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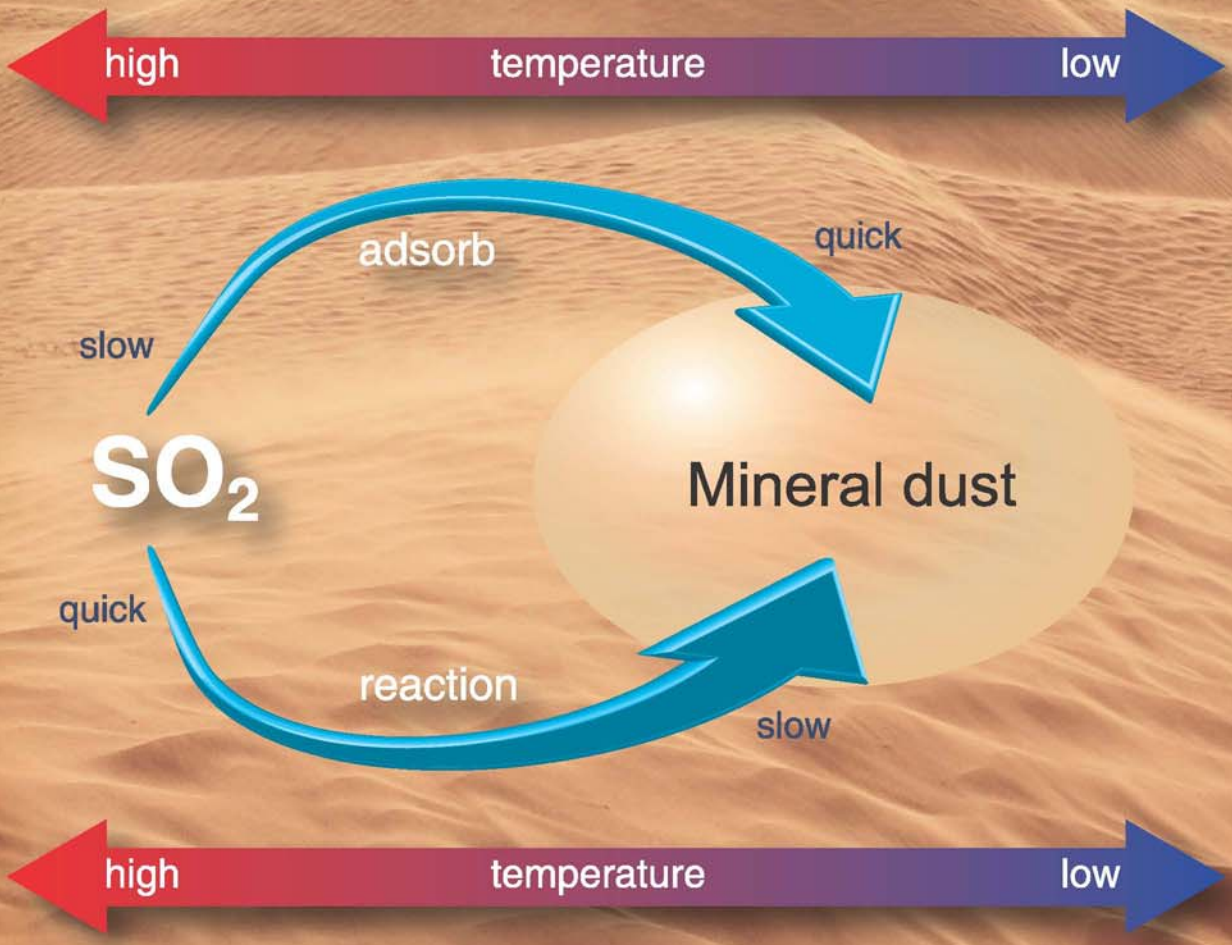

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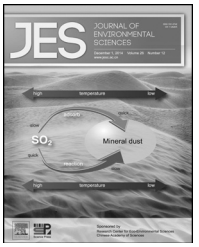

Persistent pollutants and the patchiness of urban green areas as drivers of genetic richness in the epiphytic moss *Leptodon smithii*

