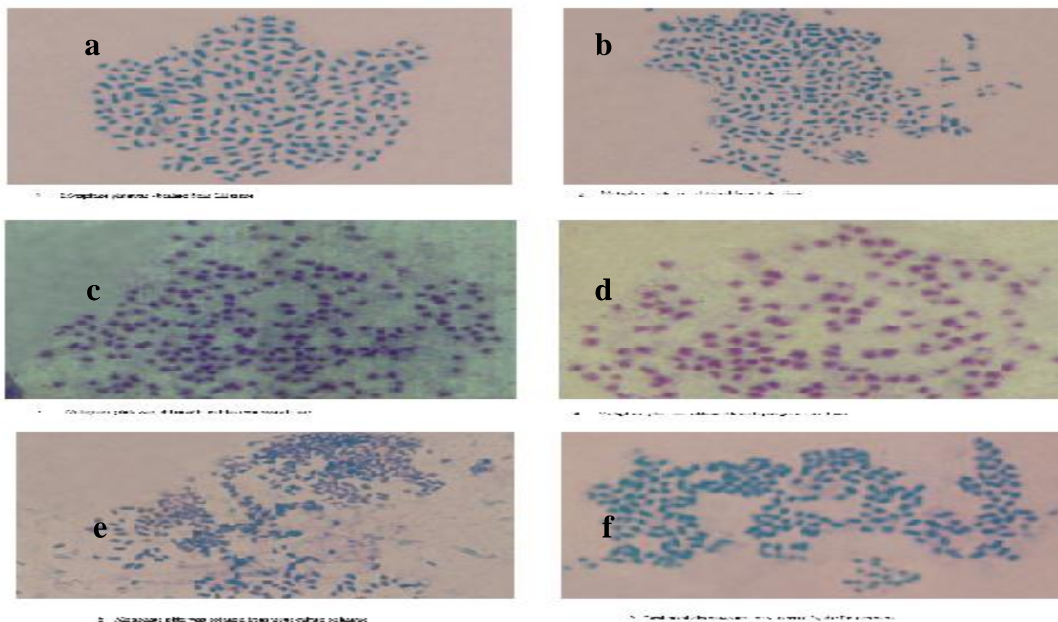

Fig 1: Showing metaphases spread from **a)** Gill; **b)** Testes; **c)** Blastema; **d)** Hepatopancreas; **e)** Tissue culture and **f)** the most condensed chromosomes

Molecular Data Analysis

A band was considered to be polymorphic if it was present in at least one genotype and absent in the others. A data matrix was generated in which each band was scored 1 if present and 0 if absent. The percentage of polymorphic loci, Nei's genetic diversity (Nei, 1978), Shannon index, average heterozygosity, and genetic distance (Nei, 1972) were calculated using POPGENE v. 1.31. Statistica 6.0 was used to test the difference in intrapopulation genetic distance and average heterozygosity between populations. A dendrogram of the 6 populations was constructed based on the unweighted pair-group method with arithmetic means (UPGMA) in POPGENE 3.2.

Selectively amplified results of 10 primer combinations were analyzed in this study. A total of 159 bands were identified in the three populations of crayfish, and the average was 15.9 bands for each primer combination. The average heterozygosity and percentage of polymorphic loci for each population are summarized in Table 3 and Fig.2, 3 & 4. Great variation in genetic diversity was observed among populations, as indicated by the average heterozygosity and percentage of polymorphic loci. The greatest percent polymorphism (70.25%) was that of the Sharkia population, while, the two other populations the percentage of polymorphism reach to 65.37%. A *GST* analysis was performed to estimate differences among locations. The estimated *GST* value averaged over all polymorphic loci was 0.2884, indicating a strong population structure, Table 4.

