AFLP ASSESSMENT OF *FICUS* CULTIVARS FOR IDENTIFICATION AND CONSERVATION

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ABSTRACT

The fig (*Ficus carica* L., *Moraceae*), a typical Mediterranean fruit crop, is characterized by large adaptive potentialities to various ecological areas. To create a molecular characterization for 16 cultivars of *Ficus*, five primer combinations were used to amplify these cultivar's genomic DNA, producing a total of 292 legible bands (markers) were revealed, of which 281(96%) distinct polymorphic band patterns. The genetic diversity among 16 cultivars of fig ranged between 0.2124-0.7154. The lowest genetic distance was found between Qashe kani and Arzani where as the highest genetic distance was found between Qarani rash and Ribari. Cluster analysis by Using UPGMA method based on the similarity coefficient, cultivars were separated into seven major genetic clusters which were named as (F1, F2, F3, F4, F5, F6 and F7) with many sub-clusters. The analyzed data illustrates a good variability in the genetic pool of the local common fig making it a valuable source for incorporation into potential breeding programs for the region.

Key words: Fig (Ficus carica), AFLP, genetic similarity

INTRODUCTION

Ficus carica L. (2n = 26 chromosomes) is one of the 750 are in a fit one of the 750 species of the genus Ficus (Berg, 1989). The fig tree (Ficus spp) has one of the longest histories as a fruit tree in human history (Zohary, 1988). Trees of the Ficus species belong to the family Moracea and can be easily distinguished by the presence of roots growing from the branches (Bamikole et al., 2001). It is known to have been domesticated from a group of diverse spontaneous figs occurring in the south and east of the Mediterranean region sometime in the Early Neolithic period (Zohary and Hopf, 1993). However, large fruited fig trees found in the deciduous forests of the Colchic district of northern Turkey and the Hyrcanic district of Iran and adjacent areas, which often intergrades into the Mediterranean figs, are considered by some botanists as a distinct ecotype of F. carica, and as a separate species, F. colchica Grossh and F. hyrcanica Grossh, by others (Zhukovsky 1962). According to Vavilov (1951), Transcaucasia is considered as one of the centers of origin and diversity as one could see all phases of the domestication of fig in the southern Caucasus, where wild, transition, and modern fruit growing still exists. Phylogeny studies have shown that the common fig has a monoecious ancestor (Machado et al, 2001). It evolved later in gynodioecious species with bisexual trees (functional male figs or caprifigs) and unisexual female trees. Ficus carica L. is usually considered gynodioecious rather than dioecious.

In wild populations of the Mediterranean region, caprifig and female trees occur in similar frequencies (Valdeyron and Llyod, 1979). Fig trees produce an edible inflorescence known as a syconium (fig), which consists of a fleshy cup with a small orifice at one end enclosed by interlocking scales. Within the fig are hundreds of minute flowers which, after pollination usually by wasps, develop into tiny fruit. The fruit is consumed either fresh or preserved (dried or canned). In addition to being eaten as a fruit, several studies have reported that some Ficus species possess medicinal properties. For example, the leaves of Ficus bengalensis have been found to reduce the symptoms of ulcers and the aerial roots have been used to treat gonorrhea (Satyavati, 1976). Moreover, F. carica and F. pentoniana contain flavonoid alkaloids which are known to have antioxidant properties (Subramanian and Nair, 1970). Also, the roots of the variety F. hirta when boiled produce a gel which has been shown to be an effective antipyretic (Manandhar, 1998). As a crop grown for both food consumption and a perceived medicinal value, understanding the relatedness of different species in the Ficus family could provide insight into the distribution and evolution of beneficial traits.

Many studies have reported the morphological characterization of fig cultivars and proved the sustainability of the use of morphological parameters to evaluate and to establish a description of genotypes (Chessa *et al*, 1998). Thus, it has been assumed that the most discriminant parameters were related to