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ABSTRACT

We determined genetic variation and metal and polycyclic aromatic hydrocarbon concentrations in *Leptodon smithii* moss collected in holm oak stands at cities, outskirts and remote areas of Campania and Tuscany (Italy) to investigate if anthropogenic pressure (pollutant emissions and land use change) affects moss genetic richness. In both regions, metal and polycyclic aromatic hydrocarbon concentrations reflected the trend urban > outskirts > remote areas, excepting Tuscany remote site. In both regions, the moss gene diversity increased from urban to remote areas. The findings suggest the extent and the fragmentation of urban green areas, as drivers of moss genetic richness.

Introduction

Urbanisation is an important driver of global land transformations. The progressive conversion of lands from wild and agricultural to urban and suburban areas determines habitat loss and fragmentation, environmental pollution, soil compaction and paving and changes in local climate (the "urban heat island"; Oke, 1995), with detrimental effects on biological diversity. The species occurring in urban landscapes are the combination of those colonizing novel habitats formed by urbanisation and those remaining after local extinction. As a rule, the management of urban landscapes, the environmental pollution and the extent and connectivity of urban green areas are deemed as the main factors affecting the composition/richness of species and their genetic variability in urban ecosystems. Organisms with high dispersal capability often exhibit a lower decline from rural to urban areas. Thus, communities may show a large similarity degree among cities, known as the "biotic homogenization" (Mc Kinney, 2006). This effect is usually ascribed to the common characteristics of urban environments regardless of the surrounding biome and to the transportation of similar subsets of species across geographical boundaries. Detecting the likely origin of species living in urban ecosystems will allow us to infer the broad scale processes which have led to species richness and genetic diversity in urban ecosystems (Sattler et al., 2011).

Most moss and lichen species have an extraordinary dispersal capability and can colonize the Earth's remotest regions; they have no roots and waxy cuticles, they are largely dependent on atmospheric deposition for their metabolism and the different species show a different sensitivity to atmospheric phytotoxic pollutants (Bargagli, 1998; Sim-Sim et al., 2000; Spagnuolo et al., 2011). Thus,

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based on these features, during the last century the biological diversity (mainly estimated as species richness) of cryptogam communities has been used as an indication of air quality in urban environments (Le Blanc and De Sloover, 1970). During the last decades the concentrations of some "conventional" phytotoxic pollutants, such as SO_2, are decreasing; however, other atmospheric pollutants, such as trace metals and polycyclic aromatic hydrocarbons (PAHs), are mostly present in urban environments, largely emitted by circulating vehicles, affecting the chemical composition, physiology, and molecular diversity of lichens and mosses. At present, airborne particulate matter, *e.g.*, $PM_{2.5}$ with associated metals and PAHs, is among the most harmful pollutants (EEA, 2011). Due to technical difficulties and high costs, only some of these pollutants are monitored by physico-chemical approaches. Thus, the analysis of native moss tissues can provide a time-integrated evaluation of atmospheric deposition of many persistent pollutants not measured by automatic devices, evidencing spatial differences and temporal trends (Giordano et al., 2010, 2013; De Nicola et al., 2013b; Harmens et al., 2013; Spagnuolo et al., 2013). However, to make reliable comparisons among pollutant concentrations in moss samples of the same species collected in different urban sites, analysed samples must be the same age (*i.e.*, exposure time), and must have the same growth rate and form of growth (Zechmeister et al., 2003; Aboal et al., 2010). Thus, assuming that in an urban area several environmental variables such as precipitation, temperature and radiation are rather homogenous, some authors suggest taking into account the intraspecific genetic variation in biomonitoring studies, because it may affect adaptation mechanisms to airborne pollutants (Boquete et al., 2013).

In bryophyte island populations most species are clones, *i.e.*, populations with high local abundance, and often show a minimum genetic variation; according to Cronberg (2002) this variation increases with the population age. However, moss cross-transplantation experiments between polluted and unpolluted sites (Tabors et al., 2004; Boquete et al., 2013) indicate that mosses continuously exposed to high deposition of heavy metals probably undergo a genotypic adaptive response. Therefore, the molecular diversity of mosses in urban ecosystems is probably affected by historical as well as recent events such as atmospheric pollution.

