Evaluation of genotoxicity of *Pseudevernia furfuracea* (L.) Zopf by RAPD analysis

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ABSTRACT. We investigated the suitability and applicability of *Pseudevernia furfuracea* (L.) Zopf for environmental genotoxicity assessment. *P. furfuracea* lichen specimens were collected from 10 different *Pinus* species, in every 5 km, starting from around an iron-steel factory located in the central area of Karabük Province up to Yenice Forest. The impact of the pollution sources such as iron-steel factory, roads and railroads, industry, heavy traffic, and waste treatment plants on the heavy metal accumulation in lichens is known. DNA changes in *P. furfuracea* samples exposed naturally to various polluted sites were analyzed by RAPD to know the influence of the environmental pollution on the hereditary material of the organisms. Twenty-five different primers were tested and 10 yielded clear and reproducible bands. The present study shows the suitability of the lichen samples for the detection of genotoxicity and also provides information about the level of potential genotoxic agents around a steel mill.

Key words: *Pseudevernia furfuracea*; RAPD; Genotoxicity

INTRODUCTION

Lichens are associations of fungi and green algae or cyanobacteria, which are significant for studies monitoring atmospheric heavy metal pollution. Lichens are capable of absorbing elements directly from the atmosphere far above their need and accumulating them in their tissues. Because of their great tolerance to radical environmental conditions they grow in a wide geographical distribution. They benefit from atmospheric deposition as a basic source of minerals and they are perennial organisms. Their morphologies do not show changes according to climate. These features of lichens rank them among the best bioindicators of air pollution (Ölmez et al., 1985; Bargagli, 1995; Garty et al., 1997).

In atmospheric heavy metal pollution studies, generally two basic methods are applied: either physiological effects of heavy metal accumulation are analyzed in the species that live close to the pollutants or clean lichen samples are collected and exposed to the polluted areas to detect physiological responses and bioabsorption, the latter method known as a transplant study. The lichen species which live close to the polluted areas absorb and accumulate metal pollutants for years because of their perennial characteristics. Therefore determining bioaccumulation and physiological effects of lichens exposed to natural habitats provide more realistic data about the biological effects of pollutants than transplant studies conducted in the region. The frequently used bag technique has both advantages and disadvantages. While the ability to monitor the increase in the amounts of pollutants and to know the duration of exposure are advantages, absorption differences due to climatic and environmental changes, lichen material loss because of precipitation, wind and also lack of standardization are regarded as the disadvantages of this technique (Fernandez et al., 2000).

Lichens are widely used in biomonitoring studies of air pollution, either as bioindicators of air quality or as bioaccumulators of atmospheric deposition. Lichens provide a number of advantages in biomonitoring over instrumental monitoring since they can accumulate most elements, including heavy metals, and are usable at low expense. In contrast to physical and chemical methods, biological methods allow the direct assessment of the genotoxic potential of air stressors. These advantages feature biological data for the estimation of environmental impact and the potential impact on other organisms, including humans (Piraino et al., 2006; Aras et al., 2010).

The evaluation of early-warning responses at the subcellular level may be very useful for the prevention of long-term ecological damage (Kalpaxis et al., 2004), and inducement of DNA damage is considered to be one of the earliest signs of environmental disturbance. Moreover, as a biomarker endpoint, it gains importance considering the possible consequences that genotoxicity can have on organisms and populations inhabiting contaminated environments (Depledge, 1998; Belfiore and Anderson, 2001; Jha, 2004; Stambuk et al., 2009).

Heavy metals, including essential ones, are genotoxic agents after exposure to certain dosages or for long periods. They cause damage like additions, deletions and point mutations on nucleic acids. Some of this damage might be repaired by the mutation repair mechanisms but some of it might remain. In recent studies, it has been shown that changes in DNA due to genotoxic agents could be analyzed with DNA fingerprinting methods like RAPD (Random amplified polymorphic DNA) and Amplified Fragment Length Polymorphism (AFLP) (Labra et al., 2003; Liu et al., 2005). Lichens have been used in bioindication of genotoxic heavy metals for a long time. However in these studies, physiological parameters are used to evaluate environmental damage to lichens such as photosynthesis,

respiration, chlorophyll content and degradation, membrane integrity and stress-ethylene production (Liu et al., 2005). On the other hand, although lichens are considered very good bioindicators of air pollution and numerous studies have been conducted about their heavy metal accumulation capacity, only two studies conducted in our laboratory have been published to our knowledge, about their putative genotoxicity indicator capacity (Aras et al., 2010; Cansaran-Duman et al., 2011).

