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Heavy metal accumulation and genotoxicity indicator capacity of the lichen species *Ramalina pollinaria* collected from around an iron steel factory in Karabük, Turkey

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Abstract: In the present work, *Ramalina pollinaria* (Westr.) Ach. specimens collected from around an iron steel factory in Karabük were analyzed due to their heavy metal accumulation by atomic absorption spectrometry. The specimens were also evaluated for pollution-induced DNA damage by random amplified polymorphic DNA (RAPD) assay. Genomic template stabilities were calculated from the changes in RAPD profiles and compared with heavy metal content in *R. pollinaria*. The results obtained from chemical analysis suggest that *R. pollinaria* is a particularly suitable lichen species for the detection of air quality. Results of RAPD assay showed significant differences in band patterns in *R. pollinaria* as compared to the control sample with respect to disappearance and appearance of bands. In this study, we have assessed the potential of RAPD assay as an application tool for detecting the genotoxic effect of air pollutants according to the changes in DNA band patterns in *R. pollinaria*.

Key words: Air pollution, lichen, RAPD, genotoxicity

1. Introduction

Natural environments located near urban and industrial areas are often contaminated by pollutant discharges (White & Rasmussen, 1998). These discharges may contain chemical agents that are not eliminated during effluent treatment, resulting in release of contaminants into the environment (Barbosa et al., 2010). Physical or chemical analyses are often conducted in order to detect the presence of chemical agents potentially hazardous to the environment and to human health (Barbosa et al., 2010). As such, lichens as biomonitors have a long history (Garty, 1993; Loppi, 1996; Bermudez et al., 2009; Aslan et al., 2004, 2006, 2010, 2011; Cansaran-Duman et al., 2009). Lichens do not possess roots or waxy cuticles and depend mainly on an atmospheric input of mineral nutrients. These features, combined with their extraordinary capability to grow at a large geographical range and to accumulate mineral elements far above their need, rank them among the best bioindicators of air pollution (Bermudez et al., 2009). In recent years, several studies have been carried out on the sorption ability of lichens. Lichens have also been found to bind metals in a strongly pH-dependent manner. This strong metal binding ability of lichen biomass from aqueous solutions would seem to make lichen material an ideal biosorbent for removal of heavy metals (Ekmekyapar et al., 2006; Bingöl et al., 2009).

With fast economic development and industrialization, a vast range of genotoxic chemicals are produced and distributed into the environment. These chemicals adversely affect living organisms and often lead to serious diseases in human beings. Due to the highly conserved structure of the genetic material, it is possible to use a broad variety of species, including bacteria, yeasts, animals, and plants, in genotoxicity tests (Poli et al., 1999). In recent years, lichens have been used as good bioindicators of the genetic toxicity of environmental pollutants (Aras et al., 2010; Cansaran-Duman et al., 2011). Genotoxicity as a result of metal toxicity is also described to play a major role in DNA damage induction (Halliwell, 1990). Toxic chemicals induce several cellular stress responses and damage different cellular components such as membranes, proteins, and DNA (Patra & Panda, 1998; Waisberg et al., 2003; Cenkci et al., 2010; Altınözlü et al., 2012; Tran & Popova, 2013).

Genetic bioassays for the detection of chromosome aberrations and gene mutations in higher plants have been available for many years and are now well-established systems for screening and monitoring environmental chemicals (Pérez et al., 2011). In biomonitoring environmental genotoxins, it is important to use lichens because they are able to metabolize various compounds

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absorbed from the environment. In addition, the effects of heavy metals on lichen species are strongly dependent on 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).

Shugart and Theodorakis (1994) showed that genotoxic agents not only disrupt the integrity of the genome but also affect the expression of DNA directly or indirectly. These effects will lead to an increase in the incidence of different types of gene mutations and, in the long term, result in genetic variability of the exposed populations. These facts make it imperative to establish rapid and sensitive screening tests for assessment of genotoxicity (Zhou et al., 2011).

In genetic-ecotoxicology or eco-genotoxicology, the effective evaluation and proper environmental monitoring of potentially genotoxic pollutants has been improved and will continue to be improved with the development of sensitive and selective methods to detect toxicant-induced alterations in the genomes of a wide range of biota (Atienzar et al., 1999; Theodorakis et al., 2006).

Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis in eco-genotoxicology. DNA-based techniques (RFLP, RAPD, AFLP, SSR, and VNTR) are used to evaluate the variation at the DNA sequence level. Random amplified polymorphic DNA (RAPD) is one of these techniques and can be used for the detection of genetic alterations in RAPD profiles. The alterations in band patterns can clearly be detected when comparing DNA fingerprints from individuals exposed and not exposed to genotoxic agents (Savva, 1996; Atienzar et al., 1999; Enan, 2006; Theodorakis et al., 2006; Aras et al., 2010). In brief, the RAPD assay applied to genotoxicity generates DNA banding profiles that are materialized on agarose gels, and it relies on a comparison of the presence or absence of a given amplified product between the control and heavy metal-contaminated biological systems. The technique has been successfully applied to detect various types of DNA damage and mutation in plants such as *Phaseolus vulgaris* L. (kidney bean) (Enan, 2006), *Hordeum vulgare* L. (barley) (Liu et al., 2005), and *Arabidopsis thaliana* (L.) Heynh. (Conte et al., 1998).

In this study, we have described the heavy metal content of 10 samples of *Ramalina pollinaria* (Westr.) Ach. collected every 5 km from the Yenice Forest to an iron steel factory in Karabük, Turkey, by using atomic absorption spectroscopy (AAS). The region surveyed in this study suffers from substantial historical and current air contamination principally due to the presence of the steel and iron industries, which have been active there since 1925. In addition to industrial or purely urban emissions, other potential sources of atmospheric emissions in the area are waste incinerators and road transportation. In the second part of the study, we evaluate the potential of the RAPD assay to determine genotoxic effects of various pollutants in *R. pollinaria* thalli. The modifications to genomic DNA were detected by RAPD profiles through randomly primed polymerase chain reaction (PCR) reactions. In this sense, the obvious disappearance of normal bands and appearance of new bands were observed from the DNA of the thalli exposed to pollution in comparison to the control lichen samples. This is one of the first reports on the genotoxicity indicator potential (Aras et al., 2010) and the first study showing the changes in genomic template stability of *R. pollinaria* due to genotoxic effects of air pollution.