M

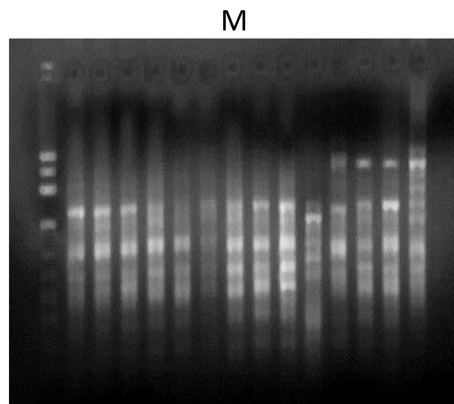

Fig.2 Random amplified polymorphic DNA (RAPD) profile generated by primer OPA20 in individual crayfish populations, Giza, Qaluabia and Sharkia Lane M = molecular marker (Φx174 DNA HaeIII digest).

M

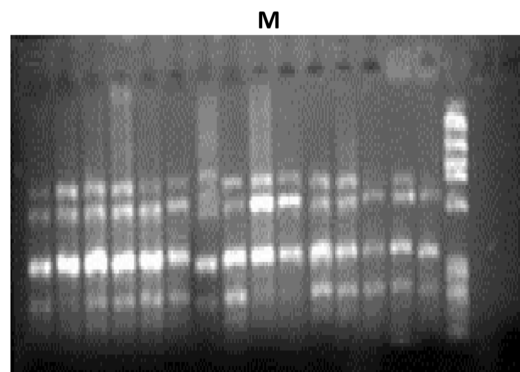

Fig.3 Random amplified polymorphic DNA (RAPD) profile generated by primer OPB14 in individual crayfish populations, Giza, Qaluabia and Sharkia. Lane M = molecular marker (Φx174 DNA HaeIII digest).

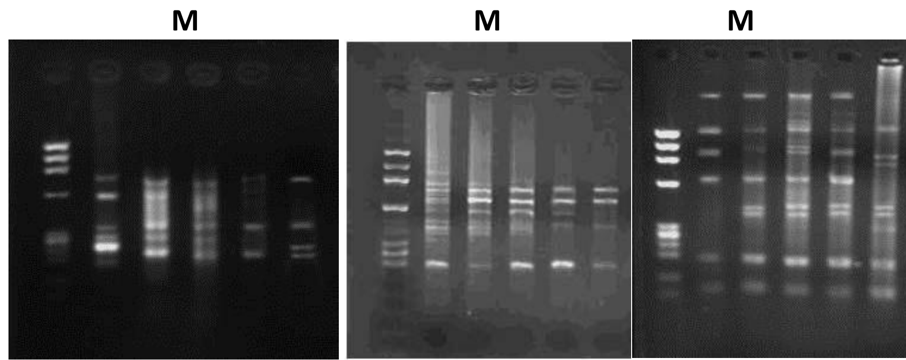

Fig.4 **Random amplified polymorphic DNA (RAPD) profile generated by primer OPB09 in individual crayfish populations, Giza, Qaluabia and Sharkia; Lane M = molecular marker (Φx174 DNA HaeIII digest).**

Table 3: Genetic diversity of the three population of *P clarkii*

Population.	No. of alleles.	Effective No. of alleles	Nei'sgene diversity	Shannon's information index	Percentage polymorphic loci
Giza	1.3137±0.4663	1.2012±0.3324	0.1180±0.1830	0.1756±0.2671	65.37 %
Qlubia	1.3137±0.4663	1.2012±0.3324	0.1180±0.1830	0.1756±0.2671	65.37 %
Sharkia	1.3725±0.4859	1.2573±0.3722	0.1467±0.1999	0.2157±0.2882	70.25 %

Table 4: Nei's analysis of gene diversity in *P clarkii* populations

Total gene diversity	**Total gene diversity**	Gene diversity within **population**	Coefficient of gene differentiation (*G*ST)	Gene flow (*N*m) *N*m = 0.5 (1 - *G*ST) / *G*ST
Mean	0.2082	0.1481	0.2884	1.2339
Standard deviation	0.0461	0.0378		