The present investigation involved the collection of ten *P. furfuracea* (L.) Zopf samples growing on *Pinus sp.* from 10 sites in and around the Karabük Steel Mill area, Karabük, Turkey and aimed to show genotoxic effects of various contaminants including heavy metals on *P. furfuracea* (L.) Zopf qualitatively by RAPD analysis. Results of the current research would provide an idea about assessment of the genotoxic potential of air pollutants around the Karabük Steel Mill in Karabük, Turkey which might provide insights for future genotoxicity monitoring studies with lichens in polluted areas.

MATERIAL AND METHODS

Area of study

The area of study is located between 40°59'03''-41º00'00''N, and 32°05'55''-32°18'15''E in the western part of the Black Sea region, and belongs to district of Yenice in the province of Karabük. From the Yenice Forest to the Karabük Steel Mill, 10 samples of *P. furfuracea* (L.) Zopf were collected every 5 km (Table 1, Figure 1). The control sample collected from the Yenice Forest was not exposed to any kind of contamination. Yenice Forest is noted for its humid and rainy climate. The annual mean temperature is 8.8°C, relative humidity is 76.2% and total precipitation is about 1200 mm. Numerous industrial activities, such as coal, iron, steel and cement, and an active intercity highway are present in the area. Besides, a railroad for shipping of coal and raw materials has existed in Karabük for many years. The regions of activity indicated are very close to the city center of Karabük, where coal is generally consumed instead of natural gas during the winter. According to the local environmental unit parameters, SO_2 and PM_{10} contamination increases to harmful levels in wintertime. A rich and large forest ecosystem in terms of species is also present in the city, which must be protected according to the World Wildlife Fund (WWF).

Table 1. The 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, Hacıömerler 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
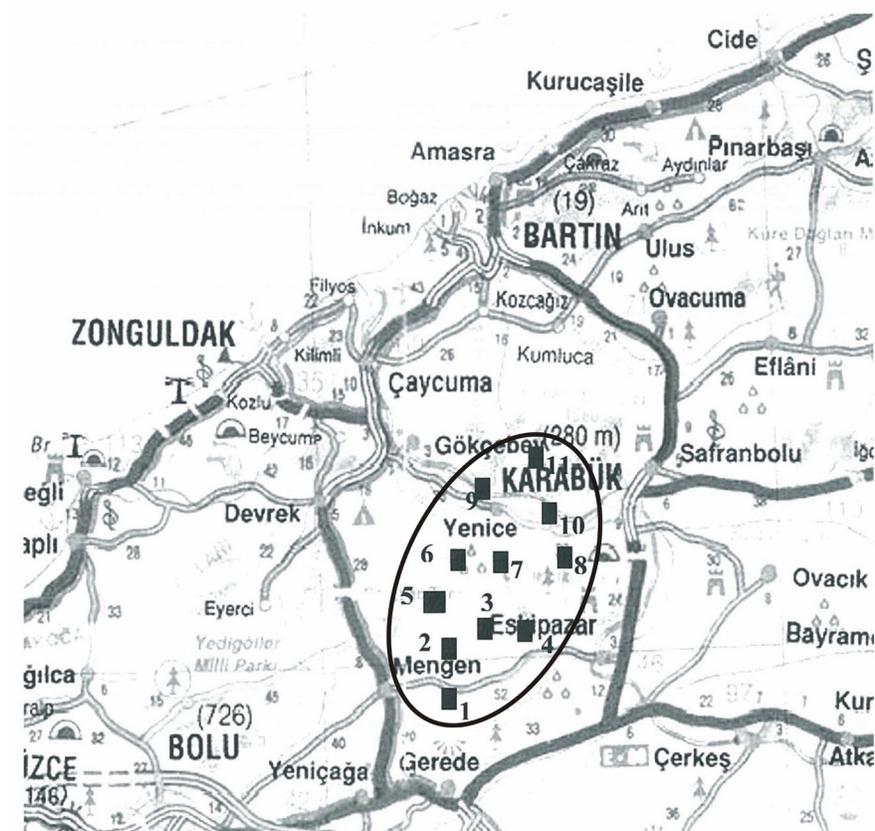
Figure 1. Regional map of the study area.

