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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

A preliminary study on micronucleus analysis and nuclear anomalies in *Pelophylax ridibundus* (Pallas, 1771) (Amphibia: Anura) specimens collected around Vize (Kirklareli) and Ida Mountains (Çanakkale, Turkey)

Vize ve Kaz Dağı çevresinden toplanan *Pelophylax ridibundus* (Pallas, 1771) (Amphibia: Anura) **örneklerinde mikronuklues analizi ve nüklear anomaliler hakkında bir ön çalışma**

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Özet: Bu çalışmada Vize (Kırklareli) ve Kazdağı (Yenice, Çanakkale) civarında toplanan *Pelophylax ridibundus* örneklerine ait eritrositlerde mikronukleus analizi yapıldı ve nuklear anomaliler tespit edildi. Bu amaçla Nisan 2011'de Vize'den 9 (5♂♂, 4♀♀), Mayıs 2011'de Kazdağı'ndan 5 (3♂♂, 2♀♀) adet *P. ridibundus* örneği toplandı. Vize'den toplanan örneklerde ortalama mikronukleus sayısı 6±4,17, frekans % 0,3; Kazdağı'ndan toplanan örneklerde ise 0,6±0,43, frekans % 0,03 olarak hesaplandı. İstatistiksel analizler neticesinde iki lokalite arasında toplam mikronukleus sayısı bakımından önemli fark bulunduğu belirlendi (p≤0,05). Çalışmada binuklear, çentikli, tomurcuklu ve küçük loplu olmak üzere 4 tip nuklear anomali saptandı. Vize ve Kazdağı örnekleri arasında toplam nuklear anomali sayısı bakımından fark tespit edilmedi (p=0,31). Araştırmamız sonunda elde edilen bulgulara göre, Vize örneklerinde mikronukleus frekansının daha yüksek bulunması, bölgedeki yoğun tarımsal faaliyetlerde kullanılan genotoksik pestisitlere maruz kalınmayla ilişkilendirilebilir.

Anahtar kelimeler: *Pelophylax ridibundus*, Mikronukleus, Nuklear anomaliler, Vize, Kaz Dağları

Abstract: In this study, a micronucleus analysis was made on the erythrocytes of the *Pelophylax ridibundus* specimens collected around Vize (Kırklareli) and Ida Mountains (Yenice, Çanakkale), nuclear anomalies were detected. For this purpose, 9 (5♂♂, 4♀♀) *P. ridibundus* specimens were collected from Vize in April 2011 and 5 (3♂♂, 2♀♀) *P. ridibundus* specimens were collected from Ida Mountains in May 2011. The mean number of micronuclei was calculated as 6±4.17 and frequency as 0.3% in the specimens collected from Vize, while the mean number of micronuclei was calculated as 0.6±0.43 and frequency as 0.03% in the specimens collected from Ida Mountains. As a result of the statistical analyses, it was determined that there was a significant difference in the total number of micronuclei between both localities (p≤0.05). Some 4 types of nuclear anomalies, i.e. binucleate, notched, blebbed and small-lobed, were detected in the study. No difference in the total number of nuclear anomalies was detected between the specimens of Vize and Ida Mountains (p=0.31). According investigation to the results, Vize samples have a higher frequency of micronuclei associated with intensive agricultural activities used genotoxic exposure to pesticides.

Keywords: *Pelophylax ridibundus*, Micronucleus, Nuclear anomalies, Vize, Ida Mountains

INTRODUCTION

One of the most important causes of reductions in the populations of amphibians distributed around the agricultural areas is pesticide contamination (Sparling *et al.*, 2001). Pesticides are effective on non-target organisms besides target organisms. These chemicals accumulate in different environmental parts (groundwater, surface water and sediment) in time and pose a threat for many species (Finizio *et al.*, 2001).