2. Materials and methods

2.1. Study area

The study area is located between 40°59′03″N and 41°00′00″N and between 32°05′55″E and 32°18′15″E in the western part of the Black Sea region; it belongs to the Yenice district in the province of Karabük. The samples of *Ramalina pollinaria* were collected from 10 sites every 5 km from the Yenice Forest to the Karabük iron steel factory and a control sample was collected from Yenice Forest (Table 1). The samples were taken from a few trees at each station where is not any kind of contamination. The 10 sampling sites were chosen in order to identify the genotoxic effects of local atmospheric deposition. There are numerous industrial activities, such as coal production, the iron and steel industry, the cement industry, and an active intercity highway, in the area. In addition, a railroad transporting coal and crude materials has existed in Karabük for many years. The regions of activity indicated are very close to the city center of Karabük, and coal is generally consumed instead of natural gas during the winter periods. According to the local environmental unit parameters, the SO_2 and PM_{10} contamination in the city center of Karabük increases to harmful levels in winter. In addition, there is a rich and large forest ecosystem in terms of species, which is protected according to the World Wildlife Fund in the city (Cansaran-Duman et al., 2011).

2.2. Lichen species

Ramalina pollinaria was chosen as a suitable bioindicator since it is of low cost and suitable for easy sampling. The samples were collected from on *Pinus* sp. at 10 sites located at various distances from the pollution sources (Table 1). Two samples were collected from each sampling point at each selected site. The samples taken from a few trees at each station were homogenized before the analysis. A control sample was collected from the forest mentioned above, where there was no source of pollution. In the laboratory, lichen samples were cleaned of contaminants

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

Locality number	GPS coordinates	Locality name	Altitude (m)	Distance (km)	Direction	Contaminants
1	44°62′N, 45°73′E	Karabük-Yenice, Kuzdağ district	1125	50	South of the ISF	Human activities, vehicular activity
2	41°15′N, 32°35′E	Karabük-Yenice, Kabaklı Kaya	1140	45	Southwest of the ISF	Setting areas, industrial activities
3	41°13′N, 32°28′E	Karabük-Yenice, Hamzakıran district	1140	40	South of the ISF	Chemical industries
4	41°14′N, 32°35′E	Karabük-Yenice, Dikilitaş	1125	35	Southeast of the ISF	Shanty area
5	41°12′N, 32°25′E	Karabük-Yenice, vicinity of Kuzdere, Hamdioğlu district	1400	30	West of the ISF	Waste incinerator
6	41°15′N, 32°34′E	Karabük-Yenice, north of Yalnızca plateau	1200	25	West of the ISF	Waste water plant
7	41°11′N, 32°27′E	Karabük-Yenice, Acısu center	1375	20	South of the ISF	Vehicular density, railway
8	41°14′N, 32°33′E	Karabük-Yenice, Kazancıoğlu district	1750	15	East of the ISF	Industrial units
9	41°12′N, 32°29′E	Karabük-Yenice, Hacıömerler district	1380	10	North of the ISF	Motor vehicles, combustion of fossil fuels
10	41°12′N, 32°29′E	Karabük-Yenice, Kızılgöz Kayası	1385	5	North of the ISF	Human activities, vehicular activity
11**	41°10′N, 32°24′E	Karabük-Yenice, vicinity of Cami district	1100	70	South of Karabük	Contamination source not available

*: Date of collection 15 November 2005. **: Control sample. ISF: Iron steel factory.

using a binocular microscope (Olympus, Germany), and consecutive washings were applied with distilled water before DNA isolation.

2.3. Element analysis

Element contents were determined using the method described by Cansaran-Duman et al. (2009). Briefly, the chemical analyses of lichen samples were conducted after extraction. A mixture of 2.0 mL of 63% HNO_3 and 1.0 mL of H_2O_2 was added to the 50-mg lichen sample and melted in Teflon-coated pots in a milestone-mark microwave oven. Deionized 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 deionized water.

Calibration curves of Mn, Zn, Fe, Pb, and Cu metals were obtained using linear regression analyses with various concentrations (0.25, 0.50, 1.00, 2.00, and 4.00 mg/L) of samples. Heavy metal concentration in these materials was determined using flame AAS (Instrument PM Avarta Model AAS, GBC Scientific Equipment, Australia). The accuracy of the process was checked with a standard extension. The calibration curves of Cd and Cr metals were obtained using linear regression analyses with various concentrations (10, 25, 40, 60, and 80 mg/L) of samples by electrothermal AAS (GBC Scientific Equipment, Australia). The accuracy of the process was checked by a standard extension (Cansaran et al., 2009).

2.4. Genomic DNA isolation and RAPD procedures

DNA extraction of the lichen samples was performed according to the protocol improved 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 260/280 nm absorbance ratio with the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA). The DNA concentrations were in the range of 1718 to 2642 ng/µL and 260/280 nm ratios were between 1.57 and 1.79. The integrity of the extracted DNAs was also evaluated by electrophoresis.

RAPD reactions were performed in a volume of 25 mL. An initial screening of 23 RAPD decamer primers was performed in order to test amplification profiles for polymorphism and reproducibility. Only 15 priming oligonucleotides yielded specific and stable results. The names of these sequences of primers are given in Table 2. The GeneRuler 100-bp DNA Ladder Plus (Fermentas) was used as a molecular weight DNA standard. Thermal cycling parameters used for PCR amplification consisted of initial denaturation at 94 °C for 30 s, annealing at 36 °C for 1 min, and extension at 72 °C for 45 s, with final extension at 72 °C for 8 min. All amplifications were conducted twice in order to evaluate the reproducibility of the polymorphic bands. A negative control, without genomic DNA, was run in parallel with every set of samples. The amplicons mixed with loading buffer were resolved electrophoretically on an agarose gel (1.2%) at 100 V for 4 h. The gel was stained with ethidium bromide (0.5 g/mL) and visualized using a gel image system.

2.5. Genomic template stability

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

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

Table 2. The sequences of the primers used in the study.

Names of primer	Sequence of primers (5′ → 3′)
OPO07	CAGCACTGAC
OPO 03	CTGTTGCTAC
OPO19	GGTGCACGTT
OPC12	TGTCATCCCC
P437	CGGATCGACA
B389	CGCCCGCAGT
BC374	GGTCAACCCT
TubeA01	CAGGCCCTTC
TubeA02	TGCCGAGCTG
TubeA03	AGTCAGCCAC
TubeB01	GTTTCGCTCC
TubeC01	TTCGAGCCAG
OPA13	CAGCACCCAC
OPA18	AGGTGACCGT
BC379	GGGCTAGGGT

where a is the average number of polymorphic bands detected in each treated sample and n is the number of total bands in the control. Polymorphism observed in RAPD profiles includes disappearance of a normal band and appearance of a new band in comparison with the profile of the control.

