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PHYLOGEOGRAPHIC INVESTIGATION OF *HORDEUM MURINUM* L. IN EUROPE BASED ON DNA MARKERS

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Hordeum murinum L. is a polyploid complex of thermophilic, annual, zoochoric grasses of Mediterranean–Irano-Turanian origin that is commonly present in Europe. *H. murinum* complex includes three annual and most often autogamous taxa: *glaucum*, *leporinum* and *murinum*. The variation of nuclear microsatellites, chloroplast microsatellites and chloroplast SNP-based PCR-RFLP markers of *H. murinum* from Europe was analyzed in order to investigate its migration. The chloroplast markers revealed three distinct haplotypes. Two of them are characteristic of *leporinum* and *murinum*. A geographical pattern of haplotypes has been detected, however it does not correspond to the known patterns of migration routes in the Holocene. Geographic distribution of genotypes defined by nuclear microsatellites has shown a geographic trend that may link the migration of *leporinum* and *murinum* with the spread of Neolithic agriculture in Europe. This study also confirms genetic distinction of *glaucum*, as well as genetic uniformity of *murinum* and *leporinum*.

Keywords: *Hordeum murinum* L., chloroplast genome, nuclear microsatellites, phylogeography

INTRODUCTION

The *Hordeum murinum* complex includes three annual and predominantly autogamous taxa: *glaucum*, *leporinum* and *murinum*. There is no consensus on the rank of those taxa. Depending on the authors, *glaucum*, *leporinum* and *murinum* are referred to as three separate species of *Hordeum*, as subspecies of *H. murinum* or even as varieties within one or two species (Giles and Lefkovitch, 1986; Jacobsen and von Bothmer, 1995). For the purpose of this study the taxa are treated as subspecies of *H. murinum* L.: subsp. *glaucum* (Steud.) Tzvelev, subsp. *leporinum* (Link) Arcang., and subsp. *murinum*. According to current data, subsp. *glaucum* is a diploid (2n = 2x = 14), subsp. *murinum* is a tetraploid (2n = 4x = 28), while subsp. *leporinum* occurs in the form of a tetra- (2n = 4x = 28) and hexaploid (2n = 6x = 42) (Giles and Lefkovitch, 1986; Jacobsen and von Bothmer, 1995).

The geographical range of the *H. murinum* complex covers the Mediterranean basin along with the areas to the east of it, reaching Kazakhstan and Pakistan, and to the north it reaches temperate Europe. During 18th and 19th centuries, the taxa of the *H. murinum* complex were introduced to South Africa and to the New World (Cocks et al., 1976; von Bothmer et al., 1995). The individual taxa differ in their range due to different environmental requirements. Thus, *glaucum* occurs in the warmest and driest regions of the Mediterranean zone, *murinum* occurs in the temperate zone of Europe, as it prefers the coldest and the most humid climate, whereas *leporinum* occupies the areas of intermediate climate. The range of *leporinum* partly overlaps with the areas where the other two subspecies occur. There are also morphological differences between the taxa of the *H. murinum* complex. The keys to the subspecies are mainly based on the traits of the spike, particularly on triplets (Cocks et al., 1976; Baum and Bailey, 1984; Giles and Lefkovitch, 1986; Jacobsen and von Bothmer, 1995). Previous molecular studies of the *H. murinum* complex revealed the allopolyploid origin of the tetra- and hexaploid forms (e.g., Jaaska, 1992; Jakob and Blattner, 2010). No differences were found between *murinum* and *leporinum* in relation to RAPD (Random Amplification of Polymorphic

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DNA) markers (De Bustos et al., 1998), hordeins (Amirouche and Misset, 2003), AFLP (Amplified Fragment Length Polymorphism) markers and sequences of a few nuclear single copy loci (Jakob and Blattner, 2010). Moreover, the latest molecular studies on the *H. murinum* complex (Ourari et al., 2011; Cuadrado et al., 2013) have shown genetic homogeneity of the tetraploids representing *murinum* and *leporinum*.

