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Investigation of mitochondrial DNA of some West Anatolian *Gammarus* species (Crustacea: Amphipoda) by PCR-RAPD techniques

[Bazı Batı Anadolu *Gammarus* (Crustacea: Amphipoda) türlerinin mitokondrial DNA'larının PZR-RAPD tekniği ile incelenmesi]

Filiz Susuz Alanyalı[1],
Leyla Açık[2],
Ahmet Özata[1],
H.Mehtap Kutlu[1]

[1]Department of Biology, Faculty of Sciences, Anadolu University, Yunusemre Campus, 26470, Eskisehir, Turkey
[2]Gazi University, Faulty of Science and Arts, Department of Biology, 06500, Ankara, Turkey

Yazışma Adresi
[Correspondence Address]

Filiz Susuz Alanyalı

Department of Biology, Faculty of Sciences, Anadolu University, Yunusemre Campus, 26470, Eskisehir TURKEY
Phone: 0 90 222 335 0580 ext. 4726
Fax: 0 90 222 320 4910
e-mail: fsusuz@anadolu.edu.tr

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ABSTRACT

Aim: Mitochondrial DNA is widely used in order to estimate the amount of genetic variability within and between different species, because of its conserved structure and organization. Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD- PCR) can be used for both systematic and genetic studies. In this study, it was aimed to investigate mitochondrial DNA variation of some West Anatolia *Gammarus* species (Crustacea: Amphipoda) by RAPD-PCR.

Material and Methods: In this study, *Gammarus* species were obtained from different localities of West Anatolia and mitochondrial DNA was extracted with the Chelex method. Random Amplified Polymorphic DNA Polymerase Chain Reaction assays were used to estimate mtDNA variation. The genetic similarities were determined by statistical analyses.

Results: In this study, mtDNA isolated from 129 individuals *of Gammarus* sp were analyzed by RAPD-PCR using 39 different random 10-mer primers, but PCR amplification were only obtained with primers M13, OPB08 and B7. The results were evaluated by Nei Genetic Distance Dendogram Program modified from Phylip 3.5 version. The tested *Gammarus* species were found as closely-related in a cluster except for *Gammarus uludagi*. These species were also shown as a distant relative to the other Gammarus sp.

Conclusion: RAPD-PCR analysis of mitochondrial DNA employed in this study reveals that there is a close relationship between studied West Anatolian *Gammarus* species. This study is also reinforce the fact that the mitochondrial DNA can be used as a powerful molecular marker in phylogenetic relationships.

Conflict of Interest: Authors declare no conflict of interest

Key Words: *Gammarus*, genetic distance, RAPD-PCR

ÖZET

Amaç: Mitokondrial DNA korunmuş yapı ve organizasyonu özelliklerinden dolayı, türlerarası genetik çeşitliliği belirlemede yaygın olarak kullanılmaktadır. Hem sistematik hem de genetik çalışmalar için, Rastgele Çoğaltılmış Polimorfik DNA Polimeraz Zincir Reaksiyonu (RAPD-PZR) tekniği kullanılabilmektedir. Bu çalışmada**,** bazı Batı Anadolu *Gammarus* (Crustacea: Amphipoda) türlerinin mitokondrial DNA'larının Rastgele Çoğaltılmış Polimorfik DNA Polimeraz Zincir Reaksiyonu tekniği ile incelenmesi amaçlanmıştır.

Gereç ve Yöntemler: Bu çalışmada, Gammarus türleri, farklı bölgelerden toplanmış, mitokondrial DNA'ları Chelex metoduna göre izole edilmiş ve Rastgele Çoğaltılmış Polimorfik DNA Polimeraz Zincir Reaksiyonu metodu ile çoğaltılmıştır. Genetik yakınlıklar, Phylip 3.5 versiyonundan modifiye edilmiş Nei Genetik Uzaklık Dendogram programıyla tespit edilmiştir.