The micronucleus test (MN) was developed in 1975 by Schmid in order to reveal the effects of genotoxic chemicals on mammalian bone marrow cells. Since then, the MN test has been used to examine the effects of chemicals with a genotoxic effect on different organisms (Orhan *et al.*, 1993; Zhuleva *et al.*, 1996; Villarini *et al.*, 1998, Çavaş and Ergene, 2005; Souza *et al.*, 2006; Saleh and Sarhan, 2007; Çelikler *et al.*, 2008; Bolognesi and Hayashi, 2011). The fact that erythrocytes are nucleated in amphibian specimens increases the usability of the MN test. There are many MN tests and nuclear anomaly studies performed in vitro and in vivo so as to determine genetic damage in amphibians (Zoll-Moreux and Ferrier, 1999; Kryukov, 2000; Saleh and Zeytinoğlu, 2001; Campana *et al.*, 2003; Lajmanovich *et al.*, 2005; Marques *et al.*, 2009; Yin *et al.*, 2009; Ahmad and Saleh, 2010).

Amphibians are the most important natural enemies of agricultural pest insects in aquatic and agricultural ecosystems (Feng *et al.*, 2004). Amphibians are bioindicator living organisms as they are quite sensitive to the changes in environmental conditions, as they spend certain periods of their lives completely in water and as their skin is quite

sensitive (Schuytema and Nebeker, 1999). *Pelophylax ridibundus* specimens used in our study belong to an amphibian species distributed quite widely in Turkey. Since it is consumed as a food substance, this species has economic significance. *P. ridibundus* specimens are water-dependent throughout their lives. Thus, they are quite sensitive to any chemical change likely to occur in the aquatic ecosystem.

In the study, Vize (Kırklareli) and Ida Mountains (Çanakkale) localities were selected as the study areas. Of the localities concerned, Vize is located in Thrace, where considerable agricultural activities are carried out. Cultivation of sunflowers and cereals are the most intense in this area. Agricultural activities in the region according to the 2006 data year, 12246.5 kg of insecticide, fungicide 64295.75 kg and 238 655 kg of herbicide used (Anonymous, 2008). Moreover, it is quite important that this locality is located within the Meriç Delta, which is reported to have been contaminated as a result of agricultural and industrial activities (Erkmen and Kolankaya, 2005). The Ida Mountains locality, however, was selected as a reference zone that was considered uncontaminated as it was located away from agricultural areas and settlements.

The aim of this study is to determine micronuclei and nuclear anomalies in the *P. ridibundus* specimens collected from Vize, which was considered contaminated in terms of pesticide contamination, and from Ida Mountains, which was considered uncontaminated in terms of pesticide contamination. Our study is important in that it contributes to the development of strategies for the protection of this species which has an essential role in ecological sense and which is economic since it is consumed as a food substance.

MATERIAL AND METHODS

In this study, 9 (5♂♂, 4♀♀) *P. ridibundus* specimens from Vize and 5 (3♂♂, 2♀♀) *P. ridibundus* specimens from Ida Mountains (Yenice, Çanakkale) were used as the animal materials. These specimens were anesthetized with ether in order to take blood. Later on, the blood taken from the heart was transferred to heparinized capillary tubes. A drop of the blood samples was put onto the clean slides and smeared as a thin layer. Some 3 preparations were made for each *P. ridibundus* specimen. The smear preparations were dried at room temperature. These preparations were fixed and stained using the May-Grunwald-Giemsa method (Lewis *et al.*, 2006). To determine the frequencies of micronuclei and nuclear anomalies, some 2000 erythrocytes from each preparation were examined under 1000x magnification using a light microscope. Preparations containing significant findings, the camera adapted Olympus BX51 light microscope, photographed using DP2-BSW software. The nuclear anomalies were determined according to Carrasco *et al.* (1990).

The non-parametric Mann-Whitney U test was used to determine the difference in the numbers of micronuclei and nuclear anomalies between both localities. The significance value was taken as $p \leq 0.05$.

RESULTS AND DISCUSSION

The mean numbers of micronuclei and nuclear anomalies in the *P. ridibundus* specimens collected from Vize and Ida Mountains are presented in Figures 1 and 2.