leaves and fruits. However, these characters are sensitive to environmental conditions; the discriminant ones are limited in number and do not allow the separation of the phenotypes into distinct groups (Valdeyron, 1976). Ficus species are represented by a large number of varieties/accessions which are facing genetic However, historical records erosion. of introductions of respective varieties are nonexistent or are highly ambiguous. As a complement to morphological, physiological and agronomic traits, all of which are purely phenotypic, genetic analyses using molecular can provide a phenotypically markers independent method for cultivar and clone characterization. Some of the difficulties with morphological traits include (i) a limited number and low heritability of phenotypic characters; (ii) difficulty in obtaining an accurate distinction between different cultivars before plants have attained the adult phase of life; (iii) an inability to distinguish cultivars from cuttings of in vitro cultured plants. To overcome these difficulties, a variety of methods have been used to fingerprint fig cultivars such as isozymes (Hedfi et al, 2003), randomly amplified polymorphic DNA (RAPD) (De Masi et al, 2003); simple sequence repeats (Khadari et al, 2003), and AFLP (Cabrita et al, 2001). Little effort has been made to establish the genetic framework and varietal relationships of figs including native variety rootstocks in this area. On the other hand, various molecular markers such as RFLPs (Khadari et al, 2005), RAPDs (Sadder and Ateyyeh, 2006), ISSRs (Salhi-Hannachi et al, 2004), AFLPs (Cabrita et al, 2001), and SSRs (Giraldo et al, 2005) have been applied widely to identify various fig cultivars, landraces, as well as population diversity and relatedness.

Other kinds of DNA markers used to a lesser extent in the identification of temperate fruit trees are AFLPs (Vos *et al*, 1995). These markers combine RFLP and PCR techniques, as they are specific PCR amplified fragments of restriction digests. Their use is more complex than that of RAPDs or SSRs since there are several steps involved beside PCR amplification and marker analysis. Angliolillo *et al*, (1999) used AFLP markers to establish genetic relationships in the genus *Olea*. Twenty-five common and four Japanese pear cultivars were also analyzed with AFLPs by Monte-Corvo *et al*, (2000) that also studied the genetic similarities among the genotypes studied.

AFLPs have proven to be extremely proficient in revealing diversity at the species level and provide an effective means of covering a wide area of the genome in a single assay (Zhu et al., 1998). AFLP technique was introduced as a reliable and reproducible marker system (Vos et al., 1995). It was preferred over other DNAbased markers mainly because of its high multiplex ratio and non-requirement of prior sequence information (Yuan et al., 2000). Comparative analysis using several PCR-based markers showed that the multi-locus AFLP technology is one of the best methods available for evaluation of germplasm (Russell et al., 1997). Aggawal et al, (1999) reported that the results of various tests done to check robustness of the dendrogram/estimates of phylogeny, clearly establish that the polymorphism revealed by AFLP is not only abundant but also stable and statistically reliable.

The objectives of this study were to: (1) optimize the AFLP conditions in *Ficus carica*, (2) estimate the level of polymorphism among the fig cultivars used in this study, (3) determine the genetic relationships and study the identification and phylogenetic analysis of cultivars of *Ficus* through AFLP markers among these genotypes using Jaccard's coefficient.

These information can be used for rational design of breeding programs, conservation of local germplasm, management and save of fig genetic resources.

MATERIALS AND METHODS

Sample collection

Leaf samples of the local Fig cultivars were collected from different districts in Kurdistan region. These cultivar samples were obtained from the Ministry of Agriculture fields in Duhok and Shaqlawa cities. The cultivars of Fig selected for this study were: (Shoshi, Baqo, Mamzhi, Maroo, Rash, Faqey shekhi, Qarani rash, Benati, Shingali, Nav sor, Shinik, Qashe kani, Katik, Arzani, Rehani and Ribari).

DNA Extraction

Genomic DNA was extracted from fresh tissue for all samples according to (Weigand *et al.*, 1993), 3g of tissue was grounded to fine powder using liquid nitrogen. The fine powder was dissolved in apre-heated (60°C) 2x CTAB extraction buffer (2x CTAB, 5M NaCl, 1M Tris-HCl, 0.5 M EDTA), and incubated at 60 °C in shaking water bath for 30 min. The mixture was

extracted with an equal volume of choloroform / isoamyl alcohol (24:1, v/v) (Maniatis *et al.*, 2001). The mixture was then centrifuged (at 4000 rpm for 30 min). The aqueous phase was transferred into another tube and precipitated with 0.66 volume of isopropanol. Precipitated nucleic acids were then dissolved in TE-buffer and stored at -20°C until use.