2.6. Statistical analyses

The results of the chemical analysis of *Ramalina pollinaria* were evaluated by one-way analysis of variance (ANOVA) to display the effects of the pollution on the bioaccumulation status. In addition, the Bonferroni test for multiple comparisons was used to test for significant differences between heavy metal accumulation in lichen species and the examined sites. The ratio between the concentration of each element after and before exposure (exposed to control ratio, EC ratio) was used to evaluate bioaccumulation rates; the EC values were then classified according to Frati et al. (2005).

The results of RAPD analysis were analyzed by considering the bands that appeared in the control sample as the criterion of judgment. Polymorphism observed in RAPD profiles included disappearance of a control band and appearance of a new band (Atienzar et al., 1999; Liu et al., 2005).

3. Results

3.1. Heavy metal content

Among the *Ramalina pollinaria* samples collected from around the Karabük iron steel factory, sites 7 (24.09 μg/g), 8 (20.46 μg/g), and 9 (19.37 μg/g) revealed higher levels of zinc (Table 3). The values obtained for sites 5, 6, and 10 were close to each other (Figure 1). Results show that Zn concentration in the lichen samples was linearly related to the vehicle traffic, railway, and activity of industrial units.

Higher levels of manganese were found at sites 8 (73.26 μg/g) and 2 (31.90 μg/g) compared to the control value of 8.8 μg/g. Motor vehicles are known to be the source of Mn in urban areas (Monaci et al., 2000) and could explain the reason for elevated Mn concentrations at station 2, which is close to the main road.

Comparisons of the Pb concentrations of the *Ramalina pollinaria* specimens from polluted sites with the control yielded very significant variations, especially at sites 1 and 8 (Table 3). Sites 1 and 8 have the highest levels of human activities and high vehicular density congestion. These sites are in the central part of the city where human activities and density of traffic are very intense (Table 3).

Although the highest levels of chromium in the *Ramalina pollinaria* were found at sites 8 (2.72 μg/g) and 9 (1.67 μg/g) (Table 2), they did not show a significant difference from the control. On the other hand, Cr concentrations at sites 3 (1.73 μg/g), 4 (1.70 μg/g), 5 (1.75 μg/g), 6 (1.75 μg/g), and 7 (1.72 μg/g) were significantly

Table 3. Comparison of element concentrations in *Ramalina pollinaria* thalli with one-way ANOVA (n = 5; df = 10, 44).

Site	Zn		Cu		Mn		Fe	
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
1	5	10.483 ± 0.025 (a)	5	1.489 ± 0.022 (a)	5	22.283 ± 0.233 (a)	5	999.509 ± 19.349 (a)
2	5	11.885 ± 0.209 (b)	5	0.905 ± 0.044 (b)	5	31.904 ± 0.849 (b)	5	487.966 ± 5.369 (b)
3	5	9.648 ± 0.077 (c)	5	1.226 ± 0.003 (c)	5	14.215 ± 1.060 (c)	5	703.250 ± 0.537 (c)
4	5	14.846 ± 0.068 (d)	5	1.369 ± 0.022 (d)	5	20.779 ± 1.403 (a)	5	652.427 ± 4.303 (d)
5	5	16.215 ± 0.047 (e)	5	1.273 ± 0.007 (c)	5	21.247 ± 0.147 (a)	5	653.943 ± 10.171 (d)
6	5	16.658 ± 0.229 (f)	5	1.353 ± 0.008 (d)	5	27.662 ± 1.061 (d)	5	463.078 ± 10.127 (b)
7	5	24.092 ± 0.012 (g)	5	1.384 ± 0.042 (d)	5	16.147 ± 0.472 (e)	5	592.600 ± 13.934 (e)
8	5	20.467 ± 0.064 (h)	5	2.129 ± 0.010 (e)	5	73.266 ± 0.272 (f)	5	1932.80 ± 2.610 (f)
9	5	19.375 ± 0.084 (i)	5	1.800 ± 0.055 (f)	5	21.578 ± 0.008 (a)	5	1148.70 ± 16.867 (g)
10	5	13.195 ± 0.011 (j)	5	1.435 ± 0.013 (ad)	5	11.729 ± 0.577 (c)	5	560.891 ± 18.523 (e)
11	5	5.919 ± 0.039 (k)	5	0.378 ± 0.006 (g)	5	8.838 ± 0.013 (g)	5	515.734 ± 38.727 (b)
ANOVA								
F-ratio		15,041.886		1139.337		1894.538		2447.878
P-ratio		0.000		0.000		0.000		0.000

Site	Pb		Ni		Cr		Cd	
	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD
1	5	1.263 ± 0.011 (a)	5	2.265 ± 0.006 (a)	5	1.999 ± 0.030 (a)	5	0.262 ± 0.026 (a)
2	5	1.038 ± 0.006 (b)	5	3.039 ± 0.009 (b)	5	1.740 ± 0.023 (b)	5	0.048 ± 0.001 (b)
3	5	1.119 ± 0.009 (c)	5	1.474 ± 0.007 (c)	5	1.733 ± 0.011 (b)	5	0.020 ± 0.001 (b)
4	5	0.881 ± 0.008 (d)	5	0.260 ± 0.013 (d)	5	1.706 ± 0.042 (b)	5	0.390 ± 0.028 (c)
5	5	0.817 ± 0.009 (e)	5	0.356 ± 0.011 (e)	5	1.751 ± 0.059 (b)	5	0.243 ± 0.004 (ad)
6	5	0.924 ± 0.012 (f)	5	0.568 ± 0.009 (f)	5	1.757 ± 0.011 (b)	5	0.265 ± 0.010 (a)
7	5	0.975 ± 0.006 (g)	5	2.169 ± 0.006 (g)	5	1.727 ± 0.010 (b)	5	0.222 ± 0.012 (d)
8	5	1.733 ± 0.008 (h)	5	0.561 ± 0.011 (f)	5	2.728 ± 0.068 (c)	5	0.403 ± 0.018 (e)
9	5	1.167 ± 0.009 (i)	5	0.101 ± 0.009 (h)	5	2.203 ± 0.043 (d)	5	0.237 ± 0.008 (ad)
10	5	0.656 ± 0.008 (j)	5	2.888 ± 0.006 (i)	5	1.672 ± 0.010 (b)	5	0.207 ± 0.009 (fd)
11	5	0.833 ± 0.006 (e)	5	0.010 ± 0.001 (j)	5	1.748 ± 0.010 (b)	5	0.062 ± 0.015 (b)
ANOVA								
F-ratio		5137.915		93,398.414		454.360		412.127
P-ratio		0.000		0.000		0.000		0.000

Same letters in a column indicate the absence of significant differences at $P < 0.05$ by Bonferroni's multiple-range test.