Population structure

The individual-based similarity trees with the highest identity values, and therefore the best fit between the distance matrix and corresponding tree, were produced through UPGMA cluster analysis (Fig 5). UPGMA was carried out to demonstrate graphically the genetic similarities among crayfish populations. The genetic identity among populations ranged from a low of 0.8407 between Sharkia and Giza populations to a high of 0.9924 between Qaluabia and Giza populations (Table 3). Nei's genetic distances for all pair wise comparisons were significantly different from zero. The genetic distance value (0.1735) between Giza and Sharkia populations was the highest, whereas the value (0.0076) between Giza and Qaluabia populations was the lowest (see Table 3). Therefore, UPGMA dendogram generated from Nei's genetic distance of the three *P clarkii* populations is illustrated in Fig 1. The cluster analysis shows that crayfish from Giza and Qaluabia populations, which were geographically, could be closely clustered together. While, Sharkia population, which was located far from the other two populations, was clustered into separate branch.

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

pop ID	Giza	Qaluabia	Sharkia
Giza	****	0.9924	0.8407
Qaluabia	0.0076	****	0.8484
Sharkia	0.1735	0.1644	****

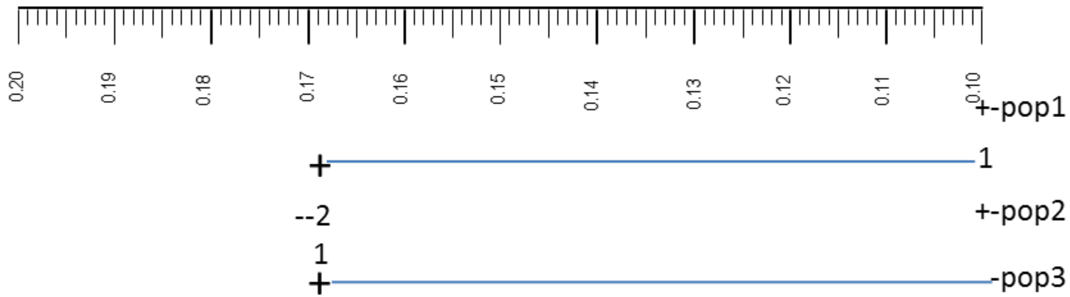

Fig 5. UPGMA dendogram generated from Nei' s genetic distances of the three populations of *P. clarkii*.

DISCUSSION

The results of our study confirm the view that rapid growing tissues are required to obtain a large number of metaphase chromosomes spread. High quality metaphase plates were obtained from the regenerated blastema and hepatopancreas tissues of *P. clarkii*. Similarly, a good quality metaphase from the blastema and hepatopancreas tissues of *Macrobrachium rosenbergii* (Lakra et al., 1997 *)* and *gill tissue of P. llamasi* (Indy et al., 2010). The data supports previous studies which demonstrated the provision of testis to be an excellent tissue for chromosomal analysis since it is not only a source of meiotic metaphases but also a source of mitotic metaphases (Chow et al., 1990; Justo Chavez et al., 1991; Xiang et al., 1994; Tan et al., 2004).The difference in the quality of metaphases in these tissues was due to using different techniques.

Preparations of tissues and slides for cytogenetic analysis are important in attaining a large number of well spread metaphases; the first step in the procedure is the treatment of the crayfish with colchicine which arrests cell division at metaphase. Treatment of blastema tissue with 0.025 % colchicine for 1 h provided good quality metaphase plates. The same concentration used in preparing of metaphases from *Macrobrachium rosenbergii* (Lakra et al., 1997). Other workers have suggested the uses of physiological saline solution with 0.01 % colchicines treatment of tissues of *P.japonicus* at room temperature for 30- 60 minutes (Hayash and Fujiwara 1988) .