Lichen material

P. furfuracea samples were collected from 10 different *Pinus* species that were located at various distances to the pollution sources (leg., det. Dr. D. Cansaran Duman) (Table 1). Each territory was determined as a station and was numbered from 1 to 10. The samples collected from a few trees from each station were homogenized before the analysis.

In the laboratory, lichen samples were cleaned from contaminants with the aid of a binocular microscope (Olympus) and consecutive washings with distilled water before DNA isolation.

DNA extraction and RAPD analysis

DNA extraction from the lichen samples was performed according to the protocol improved for various lichen species by Aras and Cansaran (2006). Concentrations of the extracted DNA samples were measured at 260 nm and the purity was estimated by measur-

ing the 260/280 nm absorbance ratio by nanodrop (NanoDrop ND-1000 Spectrophotometer). The DNA concentrations were approximately in the range of 800 ng/μL to 2100 ng/μL and 260/280 nm ratios ranged from 1.76 to 1.91. The integrity of the DNA samples was also evaluated by agarose gel electrophoresis.

PCR was performed in a reaction volume of 25 μL containing 200 ng genomic DNA, 2.5 μL 10X reaction buffer, 2.5 mM $MgCl_2$, 20 μM dNTPs, 0.2 μM primer, 0.5 U Taq polymerase (Promega) and ddH_2O, which was added to the standard volume. The PCR program had an initial cycle of 30 s at 94°C for the denaturation step, followed by 35 cycles of 1 min at 33°C for annealing, 1.45 min at 72°C for extension steps and a final extension step of 8 min at 72°C was also applied. Primer screening for RAPD analysis was performed using 25 primers and 10 of them amplified clear and reproducible bands. Amplified samples and a 100 bp DNA marker were loaded on 1.6% agarose gels containing 0.5 μL/mL ethidium bromide, and run at 5 V/cm for 4 h. Samples were visualized and analyzed under UV light by Gene Genius Bioimaging System, Syngene. The sequences of the 10 decamer primers used in the study are shown in Table 2.

Data analysis for RAPD fingerprints

Data bands appearing in the control sample are considered the criterion for judgment in the analysis of RAPD (Figure 2). Polymorphism observed in RAPD profiles included the disappearance of a normal band and appearance of a normal band in comparison to RAPD profiles in the control (Atienzar et al., 1999; Liu et al., 2005) and the total number of band changes is given in Table 3.

Estimation of genomic template stability

Genomic template stability (GTS) values were also calculated according to results of RAPD analysis. GTS implies a qualitative measure showing the obvious change to the number of RAPD profiles generated by the lichen samples collected from the polluted areas, in relation to profiles obtained from the control lichen sample. GTS % was calculated as $GTS = \left(1 - \frac{a}{n}\right) \times 100\%$, where a indicates the RAPD polymorphic profiles in each sample, and n is the number of total bands in the control. Changes in the RAPD patterns were expressed as a decrease in GTS values (Table 4).

RESULTS AND DISCUSSION

In the current study the genotoxic effects of various environmental pollutants were tested with the samples collected from their natural habitats from the Karabük region. Twenty-five different primers were tested for RAPD analysis (Table 2) and 10 of the primers yielded clear and reproducible bands. The changes in band numbers in the form of appearance and disappearance of the bands were obvious (Table 3) as can be observed in Figure 2.

Among the primers used, Tube A03 showed the highest polymorphism while Tube A02 and Tube A04 showed monomorphic band patterns. The ratio of polymorphism was calculated as the number of polymorphic bands/total bands x 100 (Table 3). According to this calculation, the appearance of a new band (a) or disappearance of an existing band in comparison with
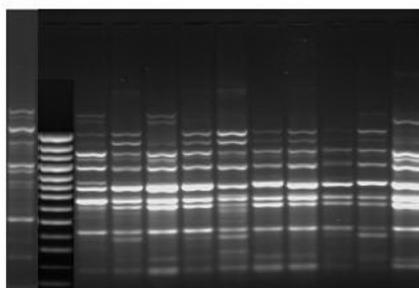
Table 2. The sequence of the primers used in the study.

