# Assessment of air pollution genotoxicity by RAPD in *Evernia prunastri* L. Ach. from around iron-steel factory in Karabük, Turkey

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**Abstract**

Lichens are widely used in biomonitoring studies of air pollution, either as bioindicators of air quality or as bioaccumulators of atmospheric deposition. Over the past decade, several molecular techniques have been developed to provide information on diversity, genotoxicology, genetic relationships, etc. The heavy metal contents of *Evernia prunastri* samples were determined by atomic absorption spectrometry. The Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) method was used to describe the pattern of DNA band variation in the samples influenced by the environmental pollution. The study was designed to describe the level of pollution in an area contaminated with smoke and waste from an iron-steel factory, and to reveal the level of potential genotoxic agents around this source of pollution. The study also examined the suitability of the lichen samples for the detection of genotoxicity.

## Introduction

Lichens and mosses are widely used for the biomonitoring of air quality, either as bioindicators of air quality or as bioaccumulators of atmospheric deposition (Conti and Cecchetti, 2001; Wolterbeek, 2002; Sczepaniak and Biziuk, 2003). These organisms are widely used because of their low cost and easily sampling, and because they allow wide areas to be monitored. Lichens are effective bioaccumulators of trace elements, and are bound to negatively charged anionic sites on the cell wall and outer surface of the plasma membrane, accumulated at intracellular sites and trapped as particles on the surface (Bargagli, 1998). Due to their resistance to environmental stress and effective accumulation capacity, toxitolerant species have been widely used for more than 30 years to assess the atmospheric deposition of trace elements and radionuclides in polluted areas (Garty and Ammann, 1987; Bennett et al., 1996; Bargagli, 1998; Nimis and Bargagli, 1999; Carreras and Pignata, 2002; Di Lella et al., 2003; Cansaran-Duman et al., 2009).

Environmental pollution is a major concern for public health, and heavy metal accumulation is considered the major component of environmental pollution. Heavy metal contamination of air and water bodies is one of the most important environmental problems in the world, and produces harmful consequences for agriculture and human health as well. Heavy metals can easily mobilise, disperse and to some extent produce toxic effects, which in turn can lead to growth inhibition and cause decline in crop yield. In cellular level, excessive amounts of toxic heavy metal ions induce several stress responses and damage to different cell components such as membranes, proteins and DNA (Jimi et al., 2004; Waisberg et al., 2003). Some genotoxic agents cannot only injure the integrity of the genome, but also directly or indirectly affect the expression of DNA. Thus, more attention should be given to investigate the genotoxic effects of the environmental pollutants.

Several studies have used the comet assay, micronucleus assay or chromosome aberration assay to measure the genotoxic effect of metals on plants (Steinkellner et al., 1999; Angelis et al., 2000). The advantage of measuring the effects of genotoxic chemicals directly on DNA is related to sensitivity and short response time. Recent advances in molecular biology such as Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) have led to the development of a number of selective and sensitive assays

for detecting DNA damage genotoxicologically (Conte et al., 1998; Savva, 1998; Atienzar et al., 2002a, 2002b; Aras et al., 2010). As mentioned in publications (Atienzar et al., 2002a; Savva, 1998), damage to the genomic DNA will then result in changes of the enzyme and binding sites and PCR products. Furthermore, it will alter the electrophoresis pattern and may potentially form the basis of novel biomarker assays for the detection of DNA damage and mutations in many different organisms (Savva, 1996; Atienzar et al., 2000). Detection of genotoxic effects using these techniques involves the comparison of profiles generated from control and exposed DNA. In a few studies conducted up to now, it has been indicated that higher plants might be more sensitive and efficient genotoxicity indicators. Although lichens are considered to be one of the best bioindicators for the determination of air pollution and numerous studies have been conducted on their heavy metal accumulation capacity, only one study has been carried out on their putative capacity as a genotoxicity indicator (Aras et al., 2010).

