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Depsides and depsidones in populations of the lichen *Hypogymnia physodes* and its genetic diversity

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The aim of this study was to determine the extent and geographical pattern of intraspecific chemical and genetic variability of the lichen *Hypogymnia physodes* by comparing populations from different habitats. We analyzed the secondary lichen substances and their relative concentrations using HPTLC and HPLC in samples collected from sites with different environmental conditions. We identified seven lichen substances: the cortical atranorin and chloroatranorin, and the medullary physodalic, physodic, protocetraric, 3-hydroxyphysodic, and 2´-*O*-methylphysodic acids. The samples were uniform qualitatively, which means that *H. physodes* has only one chemotype. We detected quantitative chemical differences between the samples without any geographical pattern. We investigated 21 samples in order to study the connection between genotypic diversity of populations and geographical distribution. We determined the sequences of five loci (*ITS*, *nucSSU*, *nucLSU*, *mitSSU*, *EF1α*). We found no significant genetic differentiation among populations collected from different areas.

Introduction

Hypogymnia physodes is a common foliose lichen in boreal and temperate forests throughout the northern hemisphere (Smith *et al*. 2009). This species occurs primarily as an epiphyte and preferentially grows on acidic bark and wood, but occasionally occurs on mosses or on soil as well. In Hungary, it is most frequently found on bark of various coniferous and deciduous trees, sometimes also on siliceous rock (Verseghy 1994). Due to its abundance and its moderate sensitivity to sulphur dioxide and heavy metals, *H. physodes* is frequently used as an indicator of air quality (Farkas *et al*. 1985, Farkas & Pátkai 1989, Pfeiffer & Barclay-Estrup 1992, Bruteig 1993, Werner 1993, Bennett *et al*. 1996, Poličnik *et al*. 2004, Rusu *et al*. 2006, Hauck 2008).

Hypogymnia physodes produces several secondary lichen substances, which are located either in the cortex or in the medulla, and are biologically active in varying ecological roles. Cortical depsides play an important role in solar radiation screening, while medullary depsidones function as antiherbivores (Solhaug *et al*. 2009). The depsidone physodic and protocetraric acids have relatively strong antimicrobial effects against numerous microorganisms, including human, animal and plant pathogens, food-spoilage organisms and producers of mycotoxins

(Ranković & Mišić 2008, Ranković *et al*. 2008). Acetone extracts of *H. physodes* exhibit a strong antifungal effect on some plant pathogenic fungi (Halama & Van Haluwyn 2004). The secondary metabolites of *H. physodes* selectively inhibit the intracellular uptake of Cu^{2+} and Mn^{2+}, whereas the uptake of Fe^{2+} and Zn^{2+} is not affected by the lichen substances (Hauck 2008). These impacts are consistent with the ecology of *H. physodes*, i.e., Cu^{2+} and Mn^{2+} might be toxic in ambient concentrations on acidic bark, but Fe^{2+} and Zn^{2+} do not limit the survival of this species. Physodalic acid is the most effective at controlling metal homeostasis (Hauck & Huneck 2007). Physodic, physodalic, and protocetraric acids increase the adsorption of Fe^{3+} (Hauck *et al*. 2007). Atranorin, physodalic, and physodic acids are responsible for allergic reactions to lichens (Thune & Solberg 1980, Cabanillas *et al*. 2006). Secondary metabolites of *H. physodes* at natural concentrations act as antiherbivore agents (Pöykkö *et al*. 2005). Physodalic acid exhibits mutagenicity against *Salmonella typhimurium* (Shibamoto & Wei 1984).