PCR Amplification of AFLP- primers:-

AFLP procedure was performed as described by (Vos et al., 1995) with minor modification 500ng of DNA from each sample was double digested with 5U each of the two restriction enzymes, *Tru91* (recognition site 5'T↓TAA3') and *PstI* (recognition site 5'CTGCA \downarrow G3'). The digestion reaction was prepared in 30µl final volume containing, 1x one- phor all buffer (Pharmacia Bioteh, Uppsala, Sweden), and incubated for three hours at 37°C. DNA fragments were then ligated to Pst I and Tru91 adapters by adding 50pmol of Tru91-adapter, 5Upmol PstI-adapter in a reaction containing 1U of T4-DNAligase, 1mM rATP and 1x of onephore-buffer and incubating for 3hr. at 37°C. After ligation, the reaction mixture was diluted to 1:5 using sterile distilled water. Preselective PCR amplification was performed in a reaction volume of 20 µl containing 50ng of each of the primers (P00, M43) corresponding to the Tru91 and Pst I adapters, 2µl of template- DNA, 1U Taq DNA polymerase, 1x PCR buffer and 5mM dNTPs. PCR amplification was performed in WMG thermal cycler using the following program: 30 cycles of 30s at 94 °C, 1min s at 60°C, 1min at 72 °C. Pre-amplification products were then diluted to 1:5 and 2µl were used as template for selective amplification. Selective amplification was conducted using Tru91 and *Pst1* selective primer combinations, (Table 1). Amplification was performed using a selective program of 36 cycles with the following profile: a 30sec. DNA denaturating step at 94°C, 30sec. annealing step, and a 1 min extension step at 72°C. The annealing temperature in this program varied in the first cycle where it was 65°C and in each subsequent cycle for the next 12 cycles it was reduced by 0.7°C (touchdown PCR). Then for the remaining 23 cycles, it was 56°C. Selective amplification products were loaded onto 6% denaturating polyacrylamid gels, and DNA fragments were visualized by silver staining kit (Promega, Madison, Wis) as described by the supplier. Silver - stained gels

were scaned to capture digital images of the gels after air drying.

Data analysis:-

The photographs of gels were used to score the data for AFLP analysis starting from higher molecular weight product to lowest molecular weight product. Presence of a product was identified as (1) and absence was identified as (0). Data were scored for all genotypes, their amplification product and primers. The data then entered into NTSYS-PC (Numerical Taxonomy and multivariate Analysis System), Version 1.8 (Applied Biostatistics) program (Rohlf, F. J. (1993) using the program editor. The data were analized using SIMQUAL (Similarity for Qualitative Data) routine to generate genetic similarity index (Nei and Li., 1979).

RESULTS AND DISCUSSION

In the present investigation AFLP analysis has been adapted to assay the level of polymorphism and to produce a fingerprint for 16 studied Fig cultivars. Nevertheless, the technique required initial optimization to identify primer combinations that yield interpretable and reproducible. Therefore, five primer combinations were initially tested with the 16 samples of Fig cultivars. All five tested primer combinations, yielded discernible and reproducible profiles.

Identification of varieties depending on morphological traits alone is difficult and not accurate. A variety may have different names in different plantation and genetically different varieties may have the same name (Torres and Tisserat, 1980). There are several different DNA marker analysis techniques that have been used to identify and characterize fruits to determine genetic diversity (Jubrael J. M.S., 2005). Each technique has its own requirements, sensitivity and reliability. In this study AFLP technique reliably distinguished all selected varieties and has high efficiency compared to other markers like RFLPs and RAPD.

The results of selective amplification as shown in (Figure 1) and the presence of AFLP bands across all 16 Fig cultivars clearly indicate the successful application of AFLP marker technology. AFLP analysis using five selective primer combinations generated a total of 292 fragments (bands of DNA); the number of polymorphic amplicons was 281 representing a level of polymorphism of 96% and an average number of polymorphic bands of 58.4 per AFLP primer combination. The size of the AFLP amplified fragments ranged from 50bp. to 1500bp. (Fig. 1).

Similar findings were reported by Han et al., (2000) studying tea species, they found that the number of amplified AFLP bands per assay ranged from 32 to 150 with a mean of 84.7. Le Febvre et al. (2001) analyzed 47 pepper inbred lines with 10 AFLP primer combinations and revealed 863 selectively amplified fragments of which 378 were polymorphic (34.8%). Matthes (2001) used ten AFLP et al. primer combinations with oil palm and reported that the average number of bands per primer combination was 82 which are in agreement with our results. Cervera et al., (1998) applied the AFLP technique to characterize 67 different grapevine accessions. They obtained an average of 100 amplified fragments per primer combination, of which 49% were polymorphic.