The objectives of this study were: (1) to describe the heavy metal content of *Evernia prunastri* (L.) Ach. collected in the iron-steel factory area in Karabük, Turkey; (2) to evaluate the application of RAPD as a molecular biomarker to detect DNA damage in the thallus caused by environmental pollutants and to investigate their capacity as indicators in polluted areas. The region surveyed in this study suffers from substantial historical and current air contamination, principally due to the presence of the steel and iron industry, which has been active since 1925. In addition to these industrial or purely urban emissions, other potential sources of atmospheric emissions in the area are a waste incinerator and road transportation. The preliminary investigation has involved the collection of 10 *E. prunastri* samples growing on *Pinus* sp. from 10 sites in and around the Karabük iron-steel factory area, Karabük, Turkey. The heavy metal contents of the collected samples were determined by atomic absorption spectroscopy (AAS). Following this part of the research, the same samples were used for the genotoxicity testing and tested by RAPD.

# 1 Materials and methods

## 1.1 Study area

The study area is located between $40°59'03''$–

$41°00'00''$N and $32°05'55''$–$32°18'15''$E in the western part of the Black Sea region, and belongs to Yenice District in the Province of Karabük. From Yenice Forest to the Karabük iron-steel factory, 10 samples of *E. prunastri* were collected every 5 km and a control sample was collected from Yenice Forest (Yenice-Karabük, Table 1). The control sample collected from Yenice Forest was not exposed to any kind of contamination. Sites were identified in each territory and were numbered from 1 to 10. The 10 sampling sites were chosen to identify the genotoxic effect of local atmospheric deposition. Numerous industrial activities such as those of the coal, iron and steel industry, cement industry, and an active intercity highway are present in the area. In addition, a railroad transporting coal and crude material has existed in Karabük for many years. The activity regions indicated are very close to the city centre of Karabük, and coal is generally consumed instead of natural gas during the winter periods. According to the local environmental unit parameters, $SO_2$ and $PM_{10}$ contamination increases to harmful levels in the winter. In addition, there is a rich and large forest ecosystem in terms of species which must be protected according to World Wildlife Fund (WWF) in the city.

## 1.2 Lichen material

*E. prunastri* is a common species that is widespread in the region. For this investigation, *E. prunastri* was chosen as a suitable bioindicator since its sensitivity to organic and inorganic compounds is well documented. The samples were collected from 10 different *Pinus* species located at various distances from the pollution sources (Table 1). The samples, taken from a few trees at each station, were homogenised before analysis. Control samples were collected from the forest mentioned above, where no pollution source was presented, and an allocation unit within the approximately 30 km$^2$ square area. In the laboratory, lichen samples were cleaned of contaminants using a binocular microscope (Olympus, Germany), and consecutive washings were applied with distilled water before DNA isolation.

## 1.3 Determination of element content

Element contents were determined using the method described by Cansaran-Duman et al. (2009). The chemical analyses of lichen samples were conducted after extraction

**Table 1**    Localities of the lichen samples used in the study*

| Locality No | GPS co-ordinates | Locality name | Altitude (m) |
|---|---|---|---|
| 1 | 44°62′N, 45°73′E | Karabük, Yenice, Kuzdağ District | 1125 |
| 2 | 41°15′N, 32°35′E | Karabük, Yenice, Kabaklı kaya | 1140 |
| 3 | 41°13′N, 32°28′E | Karabük, Yenice, vicinity of Hamzakıran District | 1140 |
| 4 | 41°14′N, 32°35′E | Karabük, Yenice, Dikilitaş | 1125 |
| 5 | 41°12′N, 32°25′E | Karabük, Yenice, vicinity of Kuzdere, Hamdioğlu District | 1400 |
| 6 | 41°15′N, 32°34′E | Karabük, Yenice, North of Yalnızca Plateau | 1200 |
| 7 | 41°11′N, 32°27′E | Karabük, Yenice, Acısu Center | 1375 |
| 8 | 41°14′N, 32°33′E | Karabük, Yenice, Kazancıoğlu District | 1750 |
| 9 | 41°12′N, 32°29′E | Karabük, Yenice, Hacı̈merler District | 1380 |
| 10 | 41°12′N, 32°29′E | Karabük, Yenice, Kızılgöz kayası | 1385 |
| 11** | 41°10′N, 32°24′E | Karabük, Yenice, vicinity of Cami District | 1100 |

* Date of collection on 15 November 2005. ** Control sample.

with a mixture of 2.0 mL of 63% $HNO_3$ and 1.0 mL $H_2O_2$ added into the 50 mg lichen sample and melted in Teflon-coated pots in a milestone-mark microwave oven. Deionised water (5.0 mL) was added to the melted solution and distilled through blue band paper. The final volume was adjusted to 10.0 mL with deionised water.