The basic works on the origin of the archaeophytes occurring in Poland (Zając, 1979, 1987, 1988) considered *H. murinum* sensu lato, characterizing its range as Mediterranean–Irano–Turano–Middle-European and its origin as Mediterranean–Irano-Turanian. In Central Europe *murinum* grows only in synanthropic, ruderal plant communities. Wittig (2004) in his study discussing the origin and development of the urban flora of Central Europe defines subspecies *murinum* as an anecophyte, i.e., a plant that occurs only in anthropogenic communities. It is a taxon characteristic of the *Hordeetum murini* association (Ass.) Libb. 1933 from the Sisymbrion officinalis Tüxen et al. ex von Rochow 1951 alliance (All.) (Kornaś, 1977; Matuszkiewicz, 2005). *H. murinum* is common in most European countries, it is also relatively easily identified and has recently been invasive. However, there has been no comprehensive phylogeographic study on this taxon so far. Many studies on the relationships among the taxa of the *H. murinum* complex analyzed specimens of *H. murinum* from the whole natural range. Nevertheless, these studies did not generally consider the patterns of spatial distribution of the possible forms, such as morphotypes, karyotypes, genotypes (reviewed by Bieniek and Mizianty, 2007). The most advanced phylogeographic and phylogenetic analysis of the *H. murinum* complex (Jakob and Blattner, 2010) showed no relevant variation of chloroplast DNA (cpDNA) or nuclear DNA (nDNA), thus inference about its phylogeography was impossible.

To date, the natural (glacial) refugia and the postglacial migration routes of many plant species have been described by means of cpDNA markers (e.g., Demesure et al., 1996; Dumolin-Lapègue et al., 1997; Ronikier et al., 2008; Szczepaniak, 2013). Also a few cpDNA studies on the phylogeography of synanthropic grasses published to date have yielded valuable results (Balfourier et al., 2000; Fjellheim et al., 2006; Arrigo et al., 2010). The most accurate way to analyze the variation of cpDNA is to sequence the non-coding elements of chloroplast genome, that is based on previously developed universal PCR (polymerase chain reaction) primers (Taberlet et al., 1991; Shaw et al., 2005; 2007). Another powerful tool of examining deep patterns of cytoplasmic variation are chloroplast microsatellites (cpSSRs, chloroplast Simple Sequence Repeats) (Provan et al., 1997). Previously designed primers enable the fast screening of cpSSR variation within genus *Hordeum* (Provan et al., 1999). Moreover, recently the cpSSR markers have been applied in a study of *H. murinum* (Jakob and Blattner, 2010). In this study, genetic variation within chloroplast genome of *H. murinum* was analyzed by means of newly developed substitution-based RFLP (Restriction Fragment Length Polymorphism) markers and previously described cpSSR markers. Other markers used to study phylogeography of plants are based on total DNA or nuclear genome. One of the most common total DNA markers are AFLPs. However, AFLP markers did not reveal structured variation within the *H. murinum* complex (Jakob and Blattner, 2010). Furthermore, AFLP protocol requires good quality DNA and poor quality DNA which may affect experiment results. As plant material for phylogeographic study is usually collected from a large area within a few years, it is difficult to assure proper quality of DNA. The mentioned problems associated with AFLPs have led to the search for alternative total DNA or nuclear DNA markers useful for phylogeographic studies of *H. murinum*. In contrast to AFLPs, nuclear microsatellite markers (nuSSRs, nuclear Simple Sequence Repeats) are codominant, non-anonymous and do not require high quality DNA, however they may be affected by allele homoplasy and need more careful interpretation (Skrede et al., 2009). Also nuSSRs are more cost- and time-consuming to develop and no nuSSR for *H. murinum* have been designed so far. However, there are numerous nuSSR markers developed for a closely related barley (*H. vulgare*) (Liu et al., 1996; Struss and Plieske, 1996; Ramsay et al., 2000), which have been successfully applied in phylogeographic studies of the species (Oliveira et al., 2011; Jones et al., 2012). Moreover, the transferability of barley nuSSR markers to *H. murinum* has recently been reported (Naghavi et al., 2011).

As the origin and migration of *H. murinum* in Europe remains unknown, the aim of this study was phylogeographic analysis of this complex within its European range. In order to achieve this target, it was essential to develop markers that reveal genetic structure within *H. murinum*. For the purpose of the study, chloroplast DNA (PCR-RFLP, cpSSR) and nuclear DNA markers (nuSSR) were applied in order to obtain a complex pattern of the variation.