Though several details are known about the secondary substances in *H. physodes*, we found no data on lichen substances of this species in the literature on Hungarian specimens, and only few quantitative results were found (e.g., Czeczuga *et al*. 2000, Białońska & Dayan 2005). Therefore, we studied specimens found in the three major Hungarian lichen herbaria: Hungarian Natural History Museum Herbarium (BP), Eszterházy Károly College Herbarium (EGR), Institute of Ecology and Botany of the Hungarian Academy of Sciences Herbarium (VBI). Many lichen species have different chemotypes, sometimes with specific geographical distributions (e.g., Hale 1962, Moore 1968, Sheard 1974, Filson 1981, Halvorsen & Bendiksen 1982, Culberson *et al*. 1984, Randlane & Saag 1989, Calatayud & Rico 1999). In the current study, we examined the chemical diversity of populations from different habitats (particularly from Hungary) of *H. physodes*, a toxitolerant lichen species, focusing on the possible qualitative and quantitative differences in the production of depsides and depsidones among populations.

In addition to chemical diversity in populations of a lichen species from various geographical sites, there may also be genetic variability, as is reported in the literature for some species (DePriest 1993, Beard & DePriest 1996, Zoller *et al*. 1999, Lindblom & Ekman 2006, Zhou *et al*. 2006). Therefore, we studied the genetic diversity among populations of *H. physodes* as well. The nuclear ribosomal gene complex [comprising *nucSSU* (*18S*) rDNS, *ITS* rDNS (*5.8S*, *ITS1* and *ITS2*), *nucLSU* (*28S*) rDNS, *IGS* rDNS (*5S*, *IGS1* and *IGS2*)], as well as the *mitSSU* rDNS and *EF1α* loci are suitable for population studies in lichens (e.g., Beard & DePriest 1996, Zoller *et al*. 1999, Ekman & Wedin 2000).

Material and methods

HPTLC analyses

We screened 297 *H. physodes* specimens from Hungary and 44 specimens from other countries (mostly European) by HPTLC according to Arup *et al*. (1993) in three solvent systems, most frequently in "B". We mostly analyzed dried herbarium specimens; the oldest one was from 1852. We also collected fresh material between 1997 and 2006. To study within-population chemical diversity, we analyzed specimens from the same populations as well. Additionally, we included infraspecific taxa, varieties and forms of *H. physodes*. We soaked approximately 5 mm × 5 mm thallus fragments in 0.1–0.2 ml acetone for 30 minutes in order to extract the lichen substances. We used pretreated (50 °C for 5 min, CAMAG TLC Plate Heater III), 10 cm × 10 cm thin layer chromatographic plates (Merck, Kieselgel 60 F_{254}). We applied 6–8 µl acetone extracts to each position (5 mm from each other) of the plate using a microapplicator (CAMAG Micro Applicator I). We used *Platismatia glauca* (atranorin), *Cladonia symphycarpia* (atranorin, norstictic acid), *Pleurosticta acetabulum* (atranorin, norstictic acid), and *Heterodermia leucomelos* (atranorin, zeorin) as control specimens. After chromatographic development, we examined the plates under UV light (254 and 366 nm), then sprayed (CAMAG TLC Sprayer) with a 10% sulphuric acid solution and heated at 110 °C for 5–10 minutes. Finally, we cooled the plates to room temperature and studied them under UV light (366 nm).

HPLC analyses

We analyzed 13 samples (Table 1) by HPLC with reversed-phase column and gradient elution to compare their secondary compounds. We followed the standardized method described by Feige *et al*. (1993) with some modifications. We extracted approximately 10 mg air-dried lichen thalli in 1 ml acetone. We added benzoic acid and bis-(2-ethylhexyl)-phthalate to the extraction liquid (acetone) as internal standards (20 mg of each per 1000 ml acetone). Huovinen *et al*. (1985) reported that these internal standards are suitable. We performed the analyses with a Shimadzu High Performance Liquid Chromatograph using LC-10ADvp and LC-10AS pumps, and SPD-10Avp UV/VIS Detector. We used Phenomenex® Prodigy 5 μ ODS-3V column, onto which we injected 20 μl samples. We used two solvent systems: 1% ortho-phosphoric acid in bidistilled water (solvent system A) and 100% methanol (solvent system B). We monitored the elution of the lichen substances at 245 nm. We calculated two kinds of retention indices for the lichen compounds (data not shown): *I* value (Feige *et al*. 1993) and RI (Huovinen *et al*. 1985). Concentrations of substances are given in relative terms (percentage of area: area%).