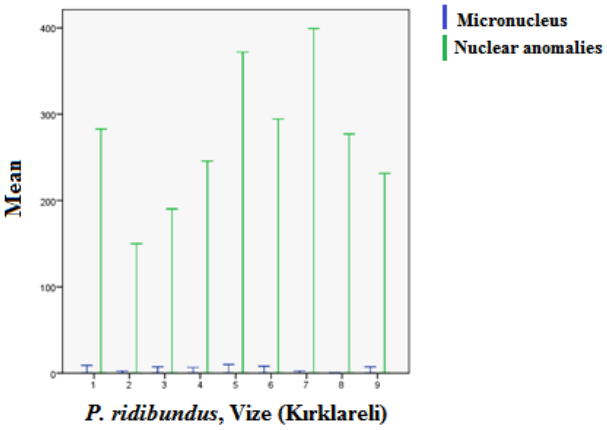

Figure 1. The mean numbers of micronuclei and nuclear anomalies in the *P. ridibundus* specimens collected from Vize (N:9)

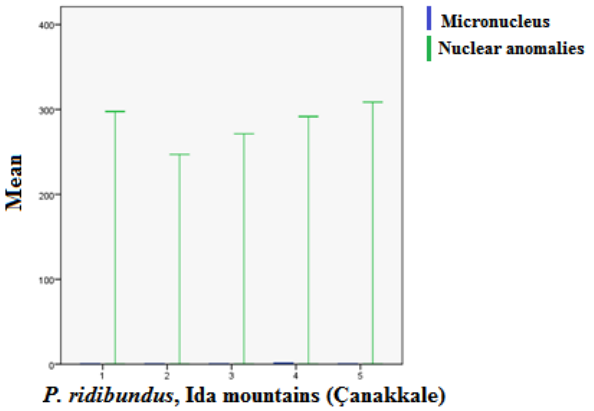

Figure 2. The mean numbers of micronuclei and nuclear anomalies in the *P. ridibundus* specimens collected from Ida Mountains (N:5)

The mean numbers and frequency of micronuclei (MN) and nuclear anomalies (NA) were found higher in the *P. ridibundus* specimens collected from Vize than in the specimens collected from Ida Mountains (Table 1).

Table 1. The mean numbers and frequency of micronuclei and nuclear anomalies in Vize and Ida Mountains

Sampling Site		Number of erythrocytes analysed	Mean±SD (MN)	% Frequency of MN	Mean±SD (NA)	% Frequency of NA
April 2011	Vize	54000	6±4.17	0.3	271.47±108.42	13.57
May 2011	Ida mountains	30000	0.6±0.43	0.03	283.27±33.64	14.16

The micronucleus, notched nucleus, blebbed nucleus, binucleate and lobed nucleus anomalies observed in *P. ridibundus* specimens and normal erythrocyte photographs are presented in Figure 3.

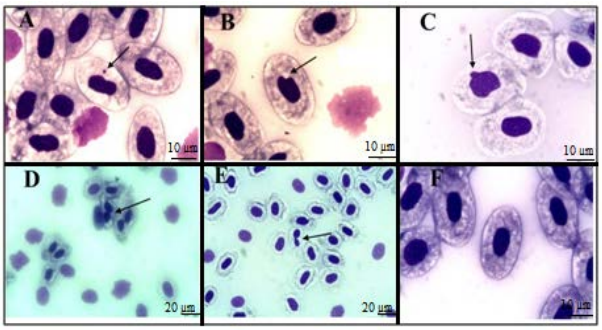

Figure 3. The micronucleus, nuclear anomalies and normal erythrocyte observed in the *P. ridibundus* specimens (A. Micronucleus, Vize sample; B. Notched nucleus, Vize sample; C. Blebbed nucleus, Ida mountains sample; D. Binucleate nucleus, Vize sample; E. Lobed nucleus, Vize sample; and F. Normal erythrocyte, Ida mountains sample). Stained by Giemsa.

The pool in the locality where the specimens of Vize were collected is located down the highly nearby sown field. In addition, considerable empty pesticide containers were seen approximately 10 meters around this locality.

It was observed that the fungicides and herbicides, in which 3 different active substances were used and which were released onto the market, were considerably used in Vize. The categories and active substances of the pesticides concerned are presented in Table 2.