Calibration curves of manganese (Mn), zinc (Zn), iron (Fe), lead (Pb) and copper (Cu) were obtained with samples of various concentrations (0.25, 0.50, 1.00, 2.00, 4.00 mg/L) using linear regression analyses. Heavy metal concentration in these materials was determined using flame atomic absorption spectroscopy (FAAS; Instrument PM Avarta model atomic absorption spectrometry, GBC Scientific Equipment, Australia). The accuracy of the process was checked with a standard extension. The calibration curves of cadmium (Cd) and chromium (Cr) metals were obtained with samples of various concentrations (10, 25, 40, 60, 80 mg/L) using linear regression analyses. Cd and Cr concentrations in *E. prunastri* samples were determined using electrothermal atomic absorption spectroscopy (ETAAS; GBC Scientific Equipment, Australia). The accuracy of the process was checked by a standard extension.

### 1.4  DNA extraction and RAPD analysis

DNA extraction from the lichen samples was performed according to the protocol developed for various lichen species by Aras and Cansaran (2006). Concentrations of the extracted DNAs were measured at 260 nm and the purity was estimated by measuring the 260 nm/280 nm absorbance ratio by nanodrop (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, USA). The DNA concentrations were approximately in the range of 1623 to 2440 ng/μL and 260 nm/280 nm ratios were between 1.59 and 1.61. The integrity of the extracted DNAs were also evaluated by electrophoresis.

Primer screening for RAPD analysis was performed using 21 primers. Thirteen out of 21 primers amplified clear and reproducible bands. PCR was performed in a reaction volume of 25 μL containing 200 ng genomic DNA, 2.5 μL 10× reaction buffer, 2.5 mmol/L $MgCl_2$, 20 μmol/L dNTPs, 0.2 μmol/L of primer, and 0.5 unit of Taq polymerase (Promega, Madison, USA), and $ddH_2O$ was added to the standard volume. The amplification reaction was carried out in a Biometra Thermal Cycle (Labrepco, Horsham, PA). The PCR programme consisted of the following steps: initial denaturation at 94°C for 30 sec, annealing at 33°C for 1 min at 35 cycles, extension at 72°C for 1.45 min and a final extension at 72°C for 8 min. Primer screening for RAPD analysis was performed using 21 primers. Amplified samples and 100 bp DNA markers were loaded on 1.6% agarose gels and run at 5 V/cm for 4 hr. Samples were visualised and analysed under UV light using the Gene Genius Bioimaging System, Syngene. The sequences of the 13 decamer primers used in the study are shown in Table 2.

### 1.5  Data analysis

The results of the chemical analyses were evaluated by

**Table 2**   Sequence of the primers used in the study

| Primer | Sequence of primer (5′→3′) | Primer | Sequence of primer (5′→3′) |
|---|---|---|---|
| B389 | CGCCCGCAGT | TubeA02 | TGCCGAGCTG |
| BC374 | GGTCAACCCT | TubeB01 | GTTTCGCTCC |
| P437 | CGGATCGACA | TubeA04 | AATCGGGCTG |
| OPO07 | CAGCACTGAC | TubeA05 | AGGGGTCTTG |
| P232 | CCGCTTGTTG | TubeC01 | TTCGAGCCAG |
| OPO04 | AAGTCCGCTC | OPO19 | GGTGCACGTT |
| TubeA01 | CAGGCCCTTC | | |

one-way analysis of variance (ANOVA) with SPSS 11.5. The ratio between the concentration of each element before and after exposure (exposed to control ratio, EC ratio) was used to evaluate bioaccumulation rates (Farati et al., 2005).

The results of RAPD analysis were determined by considering the bands which appeared in the control sample as the criterion of the judgment. Polymorphism observed in RAPD profiles included disappearance of a control band and appearance of a new band.

The results of RAPD analysis was performed by considering the bands which appear in the control sample are the criterion of the judgment. Polymorphism observed in RAPD profiles included disappearance of a control band and appearance of a new band (Liu et al., 2005; Atienzar et al., 1999).