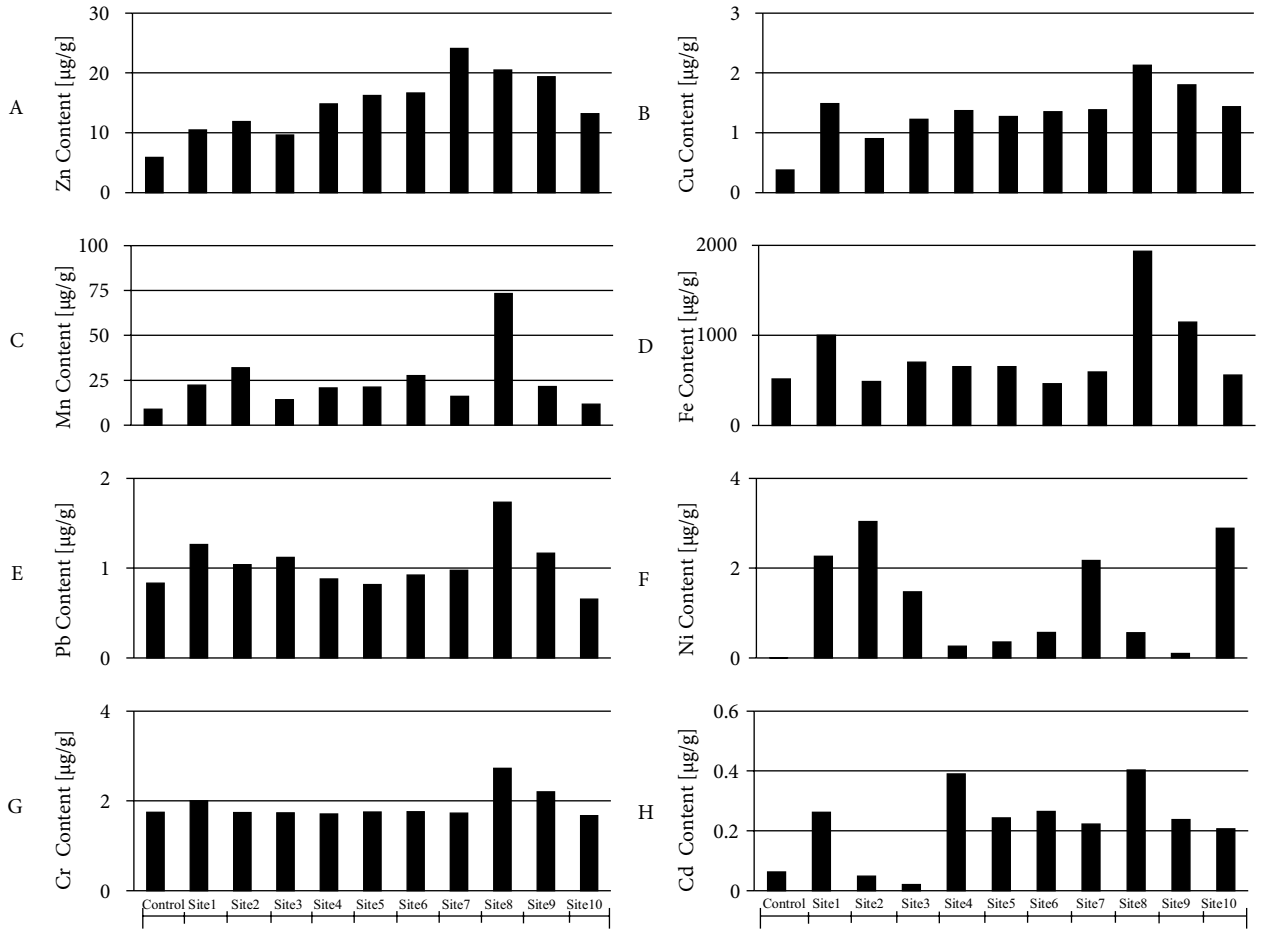

Figure 1. Graphical representation of elemental content in *Ramalina pollinaria*.

higher than at the control site (1.74 µg/g) (Table 3). The most important sources of Cr pollution are industrial activities like refining works and iron steel factories.

Copper contents in the *R. pollinaria* samples ranged from 0.90 to 2.12 µg/g. Cu content at site 8 (2.12 µg/g) in *R. pollinaria* was significantly higher than at the control site (0.37 µg/g) (Table 3; Figure 1). Nickel concentrations at sites 2 and 10 were 3.03 µg/g and 2.88 µg/g in *R. pollinaria* samples, respectively (Table 3).

The mean Cd concentrations at sites 2 (0.04 µg/g) and 3 (0.02 µg g) were slightly lower than at the control site (0.06 µg/g) (Figure 1). All sites, and especially site 8 (0.40 µg/g), showed significantly higher Cd concentrations than the control site (0.06 µg/g) in *Ramalina pollinaria* (Table 3). This might be due to the contaminants released from motor vehicles, dust raised by metal work, and other human activities.

There were some variations in the concentrations of many elements between sites when compared with the control samples (Table 4). For this reason, to allow comparison of the accumulation capacity of collected samples, EC ratios were calculated (Table 4). The use of EC ratios allowed us to correct this problem and to normalize the use of different sites. EC ratios were calculated according to Frati et al. (2005). There are 2 main approaches: calculating accumulation factors such as EC ratios (relative changes) and, more simply, calculating differences between exposed and control samples (absolute changes). Sites 1 and 8 displayed the highest metal concentrations among all sites. This result is not surprising because these localities are close to major traffic circles, industrial centers, or centers of major mining activities.

The amounts of Fe accumulated in *Ramalina pollinaria* at sites 2 and 6 were lower than those of control samples. A relatively high iron content was measured at sites 8 and 9 (Table 3). The levels of Fe accumulated in the thalli of *Evernia prunastri* (L.) Ach. and *Pseudevernia furfuracea* (L.) Zopf. from the same localities were similar to results of *R. pollinaria* (Figure 2) (Cansaran-Duman et al. 2009, 2011).

Accumulation ratios of Mn at site 2 for *Evernia prunastri*, *Pseudevernia furfuracea*, and *Ramalina pollinaria* lichen

Table 4. EC ratios for elements assayed in exposed *Ramalina pollinaria* specimens.