A previous study on the moss *Leptodon smithii* F. Weber and D. Mohr (Spagnuolo et al., 2007), a facultative epiphytic species growing on barks of *Quercus ilex* L. (a tree of the Mediterranean climax vegetation, very common also in many urban environments), showed intra-population genetic variations among samples collected in urban, extra-urban and remote areas in southern Italy. As a rule, the molecular diversity decreased along an ecological/environmental gradient, with the lowest values in the sites with the highest habitat disturbance and fragmentation. These results suggested that *L. smithii* spores probably have a limited dispersal range and the moss population age and size, together with the connectivity of urban green areas are the main drivers of its genetic diversity. A subsequent study (Spagnuolo et al., 2009) along the same gradient of anthropogenic pressure showed an inverse relationship between the moss genetic variation (given as Nei's gene diversity; Nei, 1987) and the total metal load in moss tissues. Thus, in urban environments the genetic diversity of epiphytic mosses seems to be affected by relatively recent and ongoing processes such as the atmospheric deposition of pollutants. It is known that bryophytes can develop ecotypic variants adapted to survive in polluted environments (Shaw, 1994).

In the framework of a wider project aimed at evaluating the role of urban vegetation as interceptor and accumulator of persistent atmospheric pollutants, we determined genetic variations and pollutant concentrations in populations of the epiphytic moss *L. smithii* collected in holm oak stands at urban areas, outskirts and remote areas in two Italian regions (Campania and Tuscany) with different climatic and environmental conditions (De Nicola et al., 2013b). In this article, we tested the hypothesis that anthropogenic pressure, evaluated as pollutant emissions and land use change, affects genetic richness in native moss populations.

1. Material and methods

1.1. Study areas

L. smithii is a pleurocarpous, facultative epiphytic moss, rather common on tree bark of humid and close woods in almost all continents (Nelson, 1973). In Italy this species grows on trunks of *Q. ilex* L., a common tree of Mediterranean woods, widely used in the landscaping of many Italian urban parks and gardens.

Moss samples were collected in six holm oak stands, three in Campania and three in Tuscany (Table 1): the urban environments of Naples (C-1) and Siena (T-1), the relative outskirts (C-2 and T-2) and two remote areas (C-3 and T-3). All sampling areas have a typical Mediterranean climate (slightly warmer and moister in Campania). The municipalities of Siena and Naples occupy the same area but have different population sizes (54,500 *vs.* 957,600 inhabitants respectively); moreover, the province of Naples, whose area is one third that of Siena (1171 *vs.* 3821 km^2) has a much higher number of industrial plants, motor vehicles and other anthropogenic sources of atmospheric pollutants. The urban sampling site C-1 was located in a wide and historical park that houses the former Bourbon Royal Palace and served as a hunting game reserve for the kings of Naples. It is isolated from agricultural and forest areas and is bordered by heavily trafficked roads; the dominant trees are holm oaks, chestnut trees, magnolias and elm trees. The holm oak stand of outskirts (C-2) was located in an ash ring crater (250 ha wide), a State Nature Reserve covered by dense forest vegetation and with small permanent water bodies which contribute to more favourable conditions for vegetation during summer. The C-3 remote site was in a *Q. ilex* forest located in a protected natural area at about 100 km from C-1. In Siena the urban site (T-1) was located in a private garden park with large holm oak trees, near a heavily trafficked road. This small urban park (Table 1) is connected to suburban agricultural and forest areas. The site T-2 was about 3.5 km far from Siena's city centre; it has minimal air pollution and anthropogenic disturbance and is surrounded by agricultural areas and holm oak forests. The remote site in Tuscany (T-3) was located in an ancient forest with low density of *Q. ilex* trees growing near the sea coast in very xeric conditions (yearly precipitations about 650 mm, with 5 months of water deficit; Bussotti et al., 2002).