### 1.6  Estimation of genomic template stability

Genomic template stability (GTS, %) was calculated as:

$$GTS = \left(1 - \frac{a}{n}\right) \times 100\%$$

where, *a* indicates the RAPD polymorphic profiles in each sample exposed to environmental pollution around the city of Karabuk, and *n* is the number of total bands in the control. Changes in the RAPD patterns were expressed as decreases in GTS, a qualitative measure showing the obvious change to the number of RAPD profiles generated by the lichen samples exposed to the polluted areas, in relation to profiles obtained from the control samples.

## 2 Results

### 2.1 Element content of lichen

Heavy metal concentrations of *E. prunastri* of the samples taken from sites and control group are summarised in Fig. 1. All of the stations were statistically analysed to determine their relationships with respect to each heavy metal. SPSS 11.5 analysis was used to show the relationships of the stations and some results were shown with dendrograms (Fig. 1).

Around the Karabük iron-steel factory, the highest levels of Zn in the *E. prunastri* were found at site 1 (46.6 μg/g) and site 2 (43.3 μg/g) (Fig. 1). Other sites yielded results close to each other in the *E. prunastri* specimens. Zn concentration in the lichen samples was linearly related to the vehicle traffic, railway and activity of industrial units. Gailey and Lloyd (1986) measured the heavy metal content in *Lecanora conizaeoides* collected in Armadale (central
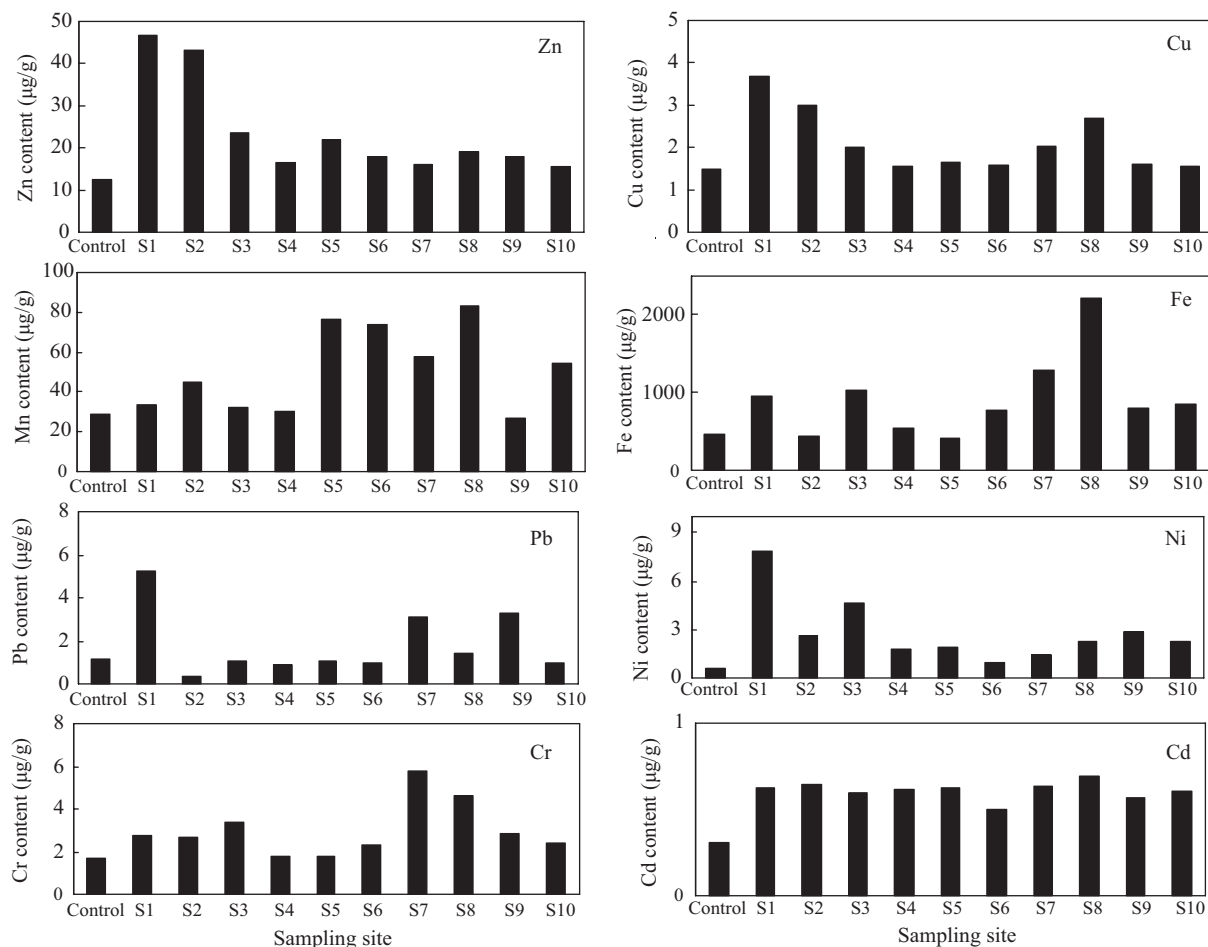
**Fig. 1** Graphical representation of elemental content in *E. prunastri*.