Bulgular: Bu çalışmada 129 örneğin mitokondrial DNA izolasyonu yapılmış ve 39 farklı rastgele primer kullanılarak Rastgele Çoğaltılmış Polimorfik DNA Polimeraz Zincir Reaksiyonu ile analiz edilmiştir. Fakat PZR çoğaltması sadece, M13, OPB08 ve B7 primerleri ile elde edilmiştir. Bu çalışmada test edilen *Gammarus* türlerinin, *Gammarus uludagi* dışında, birbiri ile yakın ilişkili türler olduğu bulunmuştur. Bu türlerin ayrıca diğer *Gammarus* türleri ile uzaktan ilişkili olduğuda gösterilmiştir.

Sonuç: Batı Anadolu Gammarus türlerinin birbirleri ile yakın ilişkili olduğu bu çalışmada uygulanan Mitokondrial DNA'nın RAPD-PZR analizi ile anlaşılmıştır. Bu çalışma ayrıca, Mitokondrial DNA'nın, filogenetik akrabalık testlerinde kuvvetli bir ayrıştırıcı moleküler belirteç olarak kullanılabileceği gerçeğini birkez daha kuvvetlendirmiştir.

Çıkar Çatışması: Yazarlar arasında çıkar catışması bulunmamaktadır

Anahtar Sözcükler: *Gammarus*, genetik uzaklık, PCR-RAPD

Introduction

Species taxonomy has enormous importance in estimating biodiversity and designing conservation strategies. The current taxonomy of many invertebrate groups is based predominantly on the similarity of a few morphological characters and has not been scrutinized scientifically and rigorously, particularly in a phylogenetic context [1]. Taxonomic identification of amphipod species (Amphipoda, Crustacea) is a complex process that frequently requires a great deal of expertise. The taxonomy of the genus *Gammarus* is one of the most problematic genus among the Amphipoda [2]. Morphology-based identification of *Gammarus* requires the examination of limited number of phylogenetically informative characters, some of which are difficult to observe, and may display a considerable amount of intraspecific variation [3].

The genus *Gammarus* (Crustacea, amphipoda) is widely distributed throughout the Northern hemisphere and includes a large number of taxa in Europe and North Africa [4-6]. This causes numerous taxonomic problems, as attested by the multiple modifications of the systematic since 1785 [3,7]. As a consequence, a large amount of relevant information regarding the diversity, distribution and ecology of *Gammarus* species has been lost, and this will continue unless new approaches are developed [2]. Recently morphological observations, hybridization experiments, and isoenzyme polymorphisms were the main criteria used to clarify the taxonomic status of the numerous species that have been classified into three groups by Karaman and Pinkster [4,5]: The *G. pulex* group includes at least 31 species; the *G. balcanicus* group includes 45 species; and the *G. roeseli* group includes at least 12 species [3].

Morphological investigation requires the examination of some characters [4-6]. But, some of these characters are difficult to observe and also considerable amount of morphological variability within species [7,8] has complicated the taxonomy, creating separate or sibling species in addition to "classical" species [9]. For these reasons the use of only morphological characters for the taxonomy in *Gammarus* appears to be difficult [10]. These kinds of difficulties may explain why no morphological data have rigorously addressed the phylogenetic concerns in the genus. Investigations of ecological characteristics may also be very useful for the taxonomic identification of some populations [11]. Hybridization experiment may solve taxonomic problems [12-15], on the other hand this way is limited by some technical problems [4,5]. Methodologies to the conventional morphology-based taxonomic identification of amphipod species are therefore urgently required, especially techniques that can be used routinely providing a simple and universal application [2]. With the rapid improvement of molecular technology and the dramatic drop in costs, DNA experiments have become the most popular choice of data for phylogenetic reconstruction. DNA data have potentials to provide nearly unlimited number of informative characters, and choices for various phylogenetic questions, since genes have different levels of variability. The advantages of DNA data and the disadvantages of morphology are much more pronounced among species groups, like the genus *Gammarus* [1]. Due to advances in molecular biology techniques, large number of highly informative DNA markers has been developed for the identification of genetic polymorphism. In the last decade, the Random Amplified Polymorphic DNA (RAPD) technique based on the Polymerase Chain Reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence. Low expense, efficiency in developing large number of DNA markers in a short time and requirement for less sophisticated equipment has made the RAPD technique valuable [16]. Also, the randomly amplified polymorphic DNA (RAPD) technique has advantages such as the screening of the large number of *loci*, the rapidity of the procedure, processing of the large number of samples and the requirement of the low amounts of specimen tissue [2].

