Detection of Genetic Relationship and Genetic Variation Four Samples of Sand Flies(Phelebotomus Papatasi) Isolated From Different Regions In Salah El-Din Governorate Using Molecular Markers

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Abstract
Objective: The genetics of sand flies phelebotomus papatasi isolated from four districts within Salah al-Din governorate using molecular markers using RAPD-PCR markers. Methods of work: Samples were collected from four districts within Salah al-Din Governorate (Dhuluiya, Balad, Al-Alam, Samarra), where they were collected from barns for raising livestock, The species were preserved using 70% ethanol alcohol and the samples were placed in special labeled plastic bottles until the DNA extraction process was performed. Then, PCR-RAPD markers were carried out using (17) primers, and the results were transmitted on agarose gel, and the bundles were photographed using a digital camera. Results: The results of the RAPD markers showed different types of bands, and the total loci identified by the primers on the samples genome were (124) loci, of which (22) are general loci and (102) are differentiated loci The initiator (P13) was distinguished by the highest number of productive sites, reaching (11) sites, while the two initiators (P3, P4) produced the least number of sites, reaching (5) loci for each. The total bands produced from those loci were 254), including (166) polymorphic bands and (88) main bands. The primer (P15) produced the highest level of bands, reaching (30) bands, while the primer produced (P3) The least number of produced primer reached (7), while the variation ratio of the produced primer was 90%.The study showed the presence of distinctive bands, the total of the distinctive bands was (70), of which (57) were unique bands and (13) were absent, the Balad sample was distinguished by the lowest number of bands (12) band, while the Dhuluiya sample got the highest number of band, reaching (21) bands. From the results of the interactions of the genetic distance, the values of the genetic distance ranged between Where the values of the genetic dimension ranged between 0.443 -0.737, where the lowest dimension was present between the two isolates, country and science, which amounted to 0.443 and the highest genetic dimension was between the Dhuluiya sample and a country with a genetic dimension of. 0.737 which indicates that they are more different within the studied species. Conclusion: There is a close degree of affinity between the four sample of sand flies, even though they belong to families, so molecular markers can be used in classifying insects, especially flies, accurately and determining their genetic fingerprint instead of adopting the phenotypic features in the classification because molecular markers are fixed traits and do not change with changing conditions.
Keywords: Genetic Relationship, Genetic Variation, sand Flies, Molecular Markers

1. Introduction

Phlebtominae sand flies belong to the order and subfamily Diptera: phlebotomidae, and it is one of the complete life-cycle insects, and each cycle has its own taxonomic and morphological characteristics that are useful in classification and distinction, but the adult stage is the most obvious. Standing in the rest period, the wings extend upwards and are dark yellowish brown. Sand fly insects are widely spread in the tropic regions of the old-world countries and subtropic regions of the new world within a temperature range of 50 N north and 40 S in the south and the temperature 6-15 m plays a role in determining the density Insects for about three months a year (Eldringe and Edman, 2000).

Genetic variation describes the nature of genetic differences between individuals of the same species and this difference may result either through mutation, which is defined as a change in the sequence of nitrogenous bases of the genetic material DNA, or through new unions resulting from crossbreeding between pure strains that occur naturally and this difference It gives flexibility for the survival of species in the face of changing environmental conditions (Kalia, 2011). And the series of these differences continues to find what is appropriate for these circumstances and is different from its origins. In addition to the importance of natural conditions in this field, the increase in

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population and the expansion of its requirements led to human intervention, directly or indirectly, in finding other ways for genetic variation in insects, including improving new breeds for different types of economically important insects, such as the silkworm-producing insect, as well as in the field of Biological control.

Molecular markers can identify specific and genetic loci by using the primer or probe, which depend on the presence or absence of complementary loci (Williams et al, 1995). Researchers have sought to develop these markers and normalize them to work since their discovery until now (Williams et al, 1990). Many of these markers, especially RAPD, have been widely used in studying the genetic variation between flies and determining the genetic signature of flies (Singh et al, 2016: Sunitha et al, 2015: Malviya et al, 2010). The aim of the research is to uncover the genetic relationship and the genetic variation of four sample of sand flies using molecular markers.