	Sites										
	1	2	3	4	5	6	7	8	9	10	Control
Zn	1.771	2.008	1.630	2.508	2.739	2.814	4.070	3.458	3.273	2.229	1.218
Cu	3.962	2.407	3.261	3.642	3.386	3.600	3.683	5.664	4.790	3.817	1.767
Mn	2.521	3.610	1.608	2.351	2.404	3.130	1.827	8.290	2.442	1.327	1.298
Fe	1.938	0.946	1.364	1.265	1.020	0.898	1.149	3.748	2.227	1.088	0.985
Pb	1.516	1.245	1.342	1.057	0.980	1.109	1.170	2.080	1.400	0.788	1.109
Ni	222.5	298.5	144.7	25.51	34.94	55.81	213.0	55.08	9.945	283.7	7.135
Cr	1.144	0.995	0.991	0.976	1.002	1.005	0.988	1.560	1.260	0.957	0.612
Cd	4.255	0.781	0.317	6.321	3.939	4.289	3.597	6.528	3.850	3.364	0.186

species were compared, revealing an order of *E. prunastri* > *P. furfuracea* > *R. pollinaria*. However, *P. furfuracea* showed the highest levels of Mn at all other sites (Figure 2). Mn could be a tracer of both wind-blown dust particles and vehicular traffic, since this element has recently been used as a substitute for Pb in additives (Ardeleanu et al., 1999).

Ramalina pollinaria collected from sites 2 and 7 had significantly higher contents of Ni (Figure 2). *Evernia prunastri* species at sites 1, 3, and 9 contained higher Ni metal concentrations than other examined lichen species. Among the 3 species compared, *Pseudevernia furfuracea* displayed significantly higher values of Cr element at sites 1, 2, 4, 5, 6, 9, and 10. *P. furfuracea* and *E. prunastri* showed significant Cr accumulations. Previous studies that considered *P. furfuracea* as a passive biomonitor in the province of Karabük showed similar concentrations of Cr in the specimens (Cansaran-Duman et al., 2009) (Figure 2).

In concordance with the previous findings on *Pseudevernia furfuracea* and *Evernia prunastri*, the current study conducted with *Ramalina pollinaria* also demonstrated the importance of heavy metal accumulation in lichen species.

Bonferroni's multiple-range test was used to assay for significant differences between heavy metal accumulation in lichen species and examined sites. Results from Bonferroni tests are given in Table 3, where the same letters in a column indicate the absence of significant differences at $P < 0.05$ by Bonferroni's multiple-range test. It was obvious that Zn and Pb values were statistically significant ($P < 0.05$) at all examined sites (Table 3). Other results of heavy metal concentrations (Cu, Mn, Fe, Ni, Cr, and Cd) were observed to be statistically insignificant (Table 3). In particular, the levels of Cr in *Ramalina pollinaria* did not show highly significant differences. In sites 2–7 and 10, differences in Cr contents in the *R. pollinaria* samples were observed to be statistically insignificant. Only Cr concentrations at sites 1, 8, and 9 were evaluated as statistically significantly different (Table 3).

3.2. RAPD profiles

The RAPD patterns showed significant differences between lichen specimens from polluted sites and control samples with apparent changes (disappearing of a normal band and/or appearing of an extra band) in the number and size of amplified DNA fragments. Table 5 summarizes the alterations detected in RAPD profiles. The profiles of one informative primer (P437) are shown in Figure 3. The highest number of band changes was detected at site 8 (104), and the total number of band changes was increased with an increase in concentration of heavy metal accumulation.

In all cases, the RAPD patterns generated for *Ramalina pollinaria* specimens from polluted sites were clearly different from those of the control group and exhibited a distinct change with increasing concentrations of heavy metals. The differences in RAPD patterns refer to loss of normal bands and appearance of new bands as compared with the control (Figure 3; Table 5). Our results suggest that low element content may lead to a low level of genotoxic effect in the *R. pollinaria*. In addition, Figure 3 also shows that RAPD patterns were optimal when PCR was performed with all the primers, confirming that the variation of bands was stable.

A total of 253 bands ranging from 317 bp (OPA13) to 2716 bp (BC374) were amplified by 15 primers that generated bands between 8 (TubeA01) and 27 (TubeA03) with an average of 17 bands per primer (Table 5). RAPD profiles displayed substantial differences between control and polluted samples. The variation in the number of amplified DNA fragments also showed an increase