Gammarus species are an invertebrate that is a member of the nourishment chain aquatic environments and increasingly gains importance as a test organism in environmental studies. [17] Molecular data have played an important role in illustrating the evolutionary history of amphipod crustaceans because morphology alone has been found to be inadequate for resolving phylogenetic relationships between closely-related and morphologically similar crustacean species [21]. Within *Gammarus*, a steadily-increasing number of studies have confirmed molecular techniques as a powerful tool for providing new insights and a better understanding of speciation and population structure [3,22,23].

Mitochondrial DNA (mtDNA) is widely used as a powerful marker to study phylogenetic relationships and molecular evolution [3]. Especially it has been one of the most widely used molecular markers for phylogenetic studies in animals, because of its simple genomic structure [24]. Though mtDNA sequence data have proved valuable in determining phylogenetic relationships, the choice of gene is also of great significance [25].

In this study, we examined the phylogenetic relationships among the populations of some West Anatolian *Gammarus* species obtained from different localities based on mtDNA and RAPD-PCR technique.

Materials and Methods

Gammarus Material

In this study *Gammarus pulex* 1758 (from Eskisehir region); *Gammarus uludagi* 1975 (from Bursa region); *Gam-*

marus komareki 1922 (from Edirne-Kırklareli region); *Gammarus arduus* 1975 (from Edirne-Kırklareli region); *Gammarus fossarum* 1836 (from Edirne-Kırklareli region); *Gammarus agrarius* 1973 (from Antalya region); *Gammarus effeltus* 1975 (from Safranbolu region) species individuals were collected and then kept in 96% alcohol.

Isolation of Mitochondria

Individual specimens were washed in distilled water and then rapidly disrupted in a Dounce homogenizer containing 1 ml NET buffer (30 nM Tris-HCl, 60 mM EDTA, 150 mM NaCl, pH 8) and Proteinase K (100 µg/ml). The homogenate was centrifuged at 1000*g* for 5 min in order to precipitate the nuclei and cellular debris. The supernatant was recentrifuged at 12000*g* for 10 min to obtain the mitochondria.

MtDNA Extraction

mtDNA was extracted with the Chelex method [18]. The mitochondrial pellet was resuspended in 200 µl of a 40% Chelex-100 (Sigma) suspension containing Proteinase K (100 µg/ml) and incubated at 56 ºC for 1 hour. Denaturation occured in a boiling water bath for 8 minutes, before rapid centrifugation to pellet the Chelex. The solution was centrifuged at 13000*g* for 10 minutes in order to precipitate the DNA. The was dried in heat block of mtDNA and then resuspended in 40 µl of 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

PCR Amplification

RAPD assays were performed according to the process recommended by Williams et al. [19]. 10-mer random primers used in this study are listed in Table 1. Amplification was carried out 45 cycles of 30 s at 96 ºC, 30 s at 30 ºC, 30 s at 72 ºC. Fragments generated by amplification were separated according to size on %2 (w/v) agarose gel run in TAE buffer at 40-90 V. Gels were visualized by UV light and photographed using Polaroid Studio B/W film. .

Determination of Genetic Distance

The genetic similarities were determined by Nei [20] Genetic Distance Dendogram Programme modified from Phylip 3.5.