Scotland) and detected Zn in the range of 5.0–64.1 μg/g, depending on the distance and wind direction from a steel factory. They found that only Fe and Zn were detected in the lichen collected in the peripheral sites of the town. The researchers concluded that the steel foundry is the main source of metal pollution in the central Scotland.

Mn is commonly used in steel production, as well as in alloys and batteries (Markert, 1992). The highest levels of Mn in the *E. prunastri* were found in site 8 (82.7 μg/g), site 5 (77.0 μg/g) and site 6 (73.7 μg/g). In order to compare the ability of the lichen species to accumulate heavy metals, they were compared with the element concentrations in the baseline material. For example, the highest levels of Mn in *E. prunastri* were found at site 8 (82.7 μg/g) (control is 28.8 μg/g) (Fig. 1).

Comparisons of the Pb concentrations of *E. prunastri* specimens from polluted sites with the control yielded very significant variations (Fig. 1). Sites 1, 7 and especially site 9, with the highest human activities, together with high vehicular density congestion, showed the highest Pb concentration of 5.17 μg/g in site 1 which was significantly higher than the control site (1.31 μg/g) (Fig. 1). It could be concluded that Pb concentration was the highest in site 1 because this is the central part of the city, where human activities and the density of traffic are very intense. Similar phenomenon was observed by Cansaran-Duman et al. (2009) while studying *Pseudevernia furfuracea* thalli as

an indicator of air pollution in the same province in Turkey.

Although the highest levels of Cr in *E. prunastri* were found at site 7 (5.75 μg/g), and site 8 (4.65 μg/g) (Fig. 1), other sites displayed slightly higher results than the control. The most important sources of Cr pollution could be indicated as industrial activities like refining works and iron-steel factories.

The Cu contents in *E. prunastri* samples ranged from 1.54 to 3.67 μg/g. Cu content in *E. prunastri* at site 1 (3.67 μg/g) was significantly higher than that in the control site (1.46 μg/g) (Fig. 1).

All sites, especially site 8 (0.69 μg/g), showed significantly higher Cd concentrations than the sample from the control site (0.30 μg/g) in *E. prunastri* (Fig. 1). The most important sources of Cd pollution were regarded as fossil fuels used by vehicles, the metal industry, plastics, house tool construction and sewers (Markert, 1992). Markert (1992) reported that the Cd levels are in the range of 0.01 and 0.30 μg/g for unpolluted natural environments and found that all of the sites studied were polluted except rural sites, which is consistent with our results.

EC ratios were calculated according to Frati et al. (2005). There are two main perspectives: (1) calculating accumulation factors such as EC ratios (relative changes) and more simply, (2) calculating differences between exposed and control samples (absolute changes). The control samples showed some variations in the concentrations of
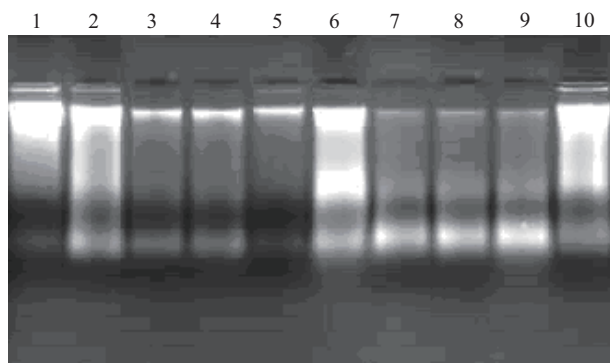
**Fig. 2**    Genomic DNA of 200 ng extracted by CTAB in *E. prunastri*.

many elements (Table 3). For this reason, to allow comparison of the accumulation capacity of collected samples, EC ratios were calculated (Table 3). The use of EC ratios allowed us to correct this problem and to normalise the use of different species. Sites 1 and 8 displayed the highest metal concentrations in all metals. It is not surprising that all these two sites are found in close proximity to major traffic islands, industrial centres or centres of major mining activities.