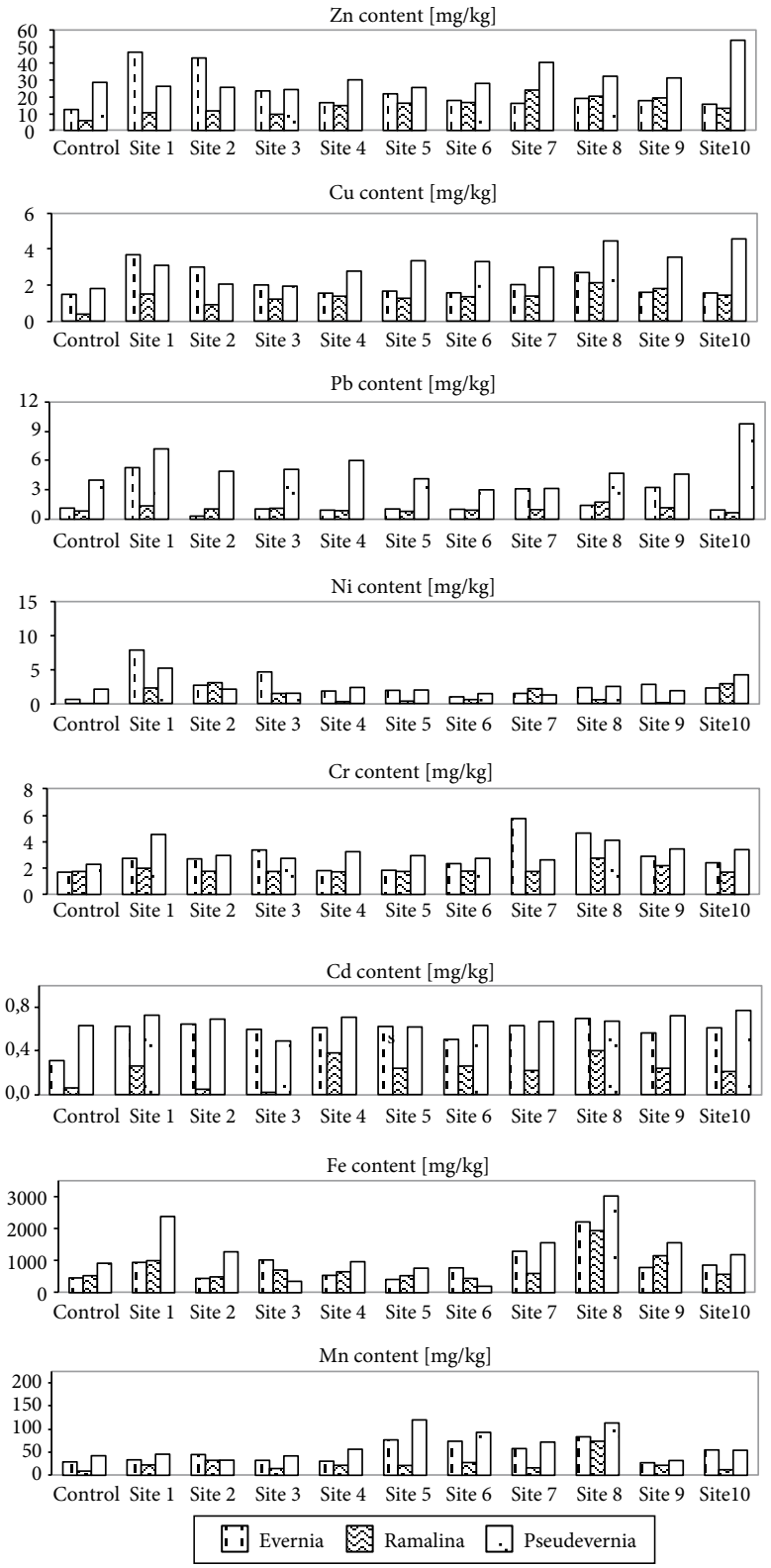

Figure 2. Comparison of *Evernia prunastri*, *Pseudevernia furfuracea* and *Ramalina pollinaria* heavy metal accumulation: Zn, Cu, Pb, Ni, Cr, Cd, Fe, and Mn.

Table 5. Changes of total bands in control, polymorphic bands, and varied bands in the specimens.

Primer	C	S1		S2		S3		S4		S5		S6		S7		S8		S9		S10	
	TB	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
OPO07	12	0	1	3	2	0	0	0	1	0	0	2	0	1	3	4	3	2	3	4	1
OPO03	9	3	3	4	3	0	1	0	0	2	0	1	1	5	4	3	1	2	1	4	2
OPO19	16	2	0	3	4	1	0	1	0	1	0	2	1	3	1	2	4	2	0	3	0
OPC12	21	2	2	5	2	2	2	3	1	0	0	2	0	2	5	3	2	1	1	4	2
P437	10	3	1	3	0	3	1	0	0	0	1	3	2	3	7	5	3	1	5	6	3
B389	19	1	2	2	1	0	2	0	0	1	2	1	1	5	5	7	2	4	2	5	1
BC374	23	0	0	4	1	2	1	2	1	3	0	0	0	6	3	1	3	4	1	4	1
TubeA01	8	2	4	3	2	3	1	2	2	0	0	0	0	4	4	5	4	3	2	3	2
TubeA02	17	3	1	2	6	0	1	3	0	1	2	1	1	3	7	8	3	2	3	2	2
TubeA03	27	1	0	3	1	0	1	1	1	3	1	3	1	2	1	5	1	5	3	9	4
TubeB01	20	2	1	3	1	1	3	0	1	0	1	0	1	1	4	3	0	7	2	5	3
TubeC01	13	3	3	2	1	0	0	2	0	0	0	1	0	0	4	4	1	4	1	6	2
OPA13	19	4	1	3	3	0	0	1	0	1	1	2	3	2	4	9	4	3	4	4	4
OPA18	25	1	1	4	2	0	1	0	0	2	0	1	3	5	2	6	2	1	3	3	2
BC379	14	0	1	3	0	2	1	1	1	1	0	0	0	2	0	1	5	2	4	1	4
		27	21	47	29	14	15	16	8	15	8	19	14	44	54	66	38	43	35	63	36
a + b	253	48		76		29		24		23		33		98		104		78		99	

C: Control sample, S: sample, a: appearance of new bands, b: disappearance of control bands, a + b: indicates polymorphic bands.

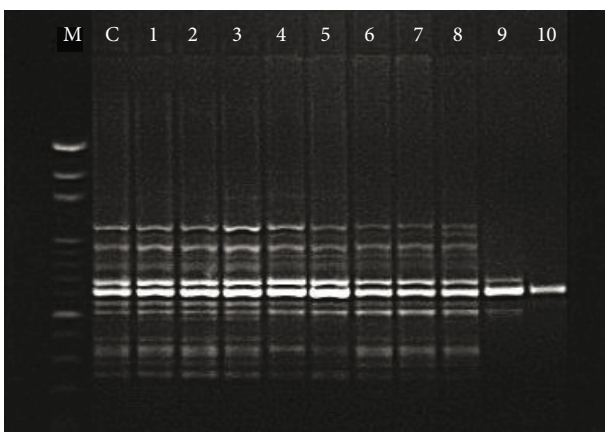

Figure 3. RAPD profiles generated by P437 primer from *Ramalina pollinaria* exposed to polluted areas around the iron steel factory in Karabük. Lane M: Molecular weight marker (100-bp ladder).

depending on the distance from the pollution sources. In total, 136 normal RAPD bands of control samples disappeared. The number of disappearing bands for primers OPC12, OPA13, and TubeA03 were high at sites 2, 8, and 10 compared with the samples from other sites (Table 5). For instance, the number of extra bands for sites from around the iron steel factory (sites 7, 8, 9, and 10) was higher (Table 5). Meanwhile, different polymorphic bands were detected for each site for the 15 primers tested. Polymorphism values (P%) of the primers are given in Table 6.

RAPD profiles showed substantial differences between control and polluted samples with apparent changes (disappearance and/or appearance) in the number of DNA bands produced by different primers. The total number of bands in the control was 253 and the sizes of the bands was ranged from 312 to 2716 bp. The changes in RAPD

Table 6. The polymorphism rates of the primers.

	TB	PB	Rate
OPO07	12	8	66.7%
OPO03	9	7	77.8%
OPO19	16	10	62.5%
OPC12	21	6	28.6%
P437	10	9	90.0%
B389	19	7	36.9%
BC374	23	8	34.8%
TubeA01	8	6	75.0%
TubeA02	17	8	47.1%
TubeA03	27	9	33.3%
TubeB01	20	10	50.0%
TubeC01	13	7	53.9%
OPA13	19	6	31.6%
OPA18	25	8	32.0%
BC379	14	8	57.2%

TB: Total bands, PB: polymorphic bands.

profiles are summarized for *Ramalina pollinaria* in Table 5 and Figure 3.

In *Ramalina pollinaria*, the number of new band appearances at sites 4 and 5 was considerably low. The number of disappeared bands was higher at site 8 and a lower number of disappeared bands was observed at site 3 (Table 5). The samples that yielded high concentrations of heavy metals in chemical analysis also displayed increased numbers of band variations in RAPD analysis (sites 2, 7, 9, and 10). The number of band variations also seemed to depend on the distance from the pollution source, like the results from chemical analysis. In this study, the highest rates of band variations were observed at sites 8 (104) and 10 (99), where human activities and density of traffic were very intense.