DNA analyses

We collected 21 lichen samples (Table 2) from different geographical sites for DNA analysis. We extracted genomic DNA from dried lichen thalli using a protocol modified from Zolan and Pukkila (1986). We used 2% sodium dodecil sulphate (SDS) as a lysis buffer. We precipitated the DNA by isopropanol, then washed the pellet with ca. 70%–87% ethanol and dried at room temperature for 1 hour. We resuspended the isolated genomic DNA in 50 μl sterile bidistilled water, and we prepared a 1:10 dilution for PCR amplification. We amplified and sequenced the following loci: internal transcribed spacer (*ITS* rDNA), nuclear small subunit ribosomal DNA (*nucSSU* rDNA), nuclear large subunit ribosomal DNA (*nucLSU* rDNA), mitochondrial small subunit ribosomal DNA (*mitSSU* rDNA), elongation factor 1 alpha (*EF1α*), RNA polymerase II

Table 1. List and characterization of *Hypogymnia physodes* samples analyzed by HPLC.

Sample	Herbarium specimen	Country	Locality	Nearest settlement or pollution source	Number of inhabitants	Distance from center of settlement	Direction from settlement*	Altitude (m)
1	no. 208/a [BP]	Hungary	Budapest, Látó-hegy	Budapest	2000000	5 km	NW	360
2	no. 192/a [BP]	Hungary	Budapest, Hosszúerdő-hegy	Budapest	2000000	10 km	NW	350
3	no. 24/C [BP]	Hungary	Budapest, Remete-hegy	Budapest	2000000	11 km	NW	375
4	EGR4383	Hungary	Zalaegerszeg	Zalaegerszeg	62000	2 km	NW	150
5	EGR1439	Hungary	Salgótarján	Salgótarján	43000	1.5 km	NE	350
6	BP89961	Hungary	Almásfüzítő-felső	Komárom	20000	8 km	E	110
7	EGR4215	Hungary	Mátra, Tarjánka	Visonta	2500	5.5 km	NNE	225
8	EGR4613	Hungary	Bükk, Kolozs-lápa	Felsőtárkány	8000	8 km	NE	605
9	EGR4598	Hungary	Csákpilis	Felsőtárkány	8000	2.5 km	ESE	500
10	EGR4239	Hungary	Egerszalók	Egerszalók	2300	0.5 km	NE	170
11	EGR1444	Hungary	Csévharaszt	Csévharaszt	2000	2 km	SW	130
12	N/A	Romania	Avenul Zgurasti	Gârda de Sus	2200	1 km	NE	850
13	EGR1445	Slovakia	Štrbské Pleso	Tatranská Štrba	1400	4 km	N	1347

* The direction of prevailing winds at the sites is NW.

Table 2. Type of DNA sequences and locality data of *Hypogymnia physodes* samples investigated. Collecting sites set in boldface are characterized in Table 1.