To determine the genetic relationships among the 16 cultivars of fig, the scoring data (1 for presence and 0 for absence) resulting from the five primer combinations were used to compute the similarity matrices according to Jaccard (Jaccard, 1908). These similarity matrices were used to generate a dendrogram using the UPGMA method. As shown in Table (2) genetic diversity among 16 cultivars of fig ranged between 0.2124-0.7154. The lowest genetic distance was found between Qashe kani and Arzani showing (81%) similarity where as the highest genetic distance was found between Qarani rash and Rebari showing (77%) similarity. These genetic relationships were reflected on the dendrogram which represents the graphical illustration of the genetic distances among the 16 fig cultivars (Figure: 2). As shown in the dendrogram, the coefficient of similarities based on AFLP fragments revealed genetic diversity between the fig genotypes under consideration (Figure: 1).

In the dendogram, it is possible to distinguish seven main genetic groups named as $(F_1, F_2, F_3,$ F_4 , F_5 , F_6 and F_7). The first genetic group include (Shoshi and Rash), the second genetic group include (Baqo, Mamzhi and Faqey shekhi), the third genetic group include (Shingali, Nav sor, Shinik, Qashe kani, Katik, Arzani and Rehani), within this main genetic group we can distinguish four sub-groups including (Shingali, Naf sor, Shinik and Rehani) while Qarani rash, Maroo, Benati and Rebari arranged in separated cluster. The total number of amplified DNA fragments may make these varieties comes in separated groups. Studying the morphology of these varieties, it is noted that they have some characters that are close to each other, for example, the shape and color of fruits. Sub-clusters separated the varieties and form distinct genetic diversity among clusters.

The analyzed data illustrates a good variability in the genetic pool of the common local fig making it a valuable source for incorporation into potential breeding programs for the region.

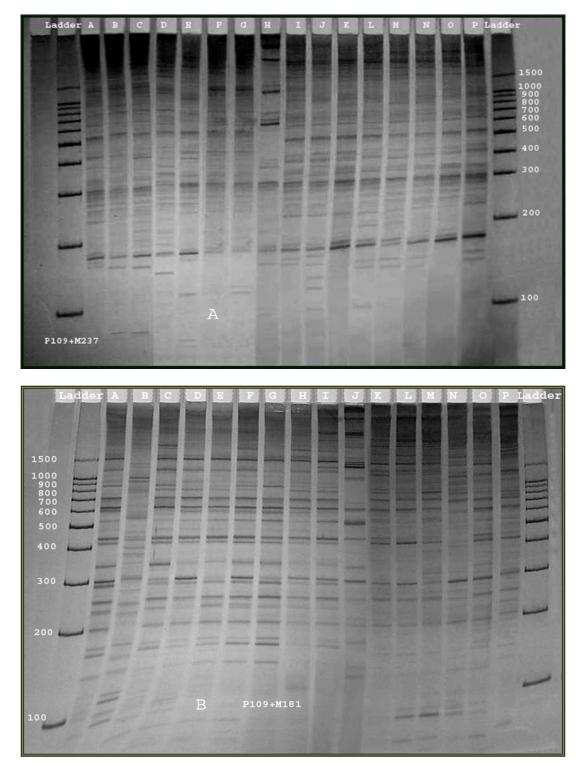


Figure 1. Profiles of the sixteen *Ficus* cultivars using the primer combinations **A** (P109 + M273) and **B**)P109 + M181). Lanes 1 through 16 refer to *Ficus* cultivars: 1. Shoshi, 2. Baqo, 3. Mamzhi, 4. Maroo, 5.Rash, 6. F. Shekhi, 7. Qarani rash, 8. Benati, 9. Shingali, 10. Navsor, 11. Shink, 12. Qashe kani, 13.Katik, 14. Arzani, 15. Rehani and 16. Rebari. Lanes **M** refer to molecular weight marker 1500-100bp.

No.	F	Pre selective primer ('53')	Selective primer ('53')			
1	POO	GACTGCGTACATGCAG	P100	GACTGCGTACATGCAGAACC		
2	M43	GATGAGTCCTGAGTAAATA	P109	GACTGCGTACATGCAGAATG		
3			P237	GACTGCGTACATGCAGGATA		
4			M181	GATGAGTCCTGAGTAACCCC		
5			M289	GATGAGTCCTGAGTAATAAA		
6			M291	GATGAGTCCTGAGTAATAAG		
7			M237	GATGAGTCCTGAGTAAGATA		

Table (1): Represents the sequences of Pre-amplification and Selective amplification primers, used in this study.