3.3. Genomic template stability

GTS is related to the level of DNA damage and the efficiency of DNA repair and replication. Therefore, a high level of DNA damage does not necessarily decrease the GTS in comparison to a low level of DNA alterations, because DNA repair and replication are inhibited by the high frequency of DNA damage.

The GTS (%), a qualitative measure reflecting the changes in RAPD profiles, was calculated for each of the 15 primers tested and is presented in Table 7. At sites 1, 8, 9, and 10, the GTS decreased to 81.28%, 58.89%, 69.17%, and 60.87% , respectively. The highest GTS value was detected at site 5 (90.9%) (Table 7).

Table 7. Changes of GTS for all primers in *Ramalina pollinaria*.

Number of the sites	GTS rate (%)	Number of the sites	GTS rate (%)
1	81.28	6	86.95
2	69.96	7	61.26
3	88.53	8	58.89
4	90.51	9	69.17
5	90.90	10	60.87

When the heavy metal contents in *Ramalina pollinaria* specimens were compared in terms of GTS values, a decrease in GTS values was observed with an increase in heavy metal content, especially in the samples from around the iron steel factory (sites 7, 8, 9, and 10) and the in the samples from around the main road (sites 1 and 2) (Table 7). GTS values were decreased obviously with an increase in heavy metal concentration in *R. pollinaria* species (Figure 4).

4. Discussion

Heavy metals induce several cellular stress responses and damage to different cellular components such as membranes, proteins, and DNA (Waisberg et al., 2003). Some studies have used the comet, micronucleus, or chromosome aberration assays to measure the effects of heavy metals and other genotoxic agents on plants (Cenkci et al., 2010). Advantages of measuring effects of genotoxic chemicals directly on DNA are mainly related to the sensitivity and short response time. Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis in the field of genotoxicology. The RAPD assay is a PCR-based technique that amplifies random DNA fragments of genomic DNA with single short primers of arbitrary nucleotide sequence under low annealing conditions (Williams et al., 1990). After proper optimization, the assay can be successfully applied to detect genomic DNA alterations induced by several DNA-damaging agents. Detection of genotoxic effect using RAPD involves the comparison of DNA amplification profiles generated from control (unexposed) and treated (exposed) samples. The changes in RAPD profiles must be interpreted carefully. Indeed, the frequency of appearance and disappearance of bands may allow a better understanding of the results. In the current study, probable DNA damages induced by various environmental pollutants and factors were

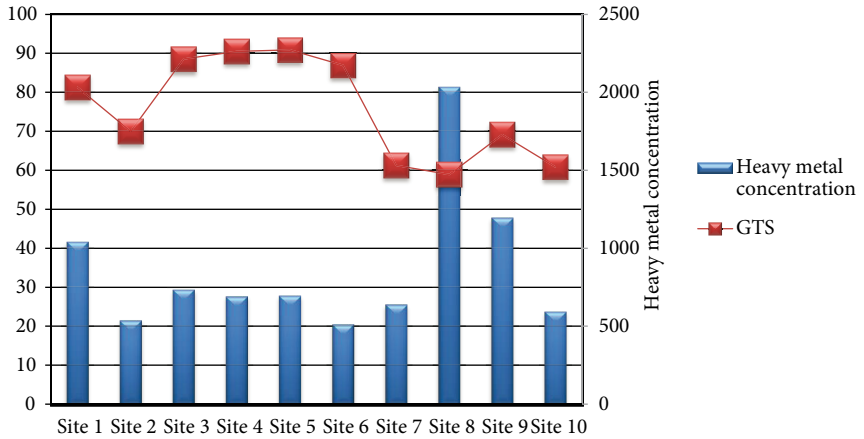

Figure 4. Comparison of total heavy metal content and GTS values in *Ramalina pollinaria*.

reflected by changes in RAPD profiles: disappearance of normal RAPD bands and appearance of new PCR products occurred in the profiles. It is proposed that alterations to RAPD profiles due to genotoxic exposure can be regarded as alterations in GTS (Atienzar et al., 1999). The results indicate that GTS in *Ramalina pollinaria* was significantly affected by the pollution around the iron steel factory in Karabük, Turkey. As reported by Liu et al. (2005), modifications of band intensity and lost bands are likely to be due to one or a combination of the following events: 1) changes in oligonucleotide priming sites, due mainly to genomic rearrangements and less likely to point mutations; 2) DNA damage in the primer binding sites; and 3) interactions of DNA polymerase in test organisms with damaged DNA. On the other hand, the appearance of a new DNA band could occur because some oligonucleotide priming sites could become accessible to oligonucleotide primers after structural change or because some changes in DNA sequence have occurred due to mutations, large deletions, and/or homologous recombination (Atienzar et al., 1999). Appearance of new bands may also be the result of genomic template instability related to the level of DNA damage and the efficiency of DNA repair and replication (Atienzar et al., 1999; Zhou et al., 2011). Another idea is that when Taq DNA polymerase encounters a DNA adduct, there are a number of possible outcomes including blockage, bypass, and the possible dissociation of the enzyme/adduct complex, which will cause the loss of bands (Atienzar & Jha, 2006).

Increased polymorphism in DNA with increased heavy metal accumulation recorded by the chemical analysis in the samples from around the iron steel factory agrees well with our previous observations (Cansaran-Duman et al., 2011), in which *Evernia prunastri* samples with high heavy metal content displayed high DNA band profile changes (Figures 2a and 2b) and reduced GTS values. On the other hand, the number of disappeared and appeared bands in *Ramalina pollinaria* was higher than the number obtained for *E. prunastri*.

Analytical studies by AAS demonstrated that accumulation of heavy metals, (Zn, Cu, Mn, Fe, Pb, Ni, Cd, and Cr) was markedly higher in thalli of the samples collected from around the iron steel factory in Karabük, Turkey. Similar results were found in a previous study conducted with *Pseudevernia furfuracea* thalli collected from the same region and same stations (Cansaran-Duman et al., 2009). The heavy metal contents in *P. furfuracea* and *Evernia prunastri* was already reported in previous studies by Cansaran-Duman et al. in 2009 and 2011, respectively. Zn contents of *P. furfuracea* at sites 4 and 10 were significantly higher than those of *E. prunastri* and *Ramalina pollinaria*. A significantly higher Zn deposition in *P. furfuracea* was observed at sites 7 and 10, since vehicular traffic is the main source of air pollution in those areas (Figure 2a). Concentration of Cd in *E. prunastri* and *P. furfuracea* was found to parallel each other at sites 5 and 7. These species also revealed higher concentrations of Cd than did *R. pollinaria*.