MATERIALS AND METHODS

SAMPLING

Plants of the *H. murinum* complex from its European range were included in this study. The research materials consisted of 473 samples representing 121 localities of *H. murinum* from 26 countries: Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, France, Germany, Hungary, Italy, Macedonia, Montenegro, the Netherlands, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Switzerland, Tunisia, Turkey, Ukraine and the United Kingdom (Tab. 1). Samples CH1, D1, H02, SRB4, T1 and Tr1 were provided by the Nordic Gene Bank (Alnarp, Sweden). All the remaining samples were collected and designated by the author of the study. Two to four samples were analyzed from each locality and the herbarium materials were collected for each locality to perform taxonomic designations. From among 121 localities under study, 85 represent *murinum*, 27 – *leporinum*, 2 – *glaucum*. Morever, within 5 localities both *murinum* and *leporinum* were found, within 1 locality both *glaucum* and *leporinum* were found and within 1 locality three subspecies were detected (Tab. 1). The samples of subsp. *leporinum* (6x) (Tr1) and subsp. *glaucum* (T1) were used as the control from outside Europe. Ploidy of the samples provided by Nordic Gene Bank is given in Table 1, according to the database of repository.

NUCLEAR MICROSATELLITE (NUSSR) GENOTYPING

Six microsatellite primer pairs designed to amplify nuclear microsatellite loci in *Hordeum vulgare* and previously applied by Naghavi et al. (2011) to genotype *H. murinum* L. were used in the study (Tab. 2). The PCR reactions were carried out in the total volume of 15 μL, including 1x PCR buffer with KCl; 0.4 U of Taq polymerase; 3.5 mM $MgCl_2$; 200 μM of each dNTP; 0.3 μg BSA (all by Fermentas); two 0.2 μM primers and 0.75 μL template DNA (about 10 ng/μL). The cycling parameters were: 94°C for 4 min; then 35 cycles of 92°C for 45 s, 56°C (loci Bmac0316, AF022725A) or 60°C (loci EBmac0415, HVM20, HVM40, GMS003) for 45 s, 72°C for 1 min; and a final extension at 72°C for 10 min. Fluorescently labeled SSR-PCR products were separated and detected using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter). The length of alleles was determined by means of Fragment Analysis Software version 8.0 (Beckman Coulter).

CHLOROPLAST DNA ANALYSIS

The basic cpDNA analysis consisted of two subsequent parts: (1) the initial screening for mutations of the cpDNA that covered a few plants and a great number of loci, performed by means of DNA sequencing; (2) the broad-scale cpDNA analysis that covered the whole plant material, based on a few variable loci and performed by means of the PCR-RFLP. In parallel with the PCR-RFLP, the analysis of chloroplast microsatellite loci was performed.

GENOTYPING OF CHLOROPLAST SEQUENCES

Eleven plants originating from different geographic locations were chosen for DNA sequencing. They represented the following locations: F16, H05, MKD01, N01, N17, P03, P08, P12, R04, C06 and U01. Eight cpDNA non-coding regions were amplified with the use of the previously published primers (Tab. 3). PCR analyses were conducted in the total volume of 12.5 μL, including 1x PCR buffer with KCl; 0.4 U of Taq polymerase; 2 mM $MgCl_2$; 200 μM of each dNTP (all by Fermentas); two 0.16 μM primers; and 0.5 μL template DNA (about 10 ng/μL). The thermal parameters of PCR were optimized experimentally and proceeded as follows: 94°C for 4 min; then 35 cycles of 92°C for 45 s, 50°C for 45 s, 72°C for 1 min; and a final extension at 72°C for 10 min. Each fragment was purified with the High Pure PCR Product Purification Kit (Roche) according to the manufacturer's protocol and was subsequently sequenced in both directions on the ABI 3100-Avant automated sequencer using the BigDye 3.1 sequencing kit (both Applied Biosystems).The obtained nucleotide sequences were aligned with the BioEdit version 7.0.9.0 (Hall, 1999). Next, the multiplied alignments were screened for mutations. The Restriction Mapper online tool (http://www.restrictionmapper.org/) was used to locate the restriction sites among the detected mutations. The obtained sequences were also compared with the GenBank resources using BLAST (Altschul et al., 1990; http://blast.ncbi.nlm.nih.gov/) to assess direction of detected mutations.

PCR-RFLP ANALYSIS OF CHLOROPLAST INTERGENIC SPACERS

Amplification of the selected intergenic spacers was carried out with PCR conditions described above. Then PCR products were digested with restriction enzymes that produce polymorphic profiles, namely: trnL-rpl32 digested with TaqI; petA-psbJ digested with EcoRI; rps16-trnK digested with HpaII (Roberts et al., 2015). The

TABLE 1. List of analyzed localities of *Hordeum murinum* complex.