Primer name	Primer sequence (5'→3')
TUBE A 01	CAGGCCCTTC
TUBE A02	TGCCGAGCTG
TUBE A03	AGTCAGCCAC
TUBE A04	AATCGGGCTG
B 389	CGCCCGCAGT
OPO 04	AAGTCCGCTC
OPA 13	CAGCACCCAC
OPA 18	AGGTGACCGT
OPB 17	AGGGAACGAG
OPF 05	CCGAATTCCC

Table 3. Changes of total bands in control, polymorphic bands and varied bands in samples.

Primer	C	S1 a	S1 b	S2 a	S2 b	S3 a	S3 b	S4 a	S4 b	S5 A	S5 b	S6 a	S6 b	S7 a	S7 b	S8 a	S8 b	S9 a	S9 b	S10 a	S10 b
Tube A01	5	1	0	0	0	1	0	0	0	1	1	1	0	2	0	1	0	0	0	0	1
Tube A02	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tube A03	8	0	1	0	1	0	0	0	0	0	0	0	1	1	1	2	0	0	1	2	0
Tube A04	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
B 389	11	0	0	0	0	0	1	1	0	0	0	2	0	0	0	0	3	0	0	0	0
Opo 04	8	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	1	0
Opa 13	10	0	1	0	1	0	1	0	1	0	1	0	1	0	0	0	3	0	1	2	1
Opa 18	8	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	0	3	0
Opb 17	7	0	0	0	0	0	0	1	0	0	0	1	0	0	2	0	0	1	0	2	0
Opf 05	9	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	2	0
a+b		3		3		4		5		5		6		8		9		9		14	

Primer	TB	PB	PR (%)	a: appearance of new bands,
				b: disappearance of control bands,
				a+b indicates polymorphic bands.
Tube A01	11	6	54.54	S: Sample
Tube A02	7	4	57.14	TB→total bands
				PB→polymorphic bands
				PR→polymorphism ratio
Tube A03	14	9	64.28	
Tube A04	8	5	62.50	
B 389	12	4	33.33	
Opo 04	9	5	55.55	
Opa 13	16	10	62.50	
Opa 18	17	8	47.05	
Opb 17	14	6	42.85	
Opf 05	12	4	33.33	

Figure 2. RAPD profiles generated by OPA 18 (5'AGGTGACCGT 3') primer from *P. furfuracea* exposed to polluted areas around the steel mill in Karabuk. *Lane M* = molecular weight marker (100 bp. ladder); *lane C* = control sample.

control was named as polymorphic. Changes in intensities of the bands were ignored as they appeared in negligible amounts. Samples which were closer to the pollution sources showed considerable polymorphism in RAPD profiles compared to the control sample (Table 3).

In the current study, among 10 stations, Station 1 is close to the pollution sources as it is located near the motorway and railway. On the other hand, Station 10 is closer to the steel milland cement factory than the other stations. Apart from Station 10, there are other stations close to potential pollution sources and it is estimated that these areas also have pollution risk. Stations number 2, 4 and 5 are also close to the motorway. Stations 8 and 9 are the closest stations to the steel mills after station 10. The Control Station is very close to Station 7 and both of them are located far away from the allocation units. In another study conducted in parallel with the same samples, some of the heavy metal concentrations were determined and the lowest levels of Mn, Pb, Cr were found in sample 3. Zn and Fe were also found relatively low amounts in sample 3 (Cansaran-Duman et al., 2009).

Heavy metals are a major component of air pollution and many studies have shown that concentrations of absorbed heavy metal elements in lichen samples rise as they get closer to polluted sites like busy motorways and steel mills and also depending on the exposure time. Pilegaard (1978, 1979), Goyal and Seaward (1981), Gailey et al. (1985), Gailey and Lloyd (1986), and Vestergaad et al. (1986) showed in their studies with various lichen species that Pb, Cu, Cd, Mn, Cr, and Ni element concentrations in lichen samples which are close to the iron and steel mills were higher than those far from industrial sites.