2. Materials and Methods of Work

Collecting and diagnosing samples: Samples were collected from four districts within Salah al-Din Governorate (Dhuluiya, Balad, Al-Alam, Baiji), where they were collected from barns for raising livestock and were collected using special traps. The types of flies were diagnosed in the Research Center and Natural History Museum \ University of Baghdad, according to the book No. 41 dated 6/12/2022. The species were preserved using 70% ethanol alcohol and the samples were placed in special labeled plastic bottles until the DNA extraction process was performed. They are four types of complete flies, as shown in Table (1):

Table (1) types of flies used in the study		
n	The scientific name	Local name
1	Phelebotomus papatasi	Samarra
2	Phelebotomus papatasi	Al-alam
3	Phelebotomus papatasi	Dhuluiya
4	Phelebotomus papatasi	Balad

DNA extraction: DNA was extracted from insects using a modified method of the first two methods mentioned by (Boyce, 1989), and the second method is (Al-Sugmiany, 2017) from (Haung, 2013).

DNA purification: The process of measuring the concentration and purity of DNA was done using a (nano drop) device, and then the sample was diluted to a concentration of 50ng / ml and preserved by freezing until use.

Gel electrophoresis: The necessary solutions, materials, and gels are prepared, and samples loaded in the electrophoresis process according to the method mentioned by (Sambrook et al. 1989: Al-Sugmiany, 2017).

RAPD - PCR Reactions: RAPD markers were performed based on (Williams *et al*, 1990) for four types of flies using (17) primers shown in Table (2), And the components of the reaction shown in Table (3).

Table (2) the primers used in the study					
no.	Primer	Sequence 5´→→→ 3´	no.	Primer	Sequence 5´→→→ 3´
1.	OP A-01	CAGGCCCTTC	10.	OP C-16	CACACTCCAG
2.	OP A-06	GGTCCCTGAC	11.	OP C-10	TGTCTGGGTG
3.	OP B-04	GGACTGGAGT	12.	OP D-03	GTCGCCGTCA
4.	OP B-12	CCTTGACGCA	13.	OP D10	GGTCTACACC
5.	OP B-14	TCCGCTCTGG	14.	OP D-18	GAGAGCAAC
6.	OP C-08	TGGACCGGTG	15	OP G-02	GGCACTGAGG
7.	OP H-16	TCTCAGCTGG	16	OP G-08	TCACGTCCAC
8.	OP J-04	CGGAACACGG	17	OP G-14	GGATGAGACC
9.	OP B-20	GGACCCTTAC			

Table (3) solutions used in the RAPD markers		
C	Components	Volume
1	Green Master mix	12.5 µ l
2	Primer	2 µ l
3	Nuclease free water	8.5µ l
4	DNA template	2µ l
5	Total Volume	25µ l

The reaction program was applied with the pre denaturation temperature (94) for a period of (4) minutes for one cycle only, after which (40) cycle consisted of denaturation heat (93) for (45) seconds and the annealing temperature (36) for (45) seconds and the extension heat (72). For (1) minute and a final heat of extension (72) for (7) minutes, one cycle. The end of the reaction time, the tubes were removed from the thermoplastic device and kept in the freezing, and (5) microliters were withdrawn from the tubes and the mixture was loaded onto the prepared acarose gel at a concentration of 1.5% and stained with the Red say dye with the Marker volumetric guide. Then the samples were removed on the acarose gel. Then the jelly was photographed with a high-resolution digital camera and the images were saved in a computer.

3. Statistical Analysis

The results of the multiplication operations of the primers used in the RAPD markers were taken and converted into tables, depending on the presence or absence of the DNA bands and comparing it between the different samples, where the presence of the bands is symbolized by the number (1) and the absence of the bands by the number (0), the genetic similarity coefficient was calculated As well as the genetic distance between the studied samples using Nei's factor 72 (Nei and Li. 197) The similarities and differences in the genetic material (DNA) that can be obtained from the application of RAPD-PCR markers can be adopted to determine the genetic distance,

which will convert the results obtained. Which appears in the gel to characterization tables by setting (1) when the beam is present and (0) when the bands is absent.

4. Results and Discussion

Results of RAPD markers The primers produced different bands distributed between monomorphic and polymorphic bands, which were detected on agarose gel in the presence of the DNA marker (DNA ladder100bp). The results of the primers showed different patterns of bands as shown in Table (5). The genome of samples is (124) sites, including (22) general sites for all samples and (102) varying sites, and the initiator (P13) was distinguished by the highest number of productive sites, it reached (11) sites, while the initiators (P3, P4) produced the least number of sites, it reached (5) sites for each. The total bands produced from those sites were 254), including (166) polymorphic bands and (88) main bands. The initiator (P15) produced the highest level of bands, reaching (30) bands, while the initiator produced (P3) The least number of produced prefixes reached (7), while the variation ratio of the produced prefixes was 84%.