	Country	Locality	Latitude	Longitude	ID	Subspecies	No. of plants
1	Austria	Durnstein	48.393850	15.523855	A01	murinum	4
2	Austria	Graz	47.051249	15.455457	A04	murinum	3
3	Austria	Linz	48.284142	14.284085	A02	murinum	4
4	Belgium	Dinant	50.261055	4.909548	B01	murinum	4
5	Bosnia and Herzegovina	Jajce	44.338783	17.273641	BiH02	murinum	4
6	Bosnia and Herzegovina	Sarajevo/Novi Grad	43.829395	18.332971	BiH03	murinum	4
7	Bulgaria	Каварна (Kavarna)	43.435291	28.347559	BG01	leporinum	4
8	Bulgaria	Шумен (Shumen)	43.281119	26.973961	BG02	leporinum	4
9	Bulgaria	Карнобат (Karnobat)	42.651444	27.00125	BG03	leporinum	4
10	Bulgaria	София (Sofia)	42.640849	23.415995	BG04	leporinum	4
11	Bulgaria	Монтана (Montana)	43.405046	23.231792	BG05	leporinum	4
12	Croatia	Kutina	45.477737	16.779385	HR02	leporinum	4
13	Croatia	Trilj	43.615782	16.72897	HR03	leporinum	4
14	Croatia	Starigrad-Paklenica	44.287399	15.453140	HR01	murinum, leporinum	3
15	Czech Republic	Brno	49.195177	16.601865	C01	murinum	4
16	Czech Republic	Buchlovice	49.084769	17.336362	C02	murinum	4
17	Czech Republic	Olomouc	49.596926	17.262537	C04	murinum	4
18	Czech Republic	Opava	49.938983	17.895856	C06	murinum	4
19	Czech Republic	Novy Jičin	49.592692	18.018076	C07	murinum	4
20	Czech Republic	České Budějovice	48.980007	14.473266	C10	murinum	4
21	Czech Republic	Plzeň	49.737813	13.387441	C11	murinum	4
22	Czech Republic	Praha	50.047357	14.440785	C12	murinum	4
23	Czech Republic	Semtín (Pardubice)	50.060445	15.728763	C13	murinum	4
24	Denmark	Svendborg	na	na	D01 NGB6543	murinum	3
25	France	Correns	43.483271	6.080176	F01	leporinum	4
26	France	Serres	44.426877	5.718263	F02	leporinum	4
27	France	Vinassan	43.212333	3.089543	F05	leporinum	4
28	France	Libourne	44.902434	-0.233953	F07	leporinum	4
29	France	Pont-de-Labeaume	44.660784	4.296784	F03	leporinum, murinum	4
30	France	Mende	44.521613	3.496259	F04	leporinum, murinum	4
31	France	Villefranche-de-Conflent	42.586325	2.365896	F06	leporinum, murinum	4
32	France	Sens	48.195159	3.29798	F13	leporinum, murinum	4
33	France	Ussel	45.549109	2.310559	F08	murinum	4
34	France	Clermont - Ferrand	45.802425	3.115295	F09	murinum	4