Sample	AFTOL no.*	Coll. or Herb. no.	Collecting site	*ITS*	*nucLSU*	*nucSSU*	*mSSU*	*RPB2* (5–7)	*RPB2* (7–11)	*RPB1* (a–f)	*RPB1* (f–g)	*EF1α*
1	1966	0524/LW	Bükk, Borzlyuk-tető	*	*	*	*	*	*	*	*	*
2		EGR1442	Szilvásvárad		*		*					
3		EGR1444	**Csévharaszt**				*					
4		EGR1445	**Strbské Pleso**				*					
5		EGR4215	**Mátra, Tarjánka-völgy**	*	*		*					
6		EGR4238	Tarnalelesz	*			*					
7		EGR4239	**Egerszalók**	*	*		*					*
8		EGR4240	Bükk, Felsőtárkány	*	*		*					*
9		EGR4241	Bükk, Oldal-völgy	*	*		*					
10		EGR4322	Mátra, Ilona-völgy	*	*		*					*
11		23-08-2005/A	Aggtelek	*	*		*					*
12		0503/LB	Bükk, Jómarci-fertő	*	*	*	*	*	*			*
13		0504/LA	Bükk, Vöröskő-völgy	*	*	*	*	*				*
14		0506/OA	Bükk, Ökrös-fertő	*	*		*	*				*
15		0507/LL	Bükk, Balázs-kő	*	*		*	*				*
16		0508/LW	Bükk, Alsó-Kecskor	*	*		*	*				*
17		0510/LC	**Bükk, Kolozs-lápa**		*		*	*				*
18		0513/LC	Bükk, Kövesdi-kilátó	*	*		*	*				*
19		0514/LB	Bükk, Kövesdi-tető	*	*		*	*				*
20		0515/LK	**Bükk, Csákpilis**		*		*	*				*
21		0520/LA	Bükk, Kolozs-tető	*	*		*	*				*

* Sequences deposited in the database of the AFTOL (Assembling the Fungal Tree of Life) project.

largest subunit (*RPB1*), and RNA polymerase II second largest subunit (*RPB2*). We sequenced *RPB1* and *RPB2* only for the AFTOL project, and not for population studies. The primers used in this study are listed in Table 3. We prepared PCR mix for a 25 μl final volume containing 1 μl 1:10 dilution of genomic DNA, 2.5 μl PCR buffer (with 15 mM $MgCl_2$, Denville), 2.5 μl dNTPs (2mM each, Denville), 2.5 μl BSA (10 mg ml^{-1}), 1.25 μl primers (10 μM), 0.15 μl Taq polymerase (5 U μl^{-1}, Choice Taq, Denville), and 13.85 μl sterile bidistilled water. We prepared a slightly different PCR mix for the protein-coding *RPB1*, *RPB2*, and *EF1α* containing 3 μl 1:10 dilution of genomic DNA, 2.5 μl PCR buffer (Invitrogen), 0.75 μl $MgCl_2$ (50 mM), 2.5 μl dNTPs (2 mM each, Denville), 2.5 μl BSA (10 mg ml^{-1}), 2 μl primers (10 μM), 0.2 μl Taq polymerase (5 U μl^{-1}, Platinum Taq, Invitrogen), and 9.55 μl sterile bidistilled water. PCR ran on a PTC-200 Peltier thermal cycler (MJ Research) under the following conditions for nuclear and mitochondrial ribosomal genes: one initial cycle of 1 min at 95 °C (denaturation), followed by 35 cycles of 45 s at 95 °C, 40 s at 52 °C (annealing), 2–4.5 min at 72 °C (elongation; time depends on fragment size), and one cycle of 10 min at 72 °C as a final extension. For protein-coding genes, there was one initial cycle of 3 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 90 s at 52 °C, 2 min at 72 °C, and one cycle of 10 min at 72 °C. We applied cloning as needed using a TOPO TA Cloning Kit (Invitrogen). We examined PCR products by gel electrophoresis using 1% agarose gel and SYBR Green I (Invitrogen) nucleic acid gel stain, then purified by a Montage PCR Centrifugal Filter Device (Millipore). We performed sequencing reaction in a 10 μl final reaction volume using 3 μl purified PCR product, 0.75 μl Big Dye (ABI PRISM ver. 3.1), 3.25 μl Big Dye buffer, 1 μl primer (10 μM), and 2 μl bidistilled water.

We assembled and edited the sequences using the software package Sequencher 4.5 (Gene Codes Corporation), and aligned them manually in MacClade 4.06 (Maddison & Maddison 2003). In order to determine the similarities of the populations, we ran Maximum Parsimony (MP) quick tests for *ITS* and *EF1α* conducted in PAUP* 4.0b10.