Table 1. PCR Primers used in this study

PrimerName	Primer(5'....3')	%G+C	PrimerName	Primer(5'....3')	%G+C
A1	CAGGCCCTTC	70	UBC572	TTCGACCATC	50
A2	TGCCGAGCTG	70	UBC573	CCCTAATCAG	50
OPB08	GTCCACACGG	70	UBC574	GCCAGACAAG	60
OPI18	TGCCCAGCCT	70	UBC575	GGAGATGTAC	50
B4	GGACTGGAGT	60	UBC576	CACCTAATGG	50
B6	TGCTCTGCCC	70	UBC577	GTCTGATGTG	50
B7	GGTGACGCAG	70	UBC578	GGTGTCCACT	60
B18	CCACAGCAGT	60	UBC579	TGGAATCGTG	50
M13	GAGGGTGGCGGTTCT	66,7	UBC581	CCCGTTAGGG	60
UBC372	CCCACTGAC	70	UBC582	GGTATAGAC	50
UBC373	CTGAGGAGTG	60	UBC586	CCGGTTCCAG	70
UBC378	GACAACAGGA	50	UBC587	GCTACTAACC	60
UBC379	GGGCTAGGGT	70	UBC589	GACGGAGGTC	70
UBC435	CTAGTAGGGG	60	UBC590	CCGGCATGTT	60
UBC440	CTGTCGAACC	60	UBC591	TCCCTCGTGG	70
UBC441	CTGCGTTCTT	50	UBC592	GGGCGAGTCC	80
UBC443	TGATTGCTCG	50	UBC596	CCCCTCGAAT	60
UBC444	GCAGCCCCAT	70	UBC598	ACGGGCGCTC	80
UBC571	GCGCGGCACT	80	UBC599	CAAGAACCGC	60
			UBC600	GAAGAACCGC	60

Results and Discussion

In this study, the evolutionary relationships between different species of genus *Gammarus* were investigated using PCR-RAPD technique. Firstly, mtDNA was isolated from 129 individuals of West Anatolia *Gammarus sp* and amplified by PCR-RAPD using 39 different random primers However, PCR amplification was only obtained with primers OPB08, B7 and M13.

As shown in Figure 1, PCR amplification with primer M13 was resulted in the different band patterns in *Gammarus* species: *G.pulex*, *G. uludagi*, *G.effeltus*, *G.agrarius*, *G.komareki*, *G. fossarum*, *G.arduus* while PCR amplification with primer OPB08 was resulted in the identical band patterns in that *Gammarus* species (Figure 2). In contrast, PCR amplification with primer B7 resulted in the identical band patterns in *G.pulex* and *G.arduus* while resulting in different band pattern in *G. uludagi* (Figure 3). On the other hand, the maximum number of unique accession-specific DNA bands were obtained with primer B7 (Figure 3) while the maximum number of polymorphic bands (8 bands) were obtained with primer the M13 (Figure 1). However, the least polymorphic DNA bands were obtained with primer OPB08 (Figure 2).

Finally, The RAPD profiles, obtained for each species, were evaluated according to Koh et al. [26]. RAPD bands were scored for their absence as 0 and presence as 1. The cluster analysis of the distribution of 28 RAPD bands is shown as a dendrogram by using Nei Genetic Distance Dendogram Programme modified from Phylip 3.5 version. As shown in Figure 4, The *Gammarus* species studied were found as closely-related in a cluster except for *Gammarus uludagi*. These species were found as a dis-