Table (2): The genetic distance values between Ficus samples studied.

Shoshi Baqo Mamzhi Maroo Rash F.shekhi Q.rash Benati Shingali NavSor Shinik Q.kani Katik Arzani Rehani Ribari

1. Shoshi	0						
2.Baqo	0.2897 0						
3.Mamzhi	0.3482 0.2296 0						
4.Maroo	0.3596 0.4706 0.	. 3935 0					
5.Rash	0.2807 0.3837 0.	.4180 0.4023 0					
6.F.shekhi	0.3859 0.2622 0.	.2404 0.4550 0.34	0 0				
7.Q.rash	0.3892 0.4902 0.	.5352 0.5521 0.38	23 0.5269 0				
8.Benati	0.5196 0.4983 0.	.5500 0.6813 0.50	1 0.4921 0.612	3 0			
9.Shingali	0.3388 0.3089 0.	.3522 0.5065 0.35)3 0.3574 0.381	4 0.5874 0			
10.Navšor	0.3298 0.3524 0.	.4266 0.4648 0.37	58 0.3972 0.392	2 0.4696 0.2926	0		
11.Shinik	0.3954 0.4696 0.	.4394 0.5499 0.36	L7 0.4255 0.371	7 0.4818 0.2927	0.2672 0		
12.Q.kani	0.3245 0.3554 0.	.3705 0.5118 0.37	38 0.4086 0.375	4 0.4322 0.2411	0.2172 0.2539	0	
13.Katik	0.4280 0.4605 0.	.4216 0.5194 0.43	LO 0.4443 0.435	5 0.5663 0.3154	0.3155 0.2478	0.2232 0	
14.Arzani	0.3606 0.3557 0.	.4232 0.5309 0.35	19 0.4190 0.390	1 0.4996 0.3047	0.2556 0.2883	0.2124 0.2943	0
15. Rehani	0.3453 0.3175 0.	.3833 0.5077 0.34	0 0.3965 0.366	0 0.5656 0.3167	0.3158 0.3584	0.2719 0.3567	0.2567 0
16.Ribari	0.6099 0.4547 0.	.5574 0.6010 0.56	53 0.4674 0.715	4 0.5825 0.5978	0.5770 0.5844	0.6406 0.6751	0.5188 0.6573 0

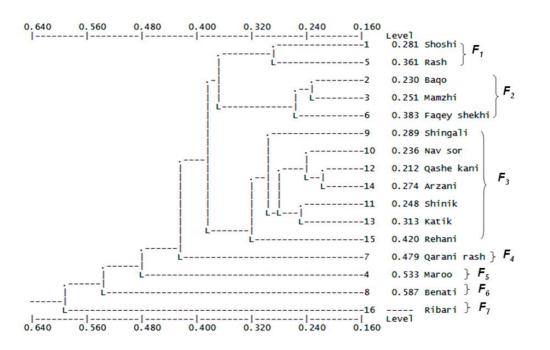


Figure 2: Represents the tree of genetic relationship between apricot varieties using AFLP markers.

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تقيم مؤشرات AFLP لاصناف التين لايجاد التنوع الوراثي و المحافظة على الاصناف

الخلاصة

يعتبر التين (L. Ficus carica) من الفاكهة المثالية التي تنمو في حوض البحر المتوسط و تتميز بقدرتما التكيفية العالية للظروف البيئية المختلفة .اسخدمت مؤشرات ال AFLP لايجاد العلاقة الوراثية بين (16) اصناف المختارة للدراسة باستخدام خمس توليفات من البرايمرات أنتحت 292 حزمة واضحة ومن بينها (%96) 281 حزم متباينة .وأدخلت البيانات التي تم الحصول عليها الى الحاسوب وتم تحليلها وفق برنامج UPGMA الاحصائي الخاص بمذا النوع من الدراسات .التنوع الوراثي بين الأصناف المدروسة تراوحت بين .(15, 200 – 2010) اقل بعد وراثي وجدت بين الصنفين قشي كاني و ارزني بينما اعلى بعد وراثي وجدت بين الصنفين قرني رش و ريباري .أظهرت نتائج التحليل التجميعي سبع مجاميع وراثية سميت , (17, 27 على بعد وراثي وجدت بين الصنفين قرني رش و ريباري .أظهرت نتائج التحليل التجميعي سبع مجاميع وراثية سميت , (17, 30 المان الحراثية المان المان المانية مين المانين قرني رش و ريباري . وحموعات ثانوية .البيانات الحراثية مين المانية مين الحراثي وجدت بين الصنفين قشي كاني و ارزيني بينما الحمان الحمان المادروسة تراوحت بين .(15, 75, 90) اقل بعد وراثي وجدت بين الصنفين قشي كاني و ارزيني بينما اعلى بعد وراثي وجدت بين الصنفين قرني رش و ريباري .أظهرت نتائج التحليل التجميعي سبع مجاميع وراثية سميت , (17, 75 الماني المانية العادة الوراثية . وحمان المانية المانية وحدت بين الصنفين قرني رش و ريباري .أظهرت نتائج التحليل التحميعي سبع مايي وراثية ميت , 18