1.2. Metal and PAH analyses

At each site, in spring 2009, small green clumps of the epiphytic moss *L. smithii* were collected from several holm oaks, at a height of 1–2 m above the ground. All clumps were carefully mixed to obtain a composite sample. At the basis of the same holm oak trees, after litter removing, surface soil subsamples (0–5 cm) were also collected by a plastic cylindrical corer and mixed to obtain a composite soil sample, in order to define the contribution of soil particles to the total metal concentrations in mosses.

Table 1 – Geographical coordinates of the sampling sites, Q. *ilex* woodland wideness, index of frequency/cover for *L. smithii*, sporophyte occurrence, and Nei's gene diversity (mean values ± S.D., *n* = 30).

Site acronyms	Coordinates	Woodland wideness (km^2)	Index of frequency/cover*	Sporophyte occurrence	Nei's gene diversity
C-1 (urban)	40°52′N–14°15′E	1.34	3	+	0.823 ± 0.033
C-2 (outskirts)	40°50′N–14°09′E	2.50	3–4	++	0.903 ± 0.029
C-3 (remote)	40°27′N–15°18′E	4.00	5	++	0.947 ± 0.017
T-1 (urban)	43°28′N–11°17′E	<0.05	3	+	0.600 ± 0.084
T-2 (outskirts)	43°18′N–11°17′E	0.40	3	+	0.784 ± 0.048
T-3 (remote)	42°50′N–10°46′E	4.5	5	++	0.897 ± 0.036

+low frequency, ++: high frequency.
* Le Blanc and De Slover, 1970.

In the laboratory, moss samples were cleaned from dead or extraneous materials under a binocular and soil samples were sorted to remove gravel and larger organic debris, homogenised, meshed at 2 mm, and dried. To detect metal concentrations, oven-dried (75°C, until constant weight) moss and soil samples were powdered in a planetary ball mill with an agate pocket (PM4, Retsch, Germany) and subsequently mineralised with the addition of 65% HNO_3 and 50% HF (2:1, *V*/*V*) in a micro-wave oven system (Ethos, Milestone, USA). Sample solutions were analysed by ICP-AES (Elan 6000, PerkinElmer, USA, for the detection of Al, Cr, Cu, Fe, Ni, Pb, V and Zn concentrations) and GF-AAS (AAnalyst 100, PerkinElmer, USA, for the detection of Cd concentrations). The percentage recoveries of each element in standard reference material (CRM 482, SRM 1572 and 1573) were used to check the accuracy of analytical procedures.

The PAH concentrations in moss samples were determined after the extraction through 3 sonications (Sonicator XL2020, Misonix Inc., USA) in a mix dichloromethane: acetone (1:1, *V*/*V*). The extracts were vacuum rotary evaporated, filtered through PTFE filters (0.2 μm pore size) and dried under a gentle nitrogen stream, as reported in De Nicola et al. (2013a). All the samples were added with of a mix of internal standard, diluted with cyclohexane and analysed using a gas cromatograph coupled with a mass spectrometer equipped with a capillary column 30 m × 0.25 mm i.d. × 0.25 μm film thickness (Agilent HP5890 and HP5971 with column HP-5MS, Hewlett Packard Inc. Agilent Technologies Inc., USA). Carrier gas was helium at flow rate of 1.11 mL/min. Oven temperature started at 70°C, with a ramp rate 20°C/min, held to 280°C for 24 min. Selected ion monitoring (SIM) of acquisition was used. Phenanthrene (Phen), fluoranthene (Flt), pyrene (Pyr), benzo[a]pyrene (B[a]P), indeno[1,2,3-c, d]pyrene (IP), and benzo[g,h,i]perylene (B[g,h,i]P) concentrations were measured after the performance of calibration curves for each PAH. The concentration of each compound was corrected for the percentage recovery (about 70%) of deuterated PAHs at known concentrations added before the extraction. The detection limit ranged from 0.001 to 0.003 ng/μL depending on each compound. Concentrations of metals (in mosses and soils) and of PAHs (in mosses) were reported as average of three replicates, on a dry weight basis. Both moss metal and PAH concentrations were normalized from 0 to 1 in relation to the maximum concentration measured in each region and summed to obtain total metals ($n\Sigma_M$) and PAHs ($n\Sigma_{PAH}$).