TABLE 1. – continued

	Country	Locality	Latitude	Longitude	ID	Subspecies	No. of plants
35	France	Bourg en Bresse	46.210283	5.234089	F10	murinum	4
36	France	Belfort	47.628899	6.85696	F11	murinum	4
37	France	Nancy	48.683739	6.19836	F12	murinum	4
38	France	Tours	47.36781	0.702991	F14	murinum	4
39	France	Le Croisic	47.296622	-2.537118	F15	murinum	4
40	France	Saint Lo	49.108068	-1.106958	F16	murinum	4
41	France	Amiens	49.898422	2.301657	F17	murinum	4
42	Germany	Magdeburg	52.128208	11.629516	N01	murinum	4
43	Germany	Brandenburg	52.404329	12.559062	N04	murinum	4
44	Germany	Bernau	52.679369	13.576294	N06	murinum	4
45	Germany	Pasewalk	53.502326	13.989926	N09	murinum	4
46	Germany	Dresden	51.073795	13.741606	N10	murinum	4
47	Germany	Jena	50.931200	11.579471	N11	murinum	4
48	Germany	Nürnberg	49.479115	11.111271	N12	murinum	3
49	Germany	Regensburg	49.012499	12.114325	N13	murinum	4
50	Germany	Passau	48.571769	13.450108	N14	murinum	4
51	Germany	Aschaffenburg	49.988021	9.143318	N15	murinum	4
52	Germany	Bad Oeynhausen	52.203428	8.806518	N16	murinum	4
53	Germany	Flensburg	54.780024	9.424735	N17	murinum	4
54	Germany	Lübeck	53.87608	10.665498	N18	murinum	4
55	Hungary	Szekszárd	46.340040	18.702250	HUN01	murinum	4
56	Hungary	Miszkolc	48.10592	20.811777	HUN03	murinum	4
57	Hungary	Kecskemét	46.889634	19.685048	HUN04	murinum	4
58	Hungary	Győr	47.694474	17.659505	HUN05	murinum	4
59	Italy	Gaeta	41.232194	13.499343	IT01	leporinum	4
60	Italy	Udine	46.051003	13.207457	IT02	leporinum	4
61	Italy	Vignole	45.910382	10.900919	IT03	leporinum	4
62	Italy	Castella	44.564212	9.946125	IT04	leporinum	4
63	Italy	Borghetto Santo Spirito	44.106842	8.238555	IT05	leporinum	4
64	Macedonia	Велес (Veles)	41.717778	21.775691	MKD01	leporinum	4
65	Montenegro	Berane	42.857658	19.875249	MNE01	leporinum	4
66	Netherlands	Arnhem	51.954184	5.894578	NL01	murinum	4
67	Poland	Choszczno	53.169176	15.416211	P02	murinum	4
68	Poland	Krosno Odrzańskie	52.055167	15.114045	P03	murinum	4
69	Poland	Paszowice k. Jawora	51.028848	16.188349	P04	murinum	4

TABLE 1. – continued

	Country	Locality	Latitude	Longitude	ID	Subspecies	No. of plants
70	Poland	Bydgoszcz	53.138139	17.966214	P05	murinum	4
71	Poland	Kędzierzyn-Koźle	50.344586	18.221349	P06	murinum	4
72	Poland	Częstochowa	50.817653	19.113587	P07	murinum	4
73	Poland	Bielsko-Biała	49.820229	19.035684	P08	murinum	4
74	Poland	Tarnobrzeg	50.573146	21.678834	P09	murinum	4
75	Poland	Sanok	49.558841	22.204476	P10	murinum	4
76	Poland	Siedlce	52.162749	22.272773	P11	murinum	4
77	Poland	Krasnystaw	50.984310	23.158680	P12	murinum	4
78	Poland	Suwałki	54.106288	22.938591	P13	murinum	4
79	Portugal	Braga	41.553018	-8.397831	PT01	leporinum	4
80	Romania	Cernavodă	44.339235	28.030989	R06	leporinum	4
81	Romania	Negreni	46.959028	22.74198	R02	murinum	4
82	Romania	Aiud	46.30934	23.717373	R03	murinum	4
83	Romania	Căciulata	45.271776	24.314652	R04	murinum	4
84	Romania	Bucureşti	44.432868	26.089061	R05	murinum	4
85	Romania	Arad	46.154827	21.321736	R07	murinum	4
86	Romania	Bacău	46.581371	26.91652	R08	murinum	4
87	Serbia	Краљево (Kraljevo)	43.722246	20.692588	SRB01	leporinum	4
88	Serbia	Јелашничка клисура (Jelasnicka Klisura)	43.279103	22.064441	SRB02	leporinum, murinum	4
89	Serbia	Београд (Belgrade)	44.772197	20.518432	SRB03	murinum	4
90	Serbia	Zeljusa	na	na	SRB04 NGB7302	murinum	2
91	Slovakia	Prešov	48.997784	21.236441	S01	murinum	4
92	Slovakia	Košice	48.713576	21.248609	S02	murinum	4
93	Slovakia	Trencin	48.893895	18.044669	S04	murinum	4
94	Slovakia	Pieštany	48.593235	17.833332	S05	murinum	4
95	Slovenia	Ljubljana	46.050342	14.494008	SLO01	leporinum	4
96	Spain	Valencia	na	na	H03	glaucum	4
97	Spain	El Chorro	36.908812	-4.760519	H01	leporinum	3
98	Spain	Cartaya	na	na	H02 NGB90136	leporinum	3
99	Spain	la Guingueta d'Aneu	42.593813	1.131809	H04	leporinum	4
100	Spain	Alcolea del Pinar	41.035292	-2.463448	H05	leporinum, glaucum	4
101	Spain	Buitrago del Lozoya	40.998245	-3.637129	H06	murinum, glaucum	4
102	Spain	Belorado	42.418194	-3.197118	H07	murinum	4
103	Spain	Erro	42.942672	-1.446373	H08	murinum	4