زيىندەگەردە تەكنيكى AFLP بۆ ناسينەوەو پاراستني جۆرەكانى ھەنجير

پـوخــته

رووكى هەنجير يەكىكە لە مىوە گونگەكانى ناوچەكانى دەرياي ناوەراست كە توانايەكى خۆگۈنجاندنى بەرزى ھەيم بۆ بارودۆخى ژينگەي جۆراوجۆر .لەم تويتژينەوەيدە زيىندەگەردە تەكنىكى AFLP بەكار ھىنىراوە بۆ ناسىنەوەو دۆزىنەوە پىيەنلەندى بۆماوەيى لەنيوان جۆرەكانى رووكى ھەنجىركە تيايدا شازدە جۆري جياوازي رووكى ھەنجىر تويتژينەوە شىيتەلكردنى بۆماوەييان بۆ ئەنجام دراوە بە بەكارھىنانى يىنج گروپى دەستېيكەر كۆى گىشتى باندە زۆركراوەكانى ترشى ناوەكى DNA بريتى بوون لە (292)باند ,كە (281) بانديان (60%) جياوازو فەشىيوەبوون .بە بەكارھىنانى بەرنامەى كۆمپيوتەرى MPGMA تايبەت بەشىتەلكىردن ولىكدانەوەى ئامارى بۆماوەيى و بە پشت بەست بە ئەنجامەكانى ئەم تويترىنەوىيە ,دوورى بۆماوەيى نيوان جۆرەكانى رووكى ھەنجىر لە زۆرترىن دووررى بۆماوەيى لە ئىوان جۆرى قەرەنى رەش وريارى بولىوەيى نيوان جۆرەكانى رووكى ھەنجىر لە بۆماوەيى و بە پشت بەست بە ئەنجامەكانى ئەم تويترىنەوىيە ,دوورى بۆماوەيى نيوان جۆرەكانى رووكى ھەنجىر لە زۆرترىن دووررى بۆماوەيى لە نيوان جۆرى قەرەنى رەش و ريبارى بوو .بە پشت بەشتەئكردن ولىكدانەوەى ئامارى زېرەرەيى دوررى بۆماوەيى لە نيوان جۆرى قەرەنى رەش و ريبارى بوو .بە پشت بەشتى بە ئەنجامەكانى شىيەلاردن بۆماوەيى دوررى بورە .بە ئەنجامەكانى ئەم تويترىنەيە دوررى بۆماوەيى لە نيوان جۆرى قەشى كانى دووكى ھەنجىر لە بورەورى دورە يە ئېماوەيى لە نيوان جۆرى قەرەنى رەش و ريبارى بوو .بە پشت بەست بە ئەنجامەكانى شىيەلاردن زېرەريىن دووررى بۆماوەيى لە نيوان جۆرى قەرەنى رەش و ريبارى بوو .بە پشت بەست بە ئەنجەمەكانى شىيەتەلكردن دۆرترىين دوررى بۆمارەيى گىت شازدە جۆرەكەي ھەنجىر لەرپىگەى ئەم تەكنىك و پېزىگرامەرە پۆلىنىكىران بۆ حەفت بۆماوەگروپى سەرەكى كە بويىتى بوون لە (14, 57, 57, 47, 57, 67, 71) و چەندىگرويىكىي بوروكى ھەنجىردە بېرەيەرەكىرەي سەرەكى كە ئەنجامەدى نەرەن بەرەرەيەرى بەرماوەيى بەش بەنىيورە بۇرەكىدى روكى دەنجىردى مەنبەر بە بۆمارەگروپى سەرەكى .ئەم ئەنجامەنە دەريانىست كەرەردەكارانى ھەنجىر بەمەبەستى ھەلبردى دىتىكىدى دەردىكىدى