Moreover, the differential rate of increase of metal concentration was stated both in samples from transplant studies and in samples exposed to heavy metals for longer periods of time. In the studies where Pb pollution was investigated, Ward (1989) and Al-Chalabi and Hawker (2000) showed that Pb pollution gradually increases in the areas close to motorways. Laaksovirta et al. (1976), Král et al. (1989), Máquas et al. (1990), Kapu et al. (1991), Garty et al. (1997), Scerbo et al. (2002), and many other researchers reported similar results in their studies carried out with lichens. The samples located in bags close to motorways or samples collected from the areas close to motorways have considerably higher Pb concentrations. Furthermore, many studies have been conducted with various types of lichen species including *P. furfuracea*, showing their capacity to absorb and accumulate atmospheric heavy metals in urban areas (Cansaran-Duman et al., 2009). Bari et al. (2001) found a significant correlation between several heavy metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) accumulated in thalli of the lichen *P. furfuracea* (L.) Zopf transplanted for 1 year in a rural site in northern Italy. All these studies prove that lichens are suitable tools for monitoring air pollution.

Despite their high tolerance to heavy metals, accumulated and absorbed metals are not totally harmless for lichens. Many studies have been carried out related to the effects of heavy metals on lichens such as chlorophyll degradation, decrease in photosynthesis, increase in the stress hormone-ethylene, decrease in ATP content, and negative effects on the integrity of the membranes (Liu et al., 2005). In addition to their physiological effects, heavy metals can cause DNA mutations, as they are genotoxic agents as well. Several studies focusing on heavy metal genotoxicity showed that those agents may cause chromosome aberrations, double helix damage, and single and double strand breaks (Tsuda and Kato, 1977; Majone and Lewis, 1979; Jacobson and Turner, 1980; Sugiyama et al., 1991; Anastassopoulou, 2003). PCR based fingerprinting methods provide an efficient tool for the investigation of the mutational changes. RAPD analysis can help determine not only the mutational effects of heavy metals but also the

mutational effects of organic and inorganic genotoxic agents on different organisms (Labra et al., 2003). Fingerprinting methods such as RAPD and AFLP are sensitive, effective, relatively cheap and simple techniques as they can be applied to various organisms directly from their DNA and they help to analyze the whole genome (Savva, 1996, 1998; Conte et al., 1998; Labra et al., 2003; Liu et al., 2005). They are quite useful, especially for pollution studies as they can compare polluted and nonpolluted samples at the same time and in relatively shorter periods (Liu et al., 2005). In the current study, lichen samples both close and far from the pollution sources were compared in order to provide genotoxicity information of mixed pollutants found in the air. The high number of polymorphic bands were observed in the samples taken from areas close to the railways and motorways (sample 1, 4, 5) and also the samples taken from a station near the steel mill (number 10) implies that a significant level of air pollution including an increase in heavy metal contamination is effective in these areas (Figure 1, 2). In accord, the results of the previous study conducted in parallel which displayed high levels of heavy metal deposition in the same samples confirm the elevated levels of environmental pollution in the area (Cansaran-Duman et al., 2009).

Additionally, the results of genomic template stability ratios (GTS) were calculated (Table 4). GTS implies a qualitative measure reflecting changes in RAPD profiles. GTS related to the level of DNA damage, the efficiency of DNA repair and replication (Atienzar et al., 1999) could explain the appearance and disappearance of bands. The lowest values were obtained in samples no. 10, 9 and 8. Generally in samples no. 8, 9 and 10, the lowest GTS values were obtained which might imply the sensitivity of lichens to genotoxic stressors near the steel mill. Previous studies have also indicated that mutations, chromosomal rearrangements and other DNA lesions could be the reason for the variation in RAPD band patterns. It was demonstrated that changes in RAPD profiles induced by pollution could be regarded as modifications in genomic template stability. Therefore, as stated by Atienzar et al. (2000) "a high level of DNA damage does not necessarily decrease the genomic template stability because DNA repair and replication are inhibited by the high frequency of DNA damage".

Table 4. Genomic template stability (GTS) ratios of samples.

Name of the sample	GTS ratios (%)
S1	96.0
S2	96.0
S3	94.6
S4	93.3
S5	93.3
S6	92.0
S7	89.3
S8	88.0
S9	88.0
S10	81.3

As a result, even a one-band change and a slight decrease in GTS might be meaningful in an RAPD assay. It was also indicated that genomic template stability, a qualitative measure of the genotoxic effect, could also be directly compared with variations in other parameters. It has been reported that GTS was more sensitive than soluble protein content in root tips and was at least as sensitive to root growth (Liu et al., 2005).