1.3. Intraspecific biodiversity

At each site, three different *Q. ilex* trees were selected, choosing those with higher *L. smithii* cover values. According to the different situations occurring at each site, two trees were within 10–15 m from each other, and the third was at about 100 m from the other two. Small moss clumps were collected from each tree, every 15–20 cm along the trunk, and ten shoots with similar size, per tree, were selected for DNA extraction. Thus, molecular analysis was performed on 30 shoots per site, for a total of 180 shoots. The shoots were cleaned, DNA-extracted and amplified through Polymerase

Table 2 – Mean metal concentrations (mg/kg dry weight) ± SE measured in moss from holm oak woodlands of Campania and Tuscany located in the urban area of Naples and Siena (C-1 and T-1), in the relative outskirts (C-2 and T-2) and in the remote areas (C-3 and T-3).

	Al	Cd	Cr	Cu	Fe	Ni	Pb	V	Zn
C-1	10724.89	0.48	15.89	60.65	7920.64	8.18	69.71	2.42	247.85
	±134.82	±0.04	±0.65	±1.13	±164.56	±0.22	±2.18	±0.07	±7.64
C-2	3617.45	0.05	3.83	15.35	2858.09	1.21	12.73	0.79	86.42
	±596.49	±0.03	±1.27	±1.60	±582.32	±0.48	±3.36	±0.14	±6.61
C-3	3913.73	0.26	5.99	7.25	2378.68	1.34	2.70	0.79	70.45
	±44.73	±0.01	±0.74	±0.34	±73.99	±0.33	±0.46	±0.04	±2.85
T-1	6776.33	0.36	22.47	52.76	5914.98	9.40	68.66	1.67	158.86
	±458.66	±0.02	±1.05	±3.66	±411.96	±0.77	±5.21	±0.13	±10.96
T-2	3069.40	0.14	7.06	23.07	2346.64	3.81	6.26	0.75	80.90
	±161.39	±0.00	±0.42	±1.00	±126.97	±0.29	±0.18	±0.05	±3.67
T-3	6330.27	0.74	21.30	13.65	6714.84	15.66	32.05	2.57	147.50
	±349.17	±0.14	±2.44	±0.98	±595.83	±1.65	±5.90	±0.17	±13.34

Chain Reactions (PCR) by each ISSR primers (CHR: 5′-CACAC ACACACACAYG-3′; DAT: 5′-GAGAGAGAGAGAGARC-3′; HAD: 5′-CTCCTCCTCCTCRC-3′; 17: 5′-CAGCACACACACACACAC-3′; 18: 5′-GTGCACACACACACACAC-3′), following the protocol reported in details in Spagnuolo et al. (2007). PCR was carried out twice for each sample to confirm the reproducibility of banding patterns and exclude possible artefacts. A total of 25 reproducible bands were scored; then, the banding profiles of all individuals were translated into a binary data matrix (haplotype matrix) and analysed by Arlequin 3.1 to calculate Nei's gene diversity (Nei, 1987) reported in Table 1.

2. Results

Average metal concentrations in native moss *L. smithii* from urban areas, outskirts and remote sites of Campania and Tuscany are given in Table 2. All samples, and especially those from the two urban environments and from the remote site in Tuscany (T-3), had high concentrations of litophilic elements (such as Al, Fe) and of most other analysed metals. Mosses from urban site C-1 showed the maximum concentrations for all the nine analysed metals, and samples from urban site T-1 showed five maxima (Al, Cr, Cu, Pb and Zn) and overall, high values of the other elements; accordingly, mosses from urban sites showed the highest $n\Sigma_M$ (Fig. 1). Four maxima (Cd, Fe, Ni and V) and high values of other metals were observed in mosses from T-3, showing indeed $n\Sigma_M$ similar to T-1 (Fig. 1).

The metal concentrations in topsoils (Table 3) reflected the different lithology of the two regions (*e.g.*, higher Al, Fe and V concentrations in soils from Campania and higher Cr and Ni concentrations in those from Tuscany) as well as the impact of anthropogenic pollutants in urban environments, such as highlighted by the concentrations of Cu, Pb and Zn in C-1 and T-1.