Injection of 0.01 - 0.1 % colchicine to *P. clarkii* adults for 5- 6 h at room temperature was found effective for obtaining mitotic chromosomes from hepatopancreas. However, longer incubation led to a greater chromosome condensation. In this study, the most condensed chromosomes were obtained by the 7hr treatment. Successfully obtained mitotic chromosomes in gonadal tissues when injected 0.01-0.1 % colchicine at a rate of 0.1 ml / g bwt to *Macrobrachium rosenbergii* males for 3-4 h (Lakra et al., 1997). Also Tan et al., (2004) obtained chromosomes from testis tissues by injecting the colchicine 2.0 ug / g BW after 5-6 h maintenance period at 25 °C. However, for antennal gland and testis tissues, the

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most effective colchicine injection dose was 1-2 ug /g BW for 8-12 at 23 °C (Justo Chavez et al., 1991). Other investigators obtained metaphases from gill tissue by incubating crayfish *P. llamasi* for five hours in a well-aerated plastic bottle containing 10 ml freshwater mixed with 1.0 ml of 1.0% colchicine solution (Indy et al., 2010). These studies suggest that colchicine concentrations of 1-2 ug /g BW can effectively arrest dividing cells in metaphase in tissues, but the maintenance periods may vary according to the species.

The hypotonic solution used in the present study was 0.75 % potassium chloride (KCl) or sodium citrate 0.9% for 1 h at room temperature. The potassium chloride gave satisfactory results. Certainly, many investigators have used potassium chloride (KCl) or sodium citrate such as 0.075 M and 0.01 M KCl for 30 minutes and 11-12 minutes in *P. japonicus* and *Macrobrachium rosenbergii*, respectively (Hayashi and Fujiwara (1988) and Justo Chavez et al. (1991), distilled water as hypotonic solution for 10 – 15 minutes in a chromosomal study on *P. aztecus*, *P. duoraum* and *P. setiferus* (Chow et al., 1990), 1.0% sodium citrate for two hours in *P. llamasi* (Indy et al., 2010). Our Techniques described above resulted in slides with good chromosomal plates in *P. clarkii.* These techniques are easy to use for cytogenetic analysis in field condition.

All the freshwater crayfish so far belong to three families under Astacidea (Astacidae, Cambaridae, and Parastacidae). The family Cambaridae is native to North America (Huner, 1997). It is the first study in which the chromosomes of the species *P. clarkii* in Egypt have been analyzed. The number of chromosomes in *P. clarkii* was 2n=190. Also two reported diploid numbers has been dated for similar species with 2n=192 (Niiyama 1959) and 2n=188 (Murofushi et al., 1984). This little variation in the number verifies geographic difference in chromosomes number among species of *P. clarkii.* Meanwhile in Cambaridae family the number of chromosomes is nearly similar as in *Cambarus immunis* where 2n=208, (Fasten, 1914), while in *Cambaroides japonicas;* the 2n= 196, (Niiyama, 1934). But the chromosomes number of species *P. digueti* is very smaller than other species in which the 2n=102, (Diupolex Chong et al., 1997). Also in Mexico *P. llamasi* has small number as the 2n=120, (Indy et al., 2010). However family Parastacidae, crayfish *Cherax quadricarinatus* has diploid number; 2n=200 (Tan et al., 2004), Australian crayfish *Cherax destructor;* 2n = 188, (Scalici et al., 2010) and European crayfish, *Astacus astacus*; 2n=176 (Mlinarec et al., 2011). These studies suggest that the ranges of chromosome number in freshwater crayfish families are large. Such cytological settings represent a methodological problem for cytotaxonomist who wants to employ karyotypical characters as taxonomic key for species identification.

Genetic diversity information among and within populations of the red swamp crayfish in Egypt also provides reliable information for the protection and restoration of wild resources. Our findings clearly indicated that some distinct differences exist among the 3 populations. The Sharkia population showed the most distinct difference with the highest allelic and gene diversity. High genetic diversity of introduced populations can be attributed to several factors: multiple introductions (Kolbe et al., 2004), a single introduction of a large number of individuals from different populations (Yue et al., 2010), and remote living locations and lower fishing pressure (Song et al., 2006). The scale of introduction and fishing intensity in Sharkia population of this species was relatively lower; which may cause high genetic diversity of introduced populations.