Results

HPTLC

We screened 297 *Hypogymnia physodes* specimens from Hungary and 44 specimens from various, mostly European localities by HPTLC. We identified five lichen substances: protocetraric acid, 3-hydroxyphysodic acid (conphysodic or oxyphysodic acid), physodalic acid, physodic acid, and atranorin. We detected no significant differences among specimens from different habitats, nor among specimens collected at different times. There were no significant chemical differences among the varieties and forms of this species. In some cases, the spots of protocetraric acid and atranorin were absent or very pale due to their very low concentrations. While one of

Table 3. List of primers for the loci sequenced in this study. Oligonucleotide sequences are available at http://www.lutzonilab.net/primers/index.shtml.

Locus	Primers
ITS	ITS1, ITS5, ITS1F, ITS4
nucSSU	nssu131, NS24, NS22; Sequencing primers: nssu897R, nssu1088R, SR7R, nssu1088, nssu634, SR7
nucLSU	LROR, LR5, LR6, LR7; Sequencing primers: LR3R, LR3
mitSSU	mrSSU1, mrSSU2R, mrSSU3R
RPB1 regions A–F	RPB1-A_f, VH6R
RPB1 regions F–G	VH6F_a, RPB1-$G2_r$
RPB2 regions 5–7	fRB2-5F, fRB2-7cR, RPB2-1554R
RPB2 regions 7–11	fRB2-7cF, fRB2-11aR, RPB2-3053R, RPB2-3053bR
EF1α	EF1α-1F, EF1α-1R, EF1α-2218R

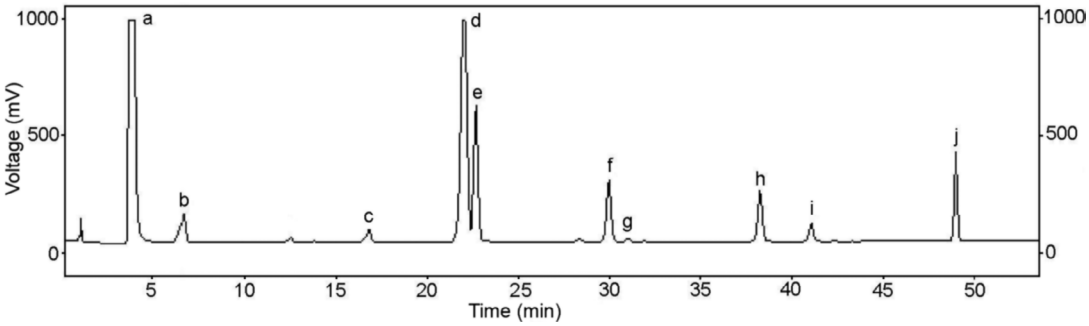

Fig. 1. HPLC chromatogram of acetone extract of *Hypogymnia physodes* (Sample 2, cf. Table 1) at 245 nm. Peaks: a: acetone, b: benzoic acid (internal standard), c: protocetraric acid, d: 3-hydroxyphysodic acid, e: physodalic acid, f: 2´-*O*-methylphysodic acid, g: physodic acid, h: atranorin, i: chloroatranorin, j: bis-(2-ethylhexyl)-phthalate (internal standard).

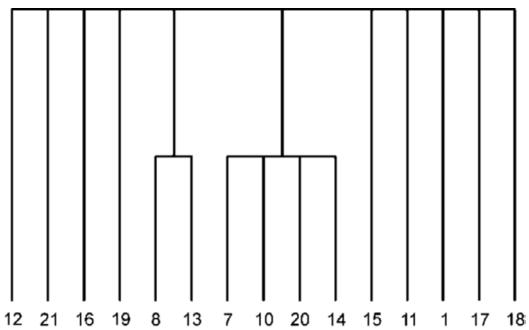

Fig. 2. Bootstrap tree resulting from Maximum Parsimony analysis of *EF1α* sequences of *Hypogymnia physodes* samples collected from various environmental conditions. Samples are indicated by numbers as in Table 2.

these substances was absent in one specimen of a certain variety or form, we detected it in other specimens of the same variety or form. We identified the cortical location of atranorin and chloroatranorin by analyzing different parts of the thallus (intact thallus, medullary layer, soralia). The other lichen substances are located in the medulla.