Average PAH concentrations in native moss from sampling sites in Campania and Tuscany are reported in Table 4. Mosses from Naples (C-1) and its outskirts (C-2) showed higher concentrations of PAHs than samples collected in Tuscany (Table 4). In both regions, the concentrations of most investigated PAHs reflected the trend urban > outskirts > remote sites, with the exception of remote site T-3, where average PAH values are in the same range or slightly higher than those in mosses from the outskirts T-2. A major presence of PAHs in moss from urban sites was observed with four maxima in C-1 and six in T-1, accordingly with the highest $n\Sigma_{PAHs}$ (Fig. 1).

In both regions, comparisons between the sum of normalised values of metal and PAH concentrations in mosses from the sampling sites and their genetic variation based on ISSR banding profile (within each site, given as Nei's gene diversity in Table 1) show a clear increase of the moss genetic diversity along the transect urban-outskirts-remote sites (Fig. 1). Although moss population from the remote site in Tuscany (T-3) had high total concentrations of some metals and PAHs, its Nei's gene diversity value was in the same range as that found in samples from C-3, with the lowest accumulation of atmospheric pollutants.

3. Discussion

Few studies have investigated the impacts of land use change (habitat fragmentation) on genetic structure of bryophyte populations, showing opposing results (Pharo and Zartman, 2007).

In agreement with previous outcomes (Spagnuolo et al., 2009), the above results show that the genetic diversity of the moss *L. smithii* growing on the bark of holm oak trees sharply decreases from remote to urban sites. This trend is shown by moss populations from both Campania and Tuscany, although Naples is located in a densely populated province (>8000 inhabitants/km^2) with many anthropogenic sources of atmospheric pollutants, whereas Siena is located in a province with a low population density (<500 inhabitants/km^2) and a territory mostly characterized by agricultural activities and forests. As indicated by Gray (1996), the genetic diversity in plant populations is mainly drawn by both the intrinsic biological properties of the species and the habitat variability. The mosses are sensitive to habitat degradation, as a consequence of their dependence from wet and dry depositions for water and nutrient supplies.

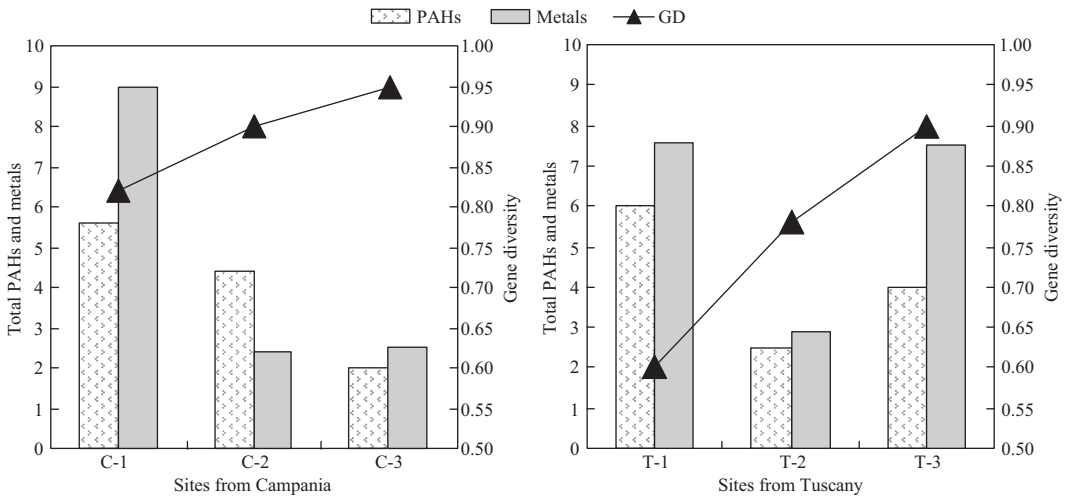

Fig. 1 – Relationships between pollutants (metals and PAHs expressed as $n\Sigma_M$ and $n\Sigma_{PAHs}$, sums of the normalised concentrations to the maximum in each region) and gene diversity (GD) measured in mosses from Campania (a) and Tuscany (b).