The study also showed the presence of distinct bands (absent bands, unique bands) as shown in Table (6). The total of distinct bands resulting from the prefixes was (70), of which (57) were unique and (13) were absent. Type (3) was distinguished by the highest percentage of unique packages, reaching (18) while type (4) produced the lowest percentage of unique packages, reaching (9) packages. As for the absent packages, type (2) obtained the least number of the absent bundles were (2) bundles, while type (1) produced the highest number of absent bundles, reaching (5) bundles. These bundles are used as a diagnostic and distinguishing feature for these types. The appearance of those bundles in one type indicates a mutation in a specific site that led to the identification The initiator of this site and the emergence of the unique package, as well as the absent packages, as a mutation occurs in the site of recognition of the initiator only in one species without the other species, which leads to the cancellation of that recognition and the disappearance of the package and this is consistent with the results of most researchers Bajpai, 2016; Brito, 2008.(

The results of the primers showed a clear discrepancy, ranging between (200-2000 (bp), where the lowest molecular size was (200 bp) in the initiator (P6) and the highest molecular size in each of the two primers (P8, p13), where the molecular size was (2000 bp) for each of them As for the efficiency of the primers used in the study, it varied among the studied species, the highest efficiency was for the (P15) it reached (811) and the lowest efficiency was for the (P3), which scored (2.7), and the discrimination powers of the primers were distinguished. The initiator (P2) had the highest discriminatory ability, which reached (10.2), while the lowest discriminatory ability was recorded for the initiator (P5).(Singh and Achint, 2017:Bajpai and Tewari, 2010).

Table (5) Results of the primers used in RAPD reactions for samples

variation ratio %	Absent bands	Unique bands	Polymorphic band number	Monomorphic bands number	Bands number	Polymorphic loci number	Monomorphic loci	Loci number	Primer Number	c
75	-	5	7	8	15	6	2	8	P1	1
100	1	4	17	-	17	9	-	9	P2	2
100	-	3	7	-	7	5	-	5	P3	3
80	1	1	8	4	12	4	1	5	P4	4
83	-	4	6	4	10	5	1	6	P5	5
86	1	2	10	4	14	6	1	7	P6	6
78	1	2	13	8	21	7	2	9	P7	7
80	2	4	14	8	22	8	2	10	P8	8
89	2	3	14	4	18	8	1	9	P9	9
71	-	3	7	8	15	5	2	7	P10	10
89	-	4	12	4	16	8	1	9	P11	11
100	2	3	14	-	14	7	-	7	P12	12
75	1	5	14	12	26	9	3	12	P14	13
90	1	4	13	4	17	9	1	10	P15	14
55	2	4	10	20	30	6	5	11	P16	15
90	14	51	166	88	254	102	22	124	Total	

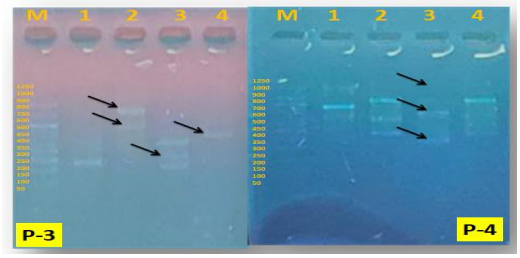
The molecular sizes of the resulting bands varied, ranging between (100-2000bp) where the lowest molecular size was (100bp) in the primer (P6) and the highest molecular size in each of the primer (P1)), where the molecular size was (2000 bp). As for the proficiency of the primers used in the study, it varied between the studied samples, so the highest efficiency was for the primer (P15) as it reached (8)) and the lowest efficiency was for the primer (P5) where it scored (2.9). As for the ability of discriminatory primer, the primer (P1) was distinguished by the highest discriminatory ability, reaching (8.7), while the least discriminatory ability of the primer was (P5), reaching (3.4) Singh and Achint, 2017) (Sultan and Qadir, 2015).

The study also showed the presence of distinct bands (Absent bands, Unique bands) as shown in Table (6). The total of distinct bands resulting from the primers

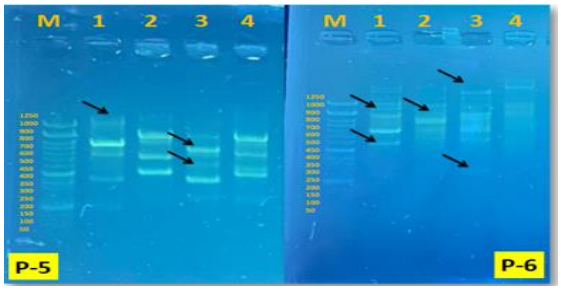
was (85) bands of which (67) were unique bands and (18) bands were absent., The metal fly was characterized by the highest percentage of unique bands, reaching (24) bands, while the horse fly got the lowest percentage of unique bands, reaching (12) bands. As for the absent bands, the house fly got the least number of absent bands, it was (3) A bands Whereas the horse fly got the highest number of absent bands as it reached (6) bands, and these bands are used as a diagnostic and distinguishing characteristic for those types, so the appearance of those bands in one type indicates a mutation in a specific loci that led to the identification of the primers of this loci and the emergence of the unique bands, as well Absent bands, as a mutation occurs in the recognition site of the initiator only in one species without the other types, which leads to the cancellation of that recognition and the bands disappears and this is consistent with the results of most researchers (Sharma et al, 2015a: Brito et a, 2008: malviya et al., 2011,2012,2015).