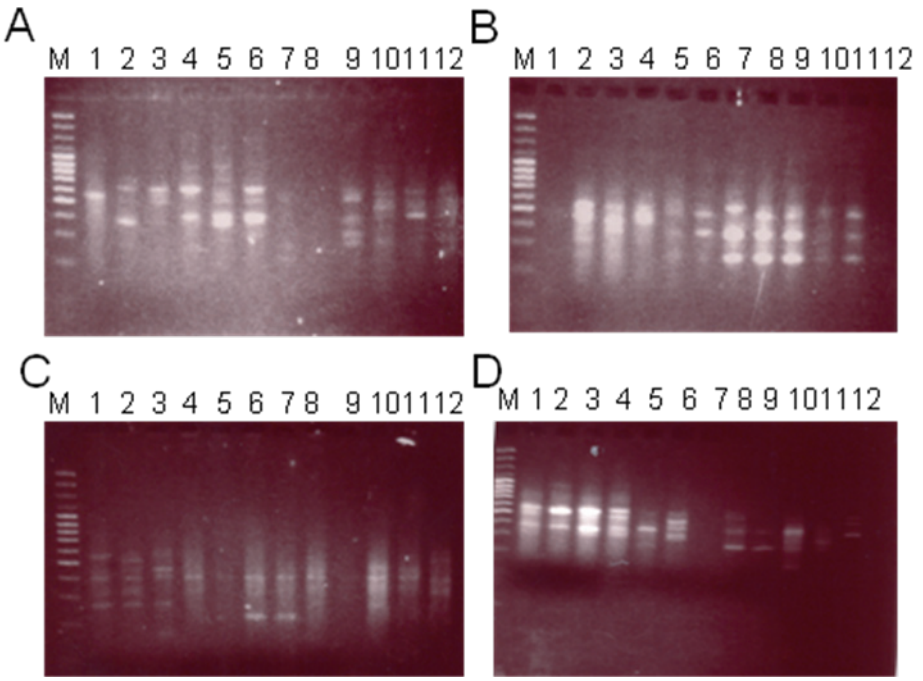

Figure 1. RAPD-PCR band patterns of *Gammarus* species amplified with primer M13. M- DNA Ladder (100 -3000 bp), **A.** *G. pulex* (Line 1-8) *G. uludagi* (Line 9-12) **B.** *G. pulex* (Line 1-6) *G. effeltus* (Line 7-12), **C.** *G. effeltus* (Line 1-10) *G. agrarius* (Line 11- 12), **D.** *G. agrarius* (Line 1-5) *G. komareki* (Line 6-8) *G. fossarum* (Line 9-10) *G.arduus* (Line 11-13)

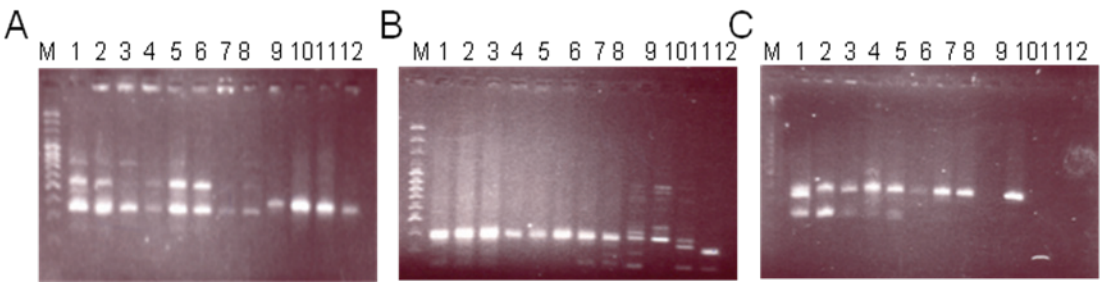

Figure 2. RAPD-PCR band patterns of *Gammarus* species amplified with primer OPB08. M- DNA Ladder (100 -3000 bp), **A.** *G. uludagi* (Line 1-9) *G. pulex* (Line 10-13) **B.** *G. effeltus* (Line 1-9) *G. agrarius* (Line 10-13) **C.** *G. komareki* (Line 1-3) *G. fossarum* (Line 4-7) *G. arduus* (Line 8-12)

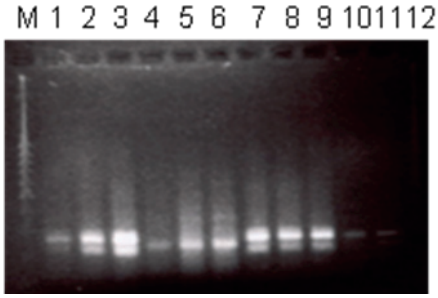

Figure 3. RAPD-PCR band results of *Gammarus* specieses amplified with primer B7. M- DNA Ladder (100 -3000 bp), *G. pulex* (Line 1-3), *G. uludagi* (Line 4-6), *G. pulex* (Line 7-9), *G. arduus* (Line 10-12).