The total concentrations of Al and Fe in mosses from the two urban environments indicated an enhanced accumulation of soil particles, likely due to dust re-suspension by wind, vehicular traffic and anthropogenic activities. The accumulation of lithophilic elements in moss samples from the remote site T-3 was probably promoted by the airy vegetation and the soil drought. The high concentrations of metals with a marked anthropogenic origin (Cd, Cr, Cu, Pb and Zn) in the urban sites C-1 and T-1 and in the remote site T-3 (except for Pb), together with the enrichment factors with respect to soil Al (Bargagli, 1998) calculable from our data, suggest an impact of human activity and atmospheric deposition on moss metal accumulations in these sites.

Like metals, also PAHs are released into the atmosphere by natural and anthropogenic sources (Harmens et al., 2013). In order to minimise pollutant contributions from natural sources, we selected PAHs with a predominant anthropogenic origin. In both regions, PAH average concentrations (Table 4) were higher in mosses from urban green areas. However, the amount of total PAHs accumulated in samples from Naples and its outskirts was about one order of magnitude higher than in mosses from T-2, reflecting the huge impact of human activities in this territory compared to that in the Siena territory.

As found for the total metal concentrations, the samples of *L. smithii* from the remote site in Tuscany (T-3) showed higher PAH concentrations (especially for phenanthrene) than mosses collected in the outskirts of Siena (T-2). The lighter PAHs, such as phenanthrene, are mostly found in the gas phase and can undergo a long-range transport; thus, the enhanced deposition of metals and PAHs in the remote holm oak forest (T-3) could be due to emissions from the industrial settlement of Piombino, about 40 km away. However, the soil is not only a sink for metals but also for persistent organic pollutants (Maisto et al., 2004, 2010); indeed, Augusto et al. (2010) found that PAH concentrations in epiphytic lichens were significantly and linearly correlated with PAH concentrations in surface soils. Thus, soil particles entrapped in mosses and especially in the samples from the two urban environments and T-3, probably contributed to the measured concentrations of pollutants.

Our results indicate that the genetic diversity of native epiphytic moss *L. smithii* decreases along gradients of anthropogenic disturbance, consistently with previous studies that have highlighted a lower molecular diversity of bryophytes and vascular plants in human-altered habitats (Patiño et al., 2010; Wyatt, 1992; Cronberg et al., 2005; Wang et al., 2006; Baucom et al., 2005). The Mediterranean region is still poorly surveyed in this respect, although numerous traits, like anthropogenic pressure, climate change and widespread natural sources of phytotoxic compounds, such as active volcanoes, fumaroles and geothermal fields, can affect the vegetation biodiversity. Mechanisms by which mosses accumulate airborne metals (Boquete et al., 2013) and PAHs (Wild et al., 2006) are not completely understood yet; however it is well known that these pollutants, together with many other persistent and volatile organic compounds and phytotoxic gases such as O_3 and SO_2, can alter the molecular and physiological status of cryptogams. The toxic pollutants, accompanied by the physical disturbances of the environment can influence survival, recruitment (by reproduction and immigration rates) and mutation, all of which, in turn, affect the genetic diversity of the exposed populations (Deng et al., 2007).

The low genetic diversity in mosses from the urban green area in Siena (with a much lower atmospheric pollution than in C-1) and the high genetic diversity in samples from T-3 (with relatively high pollutant concentrations), indicate that the history, extent and connectivity of urban green areas are probably the main drivers of intraspecific genetic variations in native moss populations. In fact, the moss of the urban holm oak wood in Naples (C-1)—with its larger extent, longer history, and more suitable microclimatic conditions—shows a higher Nei's gene diversity compared to that of the small urban *Q. ilex* stand in Siena (T-1). Similarly, also in T-3 the woodland structure seems to be the main driver of moss genetic diversity: T-3 is an ancient holm oak woodland, located in a nature Reserve, next to other large woody areas, which provides suitable conditions for moss growth and sexual reproduction that, in turn help to maintain a high level of genetic variation (*i.e.*, high Nei's gene diversity), even if different factors have determined high metal and PAH concentrations in moss.