Table 2. The pesticides observed to have been used considerably in Vize (Kırklareli) and their active ingredients

Pesticides	Active Ingredient
Fungucide	Prochloraz
Herbicide	Clodinafop-Propargyl
Herbicide	Chlorsulfuran

As a result of the Mann-Whitney U test, the difference in the total numbers of micronuclei between the specimens of Vize and Ida Mountains was found substantially significant ($p \leq 0.05$, p=0.00). As a result of the same test, however, the difference in the total numbers of nuclear anomalies between the localities concerned was not found significant (p=0.31).

This study revealed the difference between the micronucleus frequencies considered to be based on pesticide contamination in the *P. ridibundus* specimens distributed in Vize and Ida Mountains localities. Findings similar to those in our study were detected in the surveys of the MN test in which the *P. ridibundus* specimens collected from nature were used. According to Ahmad and Saleh (2010), the MN frequency increases in those regions where agricultural pollution is high. Likewise, in his study, Aymak (2010) reports that the MN frequency is higher in the region where the heavy metal pollution is particularly high out of two localities considered uncontaminated and contaminated. Furthermore, he also states in his study that the MN frequency varies by season throughout the year. The collecting of the amphibian specimens in April and May at proximate temperatures in our study prevents a possible seasonal deviation of the MN frequency. Environmental pollutants first show their effects on aquatic organisms. The pollutants concerned can show their effects on many highly organized vertebrate species including human beings by means of the food chain. The genotoxic changes in *P. ridibundus* specimens, which are of economic importance because they are consumed as food and which are exported abroad, should be taken into consideration in this context and investigated in detail by means of other studies. It is thought that similar genotoxic studies will contribute to the development of strategies for the protection of this species. In addition, the making of heavy metal and pesticide analyses in water, sediment and tissue samples in other studies to be performed will contribute to more clearly revealing the genotoxic effects concerned.

REFERENCES

Ahmad, K., Saleh, J. 2010. Clastogenic studies on Tandaha Dam water in Asser. *Journal of Black Sea/Mediterranean Environment*, 16 (1): 33-42.

Anonymous, 2008. *Meriç-Ergene Havzası Koruma Eylem Planı*. T.C. Çevre ve Orman Bakanlığı. 1-205.

Aymak, C. 2010. Determination of heavy metal pollution and its genotoxic effects by using micronucleus test in *Rana ridibunda* Pallas, 1771 (Ranidae, Amphibia) living in Mersin. Mersin University, Phd Thesis. (in Turkish)

Bolognesi, C., Hayashi, M. 2011. Micronucleus assay in aquatic animals. *Mutagenesis*, 26 (1): 205-213. doi: 10.1093/mutage/geq073

Campana, M.A., Panzeri, A.M., Moreno, V.J., Dulout, F.N. 2003. Micronuclei induction in *Rana catesbeiana* tadpoles by the pyrethroid insecticide lambda-cyhalothrin. *Genetics and Molecular Biology*, 26 (1): 99-103.

Carrasco, K.R., Tilbury, K.L., Myers, M.S. 1990. An assessment of piscine micronucleus test as an in-situ biological indicator of chemical contaminant effects. *Canadian Journal of Fisheries and Aquatic Sciences*, 47: 2723-2136. doi: 10.1139/f90-237

Çavaş, T., Ergene-Gözükara, S. 2005. Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. *Aquatic Toxicology*, 74: 264-271. doi:10.1016/j.aquatox.2005.06.00

Çelikler, S., Yıldız, G., Vatan, O., Bilaloğlu, R. 2008. In vitro antigenotoxicity of *Ulva rigida* C. Agardh (*Chlorophyceae*) extract against induction of chromosome aberration, sister chromatid exchange and micronuclei by mutagenic agent MMC. *Biomedical and Environmental Sciences,* 21: 492-498. doi: 10.1016/S0895-3988(09)60008-8

Erkmen, B., Kolankaya, D. 2005. Determination of organochlorine pesticide residues in water, sediment and fish samples from the Meriç Delta, Turkey, *International Journal of Environmental and Analytical Chemistry*, 86 (1-2): 161-169. doi: 10.1080/03067310500247926

Feng, S., Kong, Z., Wang, X., Zhao, L., Peng, P. 2004. Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell. Chemosphere*, 56 (5): 457-463.
doi: 10.1016/j.chemosphere.2004.02.010