### 2.2 DNA extraction

The concentrations of DNA samples were in the range of 1623–2440 ng/μL and 260 nm/280 nm ratios were between 1.59 and 1.61. The integrity of the extracted DNAs was also evaluated by electrophoresis. The results in Fig. 2 indicated that the CTAB method, previously improved in our laboratory for lichens, was also suitable for pollution studies and yielded good quality DNA for PCR reactions.

### 2.3 RAPD-PCR profiles of the control and polluted samples

Out of 21 decamer oligonucleotide primers tested, only 13 of them gave clear and reproducible bands. In RAPD analyses, some of the primers displayed significant differences between the control and polluted samples collected from various parts of Karabük. As Fig. 3 illustrates, the changes in band numbers in the form of appearance and disappearance were found to be obvious (Fig. 3).

The highest number of band appearance and disappearance was determined at the samples collected from the locations close to the iron-steel factory (sites 8, 9, 10) with all of the 13 primers used. The sizes of the appearing and disappearing bands were between 200 and 1500 bp. On the other hand, the other areas also displayed an

increase in band appearance and disappearance compared to the original control samples. The number of new band appearances and disappearances for each primer and for each sample DNA obtained from the different sites of the iron-steel factory are shown in Table 4.

To test the reproducibility of the RAPD-PCR, experiments were repeated at least twice for each primer and faint bands were ignored; only reproducible bands obtained in repeated experiments were taken into account. The RAPD profiles generated by the primer OPO04 are shown in Fig. 3. Among the primers used, OPO07 yielded a monomorphic band pattern. As RAPD primers scan almost the whole genome, it can be suggested that P232 and TubeA04 find the DNA regions in which alterations have occurred. Table 4 indicates the number of the changed bands observed in RAPD profiles as the disappearance and appearance of bands. Additionally, the results of genomic template stability ratios (GTS) were calculated. GTS implies qualitative measure reflecting changes in RAPD profiles (Table 5). The lowest values were obtained in the samples 2 and 6. Generally in the samples 8, 9 and 10, higher GTS values were obtained, which might imply the sensitivity of lichens to genotoxic stressors near the iron-steel factory.

To compare the sensitivity of the thallus of *E. prunastri* and RAPD profiles, changes in each factor were calculated as a percentage of their control value and changes in RAPD profiles were expressed as reductions in genomic template stability.
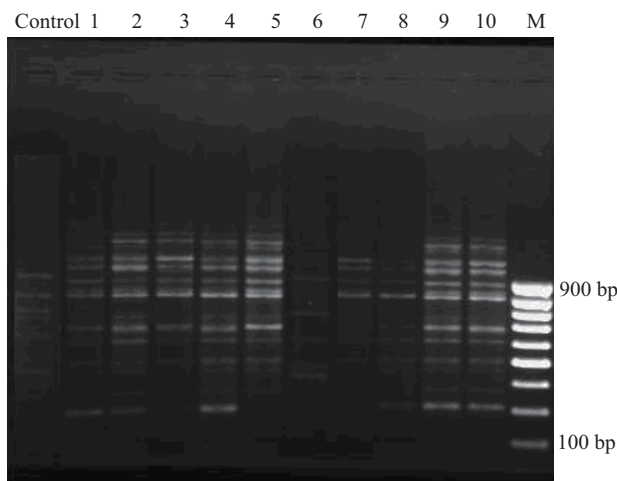


**Fig. 3**    RAPD profiles generated by OPO04 (5′-AAGTCCGCTC-3′). Primer from *E. prunastri* exposed to polluted areas around the Iron-stell Factory in Karabuk. Lane M: molecular weight marker (100 bp ladder).