Pb concentrations in *Pseudevernia furfuracea* specimens were higher than in *Evernia prunastri* and *Ramalina pollinaria* at all sites (Figure 2a). The differential accumulation of metals among lichen species can be related to a selective cation uptake, which can be attributed to a greater affinity between Pb cations and lichen cell wall exchange sites, which are probably strongly attached to binding sites on the cell wall and outer surface of the plasma membrane.

Comparison of the metal concentration levels in *Ramalina pollinaria* with those of *Pseudevernia furfuracea* and *Evernia prunastri* revealed the same of amounts of metal. Bergamaschi et al. (2007) measured 29 elements (Al, As, Br, Ca, Cd, Ce, Cl, Co, Cr, Cs, Cu, Fe, Hg, I, K,

La, Mg, Mn, Ni, Pb, Rb, Sb, Sc, Se, Sm, Th, Ti, V, and Zn) in *Hypogymnia physodes* (L.) Nyl., *P. furfuracea*, *Usnea hirta* (L.) Weber ex F.H.Wigg., and *Parmelia sulcata* Taylor. Fl. Hibern. in Italy. They found that, in general, elements did not exhibit well-defined trends, but rather showed fluctuations, and they indicated that *H. physodes*, *P. furfuracea*, *U. hirta* had a similar accumulation capacity, while that of *P. sulcata* was lower. Several studies revealed that lichens may selectively accumulate extracellular elements and metabolize or eliminate those elements that enter the cell wall (Branquinho et al., 1997; Chettri et al., 1997). Aslan et al. (2006) reported high K and Ca concentrations in *Flavoparmelia caperata* (L.) Hale samples that were also collected near agricultural areas. According to Carreras et al. (2009), their results suggested that lichen species can be successfully used to monitor air pollution. However, several other factors should be considered before making a decision about the preferred biomonitor species, such as background elemental concentration, selective uptake, or detoxification mechanisms. Generally, previous studies that used *P. furfuracea* and *E. prunastri* near an iron steel factory in the province of Karabük showed concentrations higher than those of *R. pollinaria* specimens found in the present study.

Similar results of heavy metal accumulation on lichens were reported for different lichen species by many researchers (Nyarko et al., 2008) in recent years. AAS element analyses demonstrated that the content of toxic elements was higher in *Ramalina pollinaria* than in *Evernia prunastri* (Cansaran-Duman et al., 2011). On the other hand, band disappearance in *R. pollinaria* tended to increase with increased heavy metal concentrations in the samples from sites 1, 8, and 10.

The genotoxic effects of heavy metals have been studied in many studies. It has been shown that Hg and Cr could induce DNA damage such as single- and double-strand breaks, modified bases, abasic sites, DNA–protein cross-links, oxidized bases, 8-hydroxyguanine, and even bulky adducts in organisms (Zhou et al., 2011). On the other hand, Xu et al. (2008) reported that Zn could also induce DNA strand breaks. Thus, the findings in the current study confirmed the idea that environmental pollutants, and mainly heavy metals, cause DNA damages in organisms and demonstrate the potential of RAPD analyses to monitor the level of genotoxicity in lichens.

A correlation between heavy metal content and level of heavy metal-induced DNA damage in different tissues of tobacco (*Nicotiana tabacum* L.) and potato (*Solanum tuberosum* L.) seedlings was shown by comet assay (Gichner et al., 2008). In addition, in higher plants, compared to roots, the leaf cells are better equipped with an antioxidant defense system that might protect the nuclear DNA in leaf cells from Cd-induced oxidative stress (Gichner, 2003). On the other hand, Gichner et al. (1999) reported that the nuclei isolated from tobacco leaf cells are more sensitive than the nuclei from roots to induced DNA damage. However, lichens as cryptogams that lack roots and leaves are highly open to all pollutants only with their thalli. The antioxidant defense systems and other repair systems in these organisms that remain to be studied might show differences from species to species and might explain the variations in the DNA damage level in different species, which was the case encountered in our studies.

The use of short-term bioassays, and especially genetic toxicity bioassays, to assess potent environmental pollutants has gained special attention over the last decades. These assays are capable of predicting the genotoxic potential of a pollutant under investigation by measuring gene mutations and damage to chromosomes and DNA. Plant assays are quite easy to conduct, inexpensive, and rapid and are good predictors of genotoxicity (Panda & Panda, 2002). As reported by some researchers (Savva, 1998; Atienzar & Jha, 2006), the alteration in DNA fingerprinting is a useful biomarker in eco-genotoxicology. Lichens have been used as test organisms for environmental risk and impact assessment (Nyarko et al., 2008). They are usually considered as ideal early warning indicators of air pollution (Gries, 1996; Hamada & Miyawaki, 1998). However, to our knowledge, little information is available on their potential genotoxicity indicator capacity against pollutants. In our laboratory, studies on lichen genotoxicity were begun in recent years (Aras et al., 2010; Cansaran-Duman et al., 2011), and DNA alterations in the exposed *Pseudevernia furfuracea* and *Evernia prunastri* samples were aimed to be described by RAPD analysis in order to reveal the pattern of genetic variation influenced by the various environmental pollutants. Moreover, the data obtained could demonstrate the utility of the lichens for their ability to evaluate genotoxicity under environmental stresses and may suggest which systems are more appropriate for rapid monitoring of genotoxicity. According to a previous study by Cansaran-Duman et al. (2011), the highest number of band changes in *E. prunastri* were seen at sites 8 and 10 near the iron steel factory in Karabük.

In conclusion, the data presented here support the view that RAPD analysis is a highly sensitive method for the detection of DNA damage induced by environmental pollutants like toxic chemicals. The RAPD assay is a powerful PCR-based molecular marker technique and has been used as a novel biomarker assay in surveying genomic DNA for evidence of various types of DNA damage and mutation in cells of bacteria, plants, and animals (Atienzar et al., 1999) and also in lichens (Aras et al., 2010; Cansaran-Duman et al., 2011). Moreover, as the objective in the current study was to show the existence of DNA

damage for hazard identification in risk assessment of eco-genotoxicological studies, the presence of the varied RAPD profiles can provide sufficient evidence for identification of the genotoxic effect. Lichens are widely used for the biomonitoring of air quality, either as bioindicators of air quality or as bioaccumulators of atmospheric deposition. These organisms are widely used because of their low cost and easy sampling, and because they allow wide areas to be monitored. The studies conducted by lichens in our laboratory reveal that both *Ramalina pollinaria* and *Evernia prunastri* are equally sensitive to genotoxic stresses. Based on our findings, we recommend the usage of *R. pollinaria* in addition of *E. prunastri* for identification of genotoxic effects.

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