Successful invasive species are generally thought to have high genetic diversity, which allows them to escape the harmful effects of inbreeding and adapt to their new environment (Spielman et al., 2004). However, we found that overall gene diversity varied from 0.1180 to 0.1467 in the three studied crayfish populations and was lower than that of populations in the United States (0.78) (Belfiore and May, 2003) and in China (0.1471 to 0.5829) (Zhu et al., 2013).

Barbaresi et al. (2003) have shown that low genetic diversity is due to the founder effect. Because crayfish are highly fecund and can produce more than 200 eggs at each spawning (Rodríguez-Serna et al., 2000), mating between relatives is highly possible. Villanelli and Gherardi (1998) have reported that non random mating and mate choice have been noted in some freshwater crayfish species. Therefore, we cannot conclude that the gene diversity within each population observed in this study was caused by a bottleneck effect and non random mating.

The genetic differences among the various crayfish populations may be directly related to the distance of isolation and the connectivity level of the rivers they inhabit. The genetic distance ranged from 0.1735 to 0.0076 in our study. The genetic distance between Giza and Qlubia populations was the lowest; meanwhile Giza and Sharkia populations had the greatest distance owing to their far distance.

Hedgecock et al. (1979) have reported that the population structure of freshwater crayfish is significantly influenced by several factors: land, mountains, and water level. These factors limit gene flow and therefore favour inter-population divergence .

The G_{ST} value represents the degree of genetic differentiation within populations ($0 < G_{ST} < 1$). Mickett et al. (2003) have suggested that the F_{ST} (genetic differences among population) in channel catfish (0.4456) indicates a high degree of genetic differentiation, whereas, Yue et al. (2004) have reported moderate genetic differentiation in Asian arowana, with a G_{ST} of 0.047. In our study, F_{ST} averaged over all polymorphic loci was 0.2884, which is lower to the result reported by Mickett et al. (2003) and Zhu et al. (2013). This value indicates a high degree of genetic differentiation among the three populations. Yue et al. (2010) have stated that genetic drift and selection may play important roles in genetic differentiation in *P. clarkii*. Nevertheless differences in population sizes are known to influence the degree of genetic differentiation within and between populations (Crandall, 1997; Lande, 2002). Gouin et al. (2001) have maintained that human transplantation has caused genetic differentiation in the endangered freshwater crayfish. The impact of habitat variation on genetic differentiation has been illustrated in several animals, particularly crayfish (Austin and Knott, 1996). In our study, the pattern of genetic differentiation among three populations could be explained by two hypotheses. The first is based on human transplantation, which could be responsible for the differentiation. Crayfish transplantation was a common practice as early as middle ages, and isolated populations exist because of such activities. Thus, transplantation of crayfish from populations located in different lakes may have altered the natural genetic pattern. The second hypothesis is that this genetic pattern within and between populations can be accounted for by a period of population expansion and migration or gene flow followed by contraction, isolation, and fragmentation of populations, which leads to genetic divergence mainly under the process of genetic drift

and resulted in fixation of the alternative variants in a stochastic manner. Similar patterns of genetic differentiation have been described in crayfish species using allozyme studies (Austin and Knott, 1996).

In summary, Crayfish is an extremely important economic resource in Egypt. Our study adds knowledge on the chromosome preparation of crayfish in Egypt but it also foresees the possible difficulty for polyploidy manipulation in this species due to the large number and small size of chromosomes. Moreover, the results of genetic investigation will be useful for the development of artificial propagation and genetic improvement programs for crayfish, which give populations the ability to adapt to environmental changes and stresses. Furthermore, the supply of offspring from hatcheries cannot meet the demand of the market. Our research revealed that Sharkia population has considerable genetic variety. Therefore, parental crayfish for artificial propagation should be collected from this population to ensure the conservation of wild resources and genetic diversity.

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