**Table 3**    EC ratios for elements assayed in exposed *E. prunastri* species

|    | Site 1 | Site 2 | Site 3 | Site 4 | Site 5 | Site 6 | Site 7 | Site 8 | Site 9 | Site 10 | Mean EC |
|----|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|---------|
| Zn | 3.723  | 3.457  | 1.925  | 1.318  | 1.746  | 1.426  | 1.293  | 1.519  | 1.424  | 1.238   | 1.910   |
| Cu | 2.517  | 2.039  | 1.360  | 1.054  | 1.100  | 1.062  | 1.380  | 1.834  | 1.093  | 1.070   | 1.450   |
| Mn | 1.265  | 1.568  | 1.157  | 1.055  | 2.667  | 2.483  | 2.024  | 2.898  | 0.952  | 1.967   | 1.800   |
| Fe | 2.051  | 0.962  | 2.216  | 1.181  | 0.898  | 1.681  | 2.807  | 4.799  | 1.707  | 1.872   | 2.020   |
| Pb | 5.568  | 0.329  | 1.055  | 1.072  | 1.040  | 1.000  | 3.354  | 1.425  | 2.974  | 0.887   | 1.880   |
| Ni | 13.034 | 4.416  | 7.630  | 2.976  | 3.104  | 1.655  | 2.508  | 3.785  | 3.997  | 3.454   | 4.660   |
| Cr | 1.633  | 1.646  | 2.007  | 1.069  | 1.090  | 1.395  | 3.433  | 2.732  | 1.727  | 1.432   | 1.820   |
| Cd | 2.118  | 2.136  | 1.961  | 2.267  | 2.077  | 1.675  | 2.098  | 2.323  | 1.871  | 2.027   | 2.060   |

EC: exposed control ratios.

**Table 4**   Changes of total bands in control, polymorphic bands and varied bands in samples

| Primer | C | S1 | | S2 | | S3 | | S4 | | S5 | | S6 | | S7 | | S8 | | S9 | | S10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b | a | b |
| B389 | 10 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| BC324 | 8 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 2 | 0 | 1 | 0 | 2 | 0 | 0 | 2 |
| P437 | 9 | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 |
| OPO07 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| P232 | 11 | 0 | 1 | 0 | 0 | 2 | 3 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 0 | 1 | 1 | 1 | 0 | 3 | 2 |
| OPO04 | 10 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 |
| TubeA01 | 9 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 |
| TubeA02 | 10 | 0 | 0 | 0 | 1 | 1 | 0 | 2 | 0 | 1 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |
| TubeB01 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 1 | 0 | 1 | 1 | 0 |
| TubeA04 | 11 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 2 | 1 | 0 | 0 | 3 | 0 | 1 |
| TubeA05 | 8 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| TubeC01 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 0 | 1 | 2 | 0 |
| OPO19 | 9 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| a+b | | 8 | | 9 | | 8 | | 8 | | 8 | | 9 | | 12 | | 13 | | 13 | | 16 | |

S: sample, C: control sample, a: appearance of new bands, b: dissapearance of control bands, a+b: polymorphic bands.

**Table 5**   Changes of GTS for all primer in *E. divaricata*

| Number of the sites | GTS ratio (%) | Number of the sites | GTS ratio (%) |
|---|---|---|---|
| 1 | 93.10 | 6 | 92.24 |
| 2 | 92.24 | 7 | 89.65 |
| 3 | 93.10 | 8 | 88.79 |
| 4 | 93.10 | 9 | 88.79 |
| 5 | 93.10 | 10 | 86.20 |

## 3 Discussion

Many studies have been conducted showing that the concentration of absorbed heavy metal element increases as the lichen sample gets closer to polluted sites like busy motorways and iron-steel factories, and depending on the exposure time. Some studies showed that Pb, Cu, Cd, Mn, Cr and Ni concentrations of lichen samples which are close to iron-steel factories are higher than those far from industrial sites (Pilegaard, 1978, 1979; Goyal and Seaward, 1981; Gailey and Lloyd, 1986; Gailey et al., 1985; Vestergaard et al., 1986).

The investigated area of the iron-steel factory in Karabük is one of the oldest Turkish industrial sites for steel production. Nevertheless, its impact on the genotoxic effect of air contamination has never been studied in detail. This was a perfect location to support recent results concerning the tracing of industrial activities using the RAPD technique.