HPLC

We analyzed thirteen *H. physodes* thalli growing naturally under various environmental conditions by HPLC. Seven lichen substances occurred in all thalli: the cortical β-orcinol para-depsides atranorin and chloroatranorin, the medullary β-orcinol depsidones physodalic and protocetraric acids, as well as the medullary orcinol depsidones physodic, 3-hydroxyphysodic, and 2´-*O*-methylphysodic acids (Figs. 1 and 2). According to peak areas on the HPLC chromatograms, medullary depsidones represented the major secondary compound pool, while cortical depsides occurred in substantially lower concentrations. Relative concentration (area%) of atranorin was 1.71–15.18 area% and chloroatranorin was 0.79–1.93 area%. The values for physodalic acid were 7.95–23.39 area%; protocetraric acid, 0.26–1.87 area%; physodic acid, 0.01–5.14 area%; 3-hydroxyphysodic acid, 22.22–41.72 area%; and 2´-*O*-methylphysodic acid, 4.44–15.00 area% (Table 4). There was a large range in the concentrations of various substances, especially for atranorin and physodic acid. Protocetraric acid, chloroatranorin, and physodic acid occurred in low concentration in all samples (minor lichen substances), while 3-hydroxyphysodic acid occurred at the highest concentration in all specimens, but in lower concentrations in some polluted areas (i.e., Zalaegerszeg, Salgótarján).

DNA analyses

We sequenced DNA of 21 samples collected from different populations to study the genetic diversity. DNA amplification and sequencing yielded 16 *ITS* (6 kb), 18 *nucLSU* (1.4 kb), 3 *nucSSU* (1.6 kb), 21 *mitSSU* (8 kb), 1 *RPB1* (A–D region 1.2 kb; D–G region 1.8 kb), 10 *RPB2* (5–7 region 1.2 kb; 7–11 region 9 kb), and 15 *EF1α* (1.4 kb) sequences. *ITS*, *nucLSU*,

nucSSU, *mitSUU* and *EF1α* can be informative at the population level in lichens. We found no differentiation at all among the *mitSSU* sequences. The lengths of the *nucLSU* sequences were variable, 14 specimens contained an intron (400 bps). The *nucSSU* sequences varied in the number of introns (with 200 bps length in the 342nd and 1367th positions). We detected only small differentiation among the *ITS* and *EF1α* sequences of populations, confirmed by the MP analysis (Fig. 2).

Discussion

Applying HPTLC in order to analyze the secondary compounds in the entire Hungarian population of a lichen species is unique and novel. Due to the high number of samples analyzed, various further comparative studies are possible. According to TLC and HPTLC, *H. physodes* specimens from sites of different environmental conditions do not show any qualitative differences in terms of secondary lichen compounds. Chemical contents of old and freshly collected specimens were similar, which means that herbarium specimens are suitable for taxonomic and monitoring studies after even a long period of storage.

We identified all seven lichen substances by HPLC in all specimens, including varieties and forms, thus confirmed the HPTLC results, i.e., the samples were uniform qualitatively, which means that this lichen species has only one chemotype. We detected quantitative chemical differences between samples without any geographical pattern (Table 4). From this we conclude that microclimatic conditions or age of the thalli may be responsible for the differences.

Medullary depsidones were the major secondary compound pool (with physodic and protocetraric acids as minor lichen substances) in all samples. Cortical depsides showed substantially lower concentrations. Atranorin, 3-hydroxyphysodic, physodalic, and 2´-*O*-methylphysodic acids are major compounds of this species, whereas protocetraric and physodic acids, as well as chloroatranorin are minor substances. Zeybek *et al.* (1993) showed slightly different results for *H. physodes* samples collected mainly from Turkey (Table 5).