Finizio, A., Calliera, M., Vighi, M. 2001. Rating systems for pesticide risk classification on different ecosystems. *Ecotoxicology and Environmental Safety*, 49: 262-274. doi: 10.1006/eesa.2001.2063

Kryukov, V.I. 2000. Effects of lead on the induction of micronuclei in anuran larvae. *Russian Journal of Ecology*, 31 (6): 442- 443.
doi: 10.1023/A:1026604212612

Lajmanovich, R.C., Cabagna, M., Peltzer, P.M., Stringhini, G.A., Attademo, A.M. 2005. Micronucleus induction in erythrocytes of *Hyla pulchella* tadpoles (Amphibia: Hylidae) exposed to insecticide endosulfan. *Mutation Research*, 587: 67-72. doi: 10.1016/j.mrgentox.2005.08.001

Lewis, S.M., Bain, B.J., Bates, I. 2006. *Dacie and Lewis practical hematology*. Tenth Edition Churchill Livingstone.

Marques, S.M., Antunes, S.C., Pissarra, H., Pereira, M.L., Goncalves, F., Pereira, R. 2009. Histopathological changes and erythrocytic nuclear abnormalities in Iberian green frogs (*Rana perezi* Seoane) from uranium mine pond. *Aquatic Toxicology*, 91: 187-195.
doi: 10.1016/j.aquatox.2008.04.010

Orhan, G., Ekmekci, A., Menevse, S. 1993. The effect of the male contraceptive agent gossypol acetic acid on mouse bone marrow cells in vivo: micronuclei and mitotic index. *Contraception*, 47: 377-385.
doi: 10.1016/0010-7824(93)90035-6

Saleh, K., Zeytinoğlu, H. 2001. Micronucleus test in peripheral erythrocytes of Rana ridibunda as an indicator of environmental pollution. *Anadolu University Journal of Science and Tech*nology, 2: 77- 82.

Saleh, K., Sarhan, M. 2007. Clastogenic analysis of chicken farms using micronucleus test in peripheral blood. *Journal of A pplied Sciences Research*, 3: 1646-1649.

Schmid, W. 1975. The micronucleus test. *Mutation Research*, 31: 9-15.

Schuytema, G.S., Nebeker, A.V. 1999. Effects of ammonium nitrate, sodium nitrate and urea on Red-Legged frogs.- Pacific treefrogs and African clawed frogs. *Bulletin of Environmental Contamination and Toxicology*, 63: 357-364. doi: 10.1007/s001289900988

Souza, T.S., Fontanelli, C.S. 2006. Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluents. *Mutation Research*, 605: 87-93.

Sparling, D.W., Fellers, G.M., McConnell, L.L. 2001. Pesticides and amphibian populations declines in California, USA. *Environmental Toxicology and Chemistry*, 20: 1591-1595.

Villarini, M., Moretti, M., Pasquini, R., Scassellati-Sforzolini, G., Fatigoni, C. Marcarelli, M., Monarca, S., Rodriguez, A.V. 1998. In vitro genotoxic effects of the insecticide deltamethrin in human peripheral blood leukocytes: DNA damage ('comet' assay) in relation to the induction of sister-chromatid exchanges and micronuclei. *Toxicology*, 130: 129-139.
doi: 10.1016/S0300-483X(98)00097-3

Yin, X.H., Zhu, G.N., Li, X.B, Liu, S.Y. 2009. Genotoxicity evaluation of chlorpyrifos to amphibian Chinese toad (Amphibian: Anura) by Comet assay and Micronucleus test. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 680 (1-2): 2-6.
doi: 10.1016/j.mrgentox.2009.05.018

Zhuleva, L., Umnova, N.V., Rumak, V.S. 1996. Detection of micronuclei in desquamating cells of the human oral mucosa in the territory of South Vietnam. *Genetika*, 32: 1700-1704.

Zoll-Moreux, C., Ferrier, V. 1999. The Jaylet test (Newt Micronucleus test) and the Micronucleus test in Xenopus: two in vivo tests on amphibia evaluation of the genotoxicity of five environmental pollutants and of five effluents. *Water Research*, 33 (10): 2301-2314.