Despite their high tolerance for heavy metals, accumulated and absorbed metals are not totally harmless to lichens. Many studies have been carried out related to the effects of heavy metals on lichens, such as chlorophyll degradation, a decrease in photosynthesis, an increase in the stress hormone ethylene, a decrease in ATP content and negative effects on the integrity of the membranes. In addition to the physiological effects, heavy metals can cause DNA mutations, as they are genotoxic agents as well. PCR-based fingerprinting methods provide an efficient tool for the investigation of mutational changes. RAPD analysis helps to find out not only the mutational effects of heavy metals, but also the mutational effects of organic and inorganic genotoxic agents on different organisms.

PCR-based molecular markers such as RAPD and AFLP are more sensitive, effective, relatively cheap and simple techniques, as they give evidence about DNA mutation in relation to many different organisms. They are especially useful for pollution studies, as they can compare polluted and non-polluted samples at the same time and in relatively short periods (Qi et al., 2006).

In the current study, among 10 stations, site 1 was closest to the pollution sources; it is located near the motorway and railway. On the other hand, site 10 was closer to the iron-steel and cement industries than the other stations. Apart from site 10, there were other stations close to potential pollution sources, and it is estimated that these areas also have pollution risk. Sites 2, 4 and 5 were also close to the motorway. Sites 8 and 9 were the closest stations to the iron-steel factories, with the exception of station 10. Station 3 was very close to station 7, and both of them were located far away from the allocation units. Lichen samples both close to and far from the pollution sources were compared to provide genotoxicity information related to possible heavy metal pollution. The high number of polymorphic bands observed in the samples taken from areas close to the railways and motorways (sites 1, 7, 8) and the samples taken from sites close to the iron-steel factory (sites 9, 10) implies that significant heavy metal pollution is in effect in these areas (Fig. 1).

The basic aim of biomonitoring is to supply data for an effective ecological control system. In particular, biomonitoring should act as an early warning system by providing information about the sensitivity of the population when the problem is still at sub-lethal levels. Although the measurement of bioindicator responses at lower biological organisation levels sometimes seems to be more sensitive to stress than it is at higher levels, the best way to determine heavy metal genotoxicity should be the direct quantification of the genotoxic effect (i.e., DNA damage). So far, a lot of work has been done to estimate DNA damage with the aid of cytogenetic tests or the comet assay (Steinkellner et al., 1999; Camatini et al., 1998; Miniss and Lombi, 1997). Although these investigations have produced good results, the sensitivity of these methods for genotoxicity assessment should be improved. Recently,

advances in molecular biology have led to the development of a number of selective and sensitive assays for detecting DNA damage in the field of genotoxicology (Conte et al., 1998; Savva, 1998; Labra et al., 2003, 2004; Aras et al., 2010). In our work, we used RAPD fingerprints as the bioindicator to maximise the evaluation of the DNA damage induced by heavy metals in lichens.

In this study, RAPD analysis showed that there were detectable DNA band variations when the lichens were exposed to environmental pollution. The clear correlation between heavy metal accumulation and percentage of DNA polymorphism supports the effectiveness of RAPD for investigating environmental toxicity (Conte et al., 1998; Labra et al., 2003, 2004). Changes in RAPD profiles that reflect DNA effects were investigated. DNA effects included DNA damage, as well as mutations and possibly other effects at the DNA levels, which can be induced by chemical or physical agents that directly and/or indirectly interact with genomic DNA.

The alterations were detected in an unspecific form by losses and/or gains of bands and variations in the amplification intensity. Nevertheless, the objective was to establish the existence of DNA damage, i.e., for hazard identification in risk assessment studies, the presence of any of these abnormalities in the band profiles would be enough to identify a genotoxic effect (Argelia and Concepcian, 2004).

In the current study, lichen samples both close to and far from the pollution sources were compared to provide genotoxicity information of mixed pollutants found in the air. A high number of polymorphic bands was observed in samples taken from areas close to the railways and motorways (sites 1, 4, 5) and samples taken from station near to the iron-steel factory (sites 8, 10). This implies that significant air pollution, including an increase in heavy metal contamination, is effective in these areas. Likewise, the results of RAPD analysis yielded the highest band variations in the samples from these districts.

## 4 Conclusions

In conclusion, the heavy metal contents of *E. prunastri* were analysed using AAS. The RAPD method was successfully used as a sensitive means of detecting DNA damage due to environmental pollution and shows potential as a reliable and reproducible assay for genotoxicity. Furthermore, DNA effects in conjunction with other biomarkers from higher levels of biological organisation would be a powerful ecotoxicological tool.

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