Table (6) Distinctive bands the efficiency of the primers, and the discriminatory ability

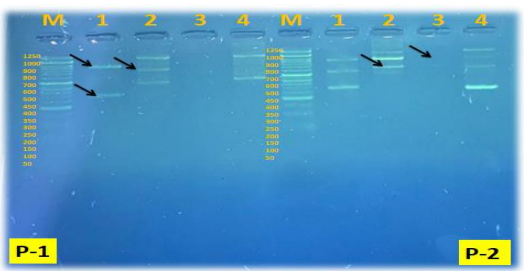
Discriminatory ability	The efficiency	Distinctive bands in flies								Molecular waght	Primer name	N
		Dhuluiya, Balad, Al-Alam, Samarra		Dhuluiya, Balad, Al-Alam, Samarra		Dhuluiya, Balad, Al-Alam, Samarra		Samarra				
		Absent	unique	Absent	unique	Absent	unique	Absent	unique			
4.2	5.9	–	1	–	-	-	3	–	1	400–1500bp	P1	1
10.2	6.6	1	1	–	2	–	1	–	3	400–1500bp	P2	2
4.2	2.7	–	–	–	3	-	1	–	2	300–1100	P3	3
4.8	4.7	-	-	1	-	–	1	–	–	350–1250	P4	4
3.4	3.6	-	1	-	1	-	2	-	-	300–1250	P5	5
6	5.5	-	-	1	2	-	-	-	-	300–1500	P6	6
7.8	8.2	-	-	-	2	-	-	1	-	300–1250	P7	7
8.4	8.6	-	-	1	1	1	1	-	2	300–2000	P8	8
8.4	7	1	1	-	1	-	-	1	1	350–1500	P9	9
4.2	5.9	-	-	-	-	-	2	-	1	350–1250	P10	10
7.2	6.2	-	-	-	2	-	2	-	-	300–1500	P11	11
8.4	5.5	-	1	-	2	-	-	2	-	250–1250	P12	12
8.4	10.2	1	2	-	-	-	1	-	2	200–1300	P13	13
7.8	6.6	-	1	-	-	-	1	1	2	300–1300	P14	14
6	11.8	-	1	-	2	1	1	-	-	300–2000	P15	15
		3	9	3	18	2	16	5	14	Total		
		12		21		18		19				
70												



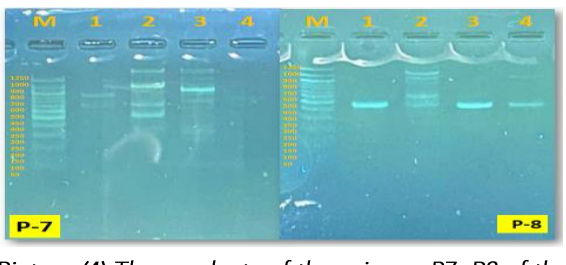
Picture (1) The products of the primers P1, P2 of the four sample of sand flies



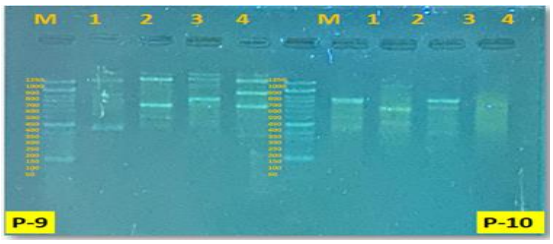
Picture (3) The products of the primers P5, P6 of the four sample of sand flies



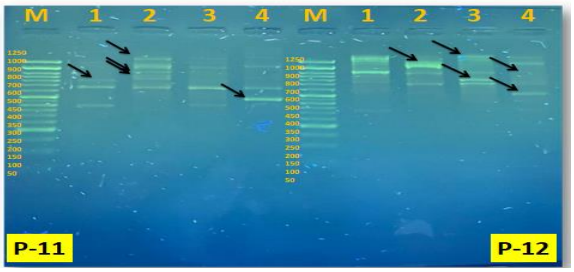
Picture (2) The products of the primers P3, P4 of the four sample of sand flies