4. Conclusions

The increasing anthropogenic pressure holds a determinant role in environmental changes; therefore, studying the effects of human impact by an integrated approach, may result useful in order to select reliable ecological tracers of environmental disturbance. In this study, the combined estimation in moss *L. smithii* of selected pollutants and genetic diversity—tracers exhibiting an opposite trend and being influenced by different temporal scales—allows the evaluation of short- and long-term effects of man-driven environmental changes.

A basic concept of biomonitoring implies the comparison of values measured from biological matrices gathered under suspected pollution conditions with "background", *i.e.*, the same plant matrices collected in "reference" areas showing

Table 3 – Mean element concentrations (mg/kg dry weight) in topsoil from holm oak woodlands of Campania and Tuscany located in the urban area of Naples and Siena (C-1 and T-1), in the relative outskirts (C-2 and T-2) and in the remote areas (C-3 and T-3).

	Al	Cd	Cr	Cu	Fe	Ni	Pb	V	Zn
C-1	39066.67	0.29	31.80	163.00	30333.33	14.13	168.00	103.44	191.33
C-2	51400.00	0.10	9.00	25.00	24800.00	5.30	87.00	78.85	78.00
C-3	5749.29	0.43	8.58	29.12	16556.00	17.57	23.43	83.55	81.55
T-1	20833.33	0.13	100.80	62.33	16066.67	32.87	121.33	41.52	102.00
T-2	10700.00	0.14	98.00	40.00	12200.00	26.10	36.00	31.62	75.00
T-3	25312.44	0.03	40.37	5.42	9746.00	26.13	30.25	23.55	42.85

Table 4 – Mean PAH concentrations (μg/kg dry weight) ± SE measured in moss from holm oak woodlands of Campania and Tuscany located in the urban area of Naples and Siena (C-1 and T-1), in the relative outskirts (C-2 and T-2) and in the remote areas (C-3 and T-3).

	Phen	Flt	Pyr	B[a]P	IP	B[g,h,i]P
C-1	47.75 ± 3.0	60.16 ± 1.36	51.06 ± 0.41	22.10 ± 0.15	28.05 ± 0.66	36.42 ± 0.35
C-2	31.77 ± 3.8	29.91 ± 5.4	23.64 ± 4.8	17.64 ± 6.5	37.04 ± 19.7	43.92 ± 20.1
C-3	15.80 ± 0.43	9.97 ± 0.58	7.73 ± 1.1	6.56 ± 4.6	19.11 ± 16.7	22.98 ± 18.2
T-1	29.08 ± 0.37	22.96 ± 1.94	19.64 ± 1.20	6.54 ± 0.84	8.87 ± 1.45	15.15 ± 1.79
T-2	18.52 ± 0.52	13.31 ± 0.79	9.07 ± 0.05	1.86 ± 0.04	2.64 ± 0.02	4.39 ± 0.07
T-3	29.20 ± 0.13	15.35 ± 0.93	11.58 ± 0.39	4.19 ± 0.11	5.79 ± 0.49	6.88 ± 0.35

similar climatic and environmental conditions, where no emission source has a local influence. Even if the reference area in Tuscany was selected far from main motorways, in a protected Reserve, it seems affected by surrounding pollution inputs, and the relative importance of each source determines metal and PAH concentrations in moss.

In the light of the present results, the difficulty in distinguishing among the different inputs, as well as the problem of finding suitable moss species and reference sites in increasingly urbanised and industrialised European countries, represent important challenges to the use of native species in environmental studies. In addition, since the genetic heterogeneity of native mosses can affect the pollutant uptake, the use of genetically homogeneous mosses is recommended. This makes in turn crucial the EU FP7 MOSSCLONE project (www.mossclone.eu), where a genetically homogeneous moss clone, with known physico-chemical characteristics, is being produced, to set up a standard protocol for active biomonitoring.

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