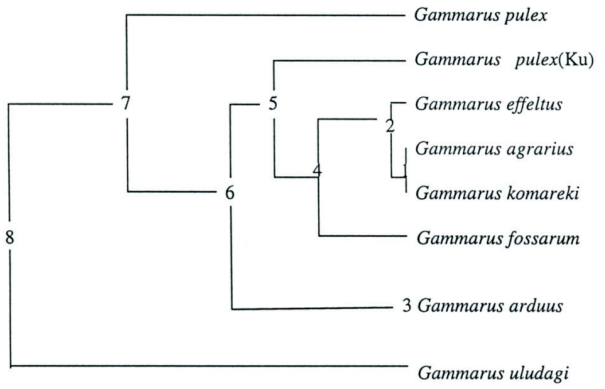

Figure 4. Dendrogram from cluster analysis of *Gammarus pulex*, *Gammarus uludagi, Gammarus komareki*, *Gammarus arduus*, *Gammarus fossarum, Gammarus agrarius, Gammarus effeltus* based on RAPD-PCR results.

tant relative to the other Gammarus sp. Interestingly, the phylogenetic and statistical analyses of the data obtained from RAPD-PCR marker systems demonstrated that *G. pulex, G.pulex (Ku), G. agrarius, G. arduus, G. effeltus, G. fossarum,* and *G. komareki* are monophyletic leaving *G. uludagi* as a separate clade.

The pairwise Jaccard's [26] coefficients for the 8 *Gammarus* accessions are also presented in this study (Table 2). These analyses clearly distinguished all the 8 accessions from each other. The accessions *G.komareki* and *G.uludagi* were grouped together as they were separated from the remaining accessions with only 14.2 percent similarity. The accessions *G. arduus* and *G. effeltus* were grouped together with a maximum similarity of 95.24 per cent, followed by *G. fossarum* and *G. komareki* which showed a similarity of 90.48 percent (Table 2).

The results of the present study clearly showed that *Gammarus* species within Turkey constitutes a broad genetic base. From the clustering pattern and genetic relationship obtained using RAPD markers, breeders can identify the diverse genotypes from different clusters and employ them in their future breeding programs. Conclusively, as in the case of easy, cost, technical labor, speed, and amount of necessary DNA, the RAPD marker system can be preferentially used in taxonomic and classification studies.

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Table 2. Similarity matrix for Jaccard's coefficients for 8 *Gammarus* accessions based on the RAPD bands obtained with primers OPB08, B7 and M13.

	Gammarus pulex	Gammarus uludagi	Gammarus pulex(Ku)	Gammarus effeltus	Gammarus agrarius	Gammarus arduus	Gammarus komareki	Gammarus fossarum
G.p	****	0.4762	0.6190	0.5714	0.6190	0.5238	0.3810	0.4762
G.u	0.7419	****	0.4762	0.5238	0.6667	0.4762	0.1429	0.2381
G.p (Ku)	0.4796	0.7419	****	0.7619	0.7143	0.7143	0.5714	0.6667
G.e	0.5596	0.6466	0.2719	****	0.8571	0.9524	0.6190	0.7143
G.ag	0.4796	0.4055	0.3365	0.1542	****	0.8095	0.4762	0.5714
G.ar	0.6466	0.7419	0.3365	0.0488	0.213	****	0.6667	0.7619
G.k	0.9651	1.9459	0.5596	0.4796	0.7419	0.4055	****	0.9048
G.f	0.7419	1.4351	0.4055	0.3365	0.5596	0.2719	0.1001	****

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