Labra et al. (2003) compared the sensitivity of DNA marker techniques such as Restriction Fragment Length Polymorphism (RFLP), RAPD and AFLP to the classical genotoxic

tests like comet and micronucleus assays that were able to detect temporary DNA changes, which, unlike mutations, may not become permanent alterations in DNA.

In conclusion, the RAPD method used in the study provides a qualitative method in which the type of DNA damage can only be speculated. AFLP, on the other hand, might provide more reliable data but still provide a qualitative means of analysis. In order to obtain quantitative data, more specific methods must be developed to analyze the products. Nonetheless, after optimizing the conditions stringently, RAPD analysis can still be used as an investigation tool to detect DNA alterations in environmental toxicology studies. The method also provides an early warning system with a higher sensitivity than the conventional techniques.

The aim of the current study was to show the genotoxic effect of mixed environmental pollutants on organisms under natural conditions. Although the samples in this study were collected from their original localities, which are very close to each other, still some other parameters might be effective for the recorded polymorphism. In this regard, induction of DNA band changes with one kind of a stressor found in air pollution might provide evidence of the genotoxic potential of air pollution. In a previous study, a controlled experiment with one type of heavy metal treatment was also conducted in our laboratory in order to show the effects of a genotoxic agent in a controlled environment (Aras et al., 2010). In the study a clean sample of *P. furfuracea* collected from the Yenice Forest was exposed to various doses of Pb in different time intervals. Two out of four primers yielded clear and reproducible bands in heavy metal treated samples. Amplification with the primer Tube A01 revealed that the samples which were exposed to Pb for 18, 24 and 48 h were different from the other series after the amplification with Tube A01. Although the polymorphism percentages were not calculated in the study obvious band changes were visualized especially 24 and 48 h after of Pb treatments. Results of the study displayed that even just one kind of a stressor (Pb) might induce DNA changes in *P. furfuracea* samples in a controlled environment (Aras et al., 2010). Thus, it might become easier to explain the level of polymorphism recorded in the current study when we consider the complexity of the atmospheric environment in which various gases and contaminants exist together.

The Cansaran-Duman et al. (2011) study on genotoxic contamination was monitored by RAPD analyses on *Evernia prunastri* lichen samples at different polluted sites in Karabük. A clear genotoxic influence is demonstrated in *E. prunastri* exposed naturally to railways, motorways and steel mills in Karabük. DNA damage assessed by RAPD analyses in *E. prunastri* near a steel mill in Karabük, Turkey exhibited similar results as the technique used in this study (Cansaran-Duman et al., 2011). The highest values of DNA damage were obtained by both lichen species (*E. prunastri* and *P. furfuracea*) exposed naturally to various polluted sites around the steel mill in Karabük. Accordingly at this site, an increase in genotoxic effect was detected compared with the steel mill. Cansaran-Duman et al. (2011) recorded high deterioration of DNA integrity in *E. prunastri* exposed at the same site in 2005, while data presented here showed medium DNA damage in *P. furfuracea* exposed at the same location. But, a direct comparison of results may be difficult due to differences in the biology of the employed species, different season and duration of exposure and because of constant alternations in quality and quantity of air pollution. However, the level of the DNA damage measured in *P. furfuracea* was lower in *E. prunastri* in the same region. Although these measurements cannot provide detailed data on a variety of specific chemicals or their interactions responsible for genotoxic impact during the investigated period, they are therefore used here only as an indication of polluted status (Stambuk et al., 2009). This may be due to adaptive mechanisms developed in

lichen species continuously inhabiting polluted environments. Nevertheless, further studies focused especially on the influence of other ecological factors on DNA damage are needed.

Air pollution represents a threat both to the environment and to human health, and it is estimated that millions of tons of toxic pollutants are released into the air each year. Mines, metal foundries, cement factories, power plants, compost factories, waste treatment facilities and busy motorways are the examples of emission sources of genotoxic agents, especially heavy metals like Cr, Ni, Mn, and Pb. Furthermore, using coal for heating in cities causes a considerable amount of heavy metal pollution. As a result detection of DNA polymorphism by RAPD analysis could be used as an investigation tool for environmental toxicology after optimizing the conditions stringently. The present study shows the suitability of the lichen samples for the detection of genotoxicity and also provides information about the level of potential genotoxic agents around a steel mill.

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