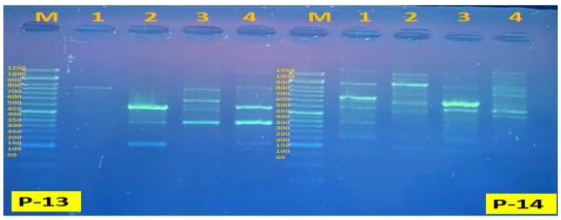
Picture (4) The products of the primers P7, P8 of the four sample of sand flies



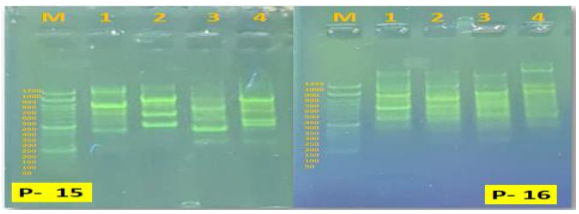
Picture (5) The products of the primers P9, P10 of the four sample of sand flies



Picture (6) The products of the primers P11, P12 of the four sample of sand flies



Picture (7) The products of the primers P13, P14 of the four sample of sand flies



Picture (8) The products of the primers P15, P16 of the four sample of sand flies

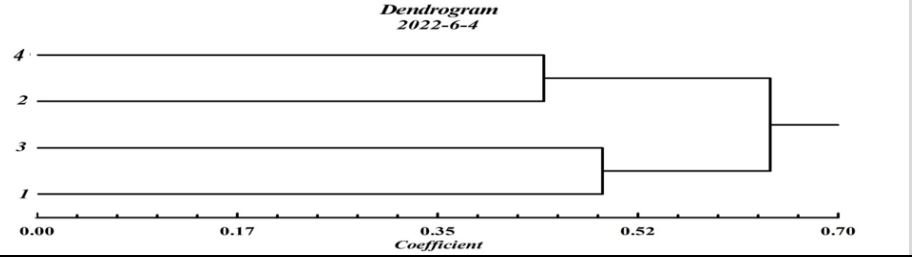
Estimating the genetic distance: The estimation of the genetic distance was performed from the results of the RAPD markers between the four types of flies using the genetic program (NTSYS-PC.version 2.10) which depends in its analyzes on the equation (Nei and Li, 1979). Table (7) shows the values of the genetic distance of the four types of flies. The study was studied using (15) random primers from the RAPD primers, it was found through the results of statistical analysis that the values of the genetic distance between 0.443 -0.737, where the lowest dimension was found between the two isolates country and science, which amounted to 0.443, and the highest genetic dimension was between the Dhuluiya sample and the country with a genetic dimension of 0.737 and this is the lowest similarity ratio between the two species within the studied species, and this applies with what he mentioned (malviya, 2011-2012-2015: Sultan and Qadir, 2015).

Cluster analysis: Based on the values of the genetic distance of the studied species obtained from the results of the RAPD, the cluster analysis group shown in chart (1) was created, where the genetic relationship through chart (2) showed that it was divided into two main groups, group (A and B), which included Group (A) the metal fly only, and this indicates that the metal fly is more different from the rest of the species, and therefore it has the highest genetic distance within the studied species, while group (B) included the other three species, and group (B) was divided into two subgroups, which are group B1 and B2, where group (B1) included the house fly only, while group B2 was divided into two subgroups (B2a, B2b) and the group (B2a) included the meat fly, while group (B2b) included the fourth type, which is the horse fly, and this explains However, group (B2), which includes both the horse fly and the meat fly, has the least genetic distance and is therefore the most similar among the species that have been studied molecularly and this applies to what was mentioned by (Sultan and Qadir, 2015) and (malviya, 2011,2012,2015).) On the genetic distance.

Conclusions

We conclude from the above results the degree of close affinity between the four types of flies, although they belong to different families, but they all belong to one order, which is the Diptera order, and this indicates that the molecular markers, including the RAPD, can be used in the classification of insects, especially flies, in a precise classification and identification The genetic imprint has instead of adopting the phenotypic traits in the classification because the phenotypic traits are unstable and change with the change of environmental conditions in addition to the great similarity between the types of flies, especially the species, including the genus, which makes the phenotypic classification process difficult and imprecise.

Table (7) the values of the genetic dimension of Molecular Data
0.000 0.590 0.000 0.443 0.616 0.000 0.619 0.494 0.737 0.000
Diagram (1) shows the genetic relationship of the four types of flies depending on the values of the genetic dimension



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