Table 4. Relative concentrations (area%) of lichen compounds in *Hypogymnia physodes* samples collected from different habitats.

Sample	Locality	Protocetraric acid	3-hydroxy-physodic acid	Physodalic acid	2´-*O*-methyl-physodic acid	Physodic acid	Atranorin	Chloroatranorin
1	Bp., Látó-hegy	1.87	40.6	23.39	11.2	0.5	2.97	1
2	Bp., Hosszúerdő-h.	1.22	39.5	16.03	4.44	5.143	15.178	1.7
3	Bp., Remete-h.	0.26	23.68	10.88	7.38	0.08	3.86	1.42
4	Zalaegerszeg	0.869	22.224	10.984	11	0.4	6	1
5	Salgótarján	1.242	25.18	8.429	6.583	0.229	1.708	0.9
6	Almásfüzitő-felső	1.27	38.65	17.37	6.39	3.68	6	1.67
7	Mátra, Tarjánka	1.32	50.75	7.95	9.45	0.29	9.92	1.93
8	Bükk, Kolozs-lápa	1.15	41.72	17.38	9.37	0.34	4.91	1.22
9	Bükk, Csákpilis	1.03	27.4	11.78	7.84	0.007	4.581	0.887
10	Egerszalók	1.001	37.1	11.28	15	0.1	7.908	1.333
11	Csévharaszt	1.095	24.151	10.121	6.3	0.265	4.52	1.32
12	Avenului Zgurăşti	1.08	25.74	10.296	11.43	0.25	1.87	0.79
13	Štrbské Pleso	1.12	30.75	11.254	10.816	0.37	7.45	0.99
Mean		1.117	32.880	12.857	9.015	0.896	5.913	1.243
SD		0.351	8.996	4.408	2.873	1.594	3.650	0.358

The high relative concentrations of physodalic acid in our samples growing naturally in polluted sites (Budapest: Hosszúerdő-hegy, Látó-hegy, Remete-hegy, Almásfüzitő-felső, Salgótarján) are not surprising, since this substance might be effective against pollution stress as Białońska and Dayan (2005) suggested based on increased levels in transplanted *H. physodes* samples. The samples with the highest levels of physodalic acid in the present investigation in Budapest were collected two decades ago when pollution by heavy metals and acidic inorganic sulphur compounds was high (Farkas *et al.* 1985). However, the results for other substances cannot be compared with that study. Białońska and Dayan (2005) found remarkable changes in the levels of secondary compounds in *H. physodes* thalli transplanted to areas polluted with heavy metals and acidic inorganic sulphur compounds, while the current study showed various concentrations without regard to pollution level. Many details about the secondary metabolism of this widespread toxitolerant lichen species are still not entirely understood, and further studies are needed to determine its usefulness in bioindication research. Moreover, the study of other more sensitive and rarer lichen species are necessary as well, in order to determine whether natural environmental factors influence the biosynthesis of lichen compounds, and to identify those substances. Statistical sampling and experimental conditions might also be used for more detailed studies.

We found no significant genetic differentiation among populations collected from different areas, indicating high gene flow and/or widespread clonal reproduction. We detected only very low variability in the *ITS*, *nucLSU*, *nucSSU*, and *EF1α* sequences of populations from different habitats, without any geographical patterns. Haplotype network would also be a possibility for presenting our results as was suggested recently (B. McCune pers. comm.). We are going to check the results by further investigation of additional specimens and other genetic markers, such as microsatellites and minisatellites.

Table 5. Quantity of lichen substances established in the current study and in Zeybek *et al.* (1993).

Lichen substances	Samples	
	This study	Turkish
Protocetraric acid	minor–trace	minor
3-hydroxyphysodic acid	major	major
Physodalic acid	major	major
2´-*O*-methylphysodic acid	major–minor	traces
Physodic acid	minor–trace	major
Atranorin	major–minor	major
Chloroatranorin	minor–trace	minor

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