TABLE 1. – continued

	Country	Locality	Latitude	Longitude	ID	Subspecies	No. of plants
104	Switzerland	Martigny	na	na	CH01 NGB6526	murinum	3
105	Tunisia	Faid	na	na	T01 NGB6872	glaucum	3
106	Turkey	Aratol	na	na	Tr01 NGB6878	leporinum (6x)	3
107	Ukraine	Сімеїз (Simeiz)	44.403741	34.002771	U16	leporinum	4
108	Ukraine	Дрогобич (Drohobych)	49.357895	23.511753	U01	murinum	4
109	Ukraine	Львів (Lviv)	49.838624	23.998124	U02	murinum	4
110	Ukraine	Івано-Франківськ (Ivano-Frankivsk)	48.902834	24.693271	U04	murinum	4
111	Ukraine	Луцк (Lutsk)	50.742769	25.319001	U05	murinum	4
112	Ukraine	Чернівці (Chernivtsi)	48.267938	25.949704	U07	murinum	4
113	Ukraine	Рівне (Rivne)	50.604484,	26.272121	U08	murinum	4
114	Ukraine	Кременець (Kremenets)	50.116204	25.720090	U09	murinum	4
115	Ukraine	Хотин (Khotyn)	48.518864	26.498445	U11	murinum	4
116	Ukraine	Ві́нниця (Vinnytsia)	49.227211	28.415730	U14	murinum	4
117	Ukraine	Вільшанка (Vilshanka)	48.235575	30.87825	U15	murinum	4
118	Ukraine	Бахчисара́й (Bakhchysarai)	44.747219	33.899989	U19	murinum	4
119	Ukraine	Миколаїв (Mykolaiv)	47.021154	32.002466	U20	murinum	4
120	Ukraine	Сарата (Sarata)	46.049527	29.718382	U21	murinum	4
121	United Kingdom	London	51.509117	-0.178695	UK01	murinum	4

digestion of PCR products was carried out with the use of Thermo Scientific restriction enzymes and buffers, according to the protocols recommended by the producer. The digestions with EcoRI, and HpaII enzymes were incubated overnight at 37°C, and with TaqI enzyme overnight at 65°C. The activity of the enzymes was stopped by cooling to 4°C and by adding 2 μl of 6x loading buffer. The restriction products were separated by agarose gel electrophoresis and stained with ethidium bromide, then visualized under UV-light and photographed.

CHLOROPLAST MICROSATELLITE (CPSSR) GENOTYPING

Four primer pairs previously used by Jakob and Blattner (2010) to analyze *Hordeum murinum* were applied (Tab. 2). The composition of PCR reactions was identical to that described above for nuSSR. The following thermal parameters of PCR were optimized experimentally: 94°C for 4 min; then 35 cycles of 92°C for 45 s, 53.5°C (loci cpSSR3, cpSSR5, cpSSR6Ri) or 56°C (locus cpSSR6Fi) for 45 s, 72°C for 1 min; and a final extension at 72°C for 10 min. Fluorescently labeled SSR-PCR products were separated and detected using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter). The length of alleles was determined by means of Fragment Analysis Software version 8.0 (Beckman Coulter).

STATISTICAL ANALYSIS

The diversity parameters were calculated by GenAlEx ver. 6.502 (Peakall and Smouse, 2006). The phylogenetic tree based on nuSSR data was constructed by the DARwin 6.0.11 software (Perrier and Jacquemoud-Collet, 2006) with the use of simple matching distance and the neighbor-joining method. The robustness of each node was evaluated

TABLE 2. Nuclear and chloroplast microsatellite loci analyzed in the study.

Locus	Primers	No. of alleles	Size range	Chromosome	Repeat
AF022725A	Ramsay et al., 2000	1	115	7H	(TG)8
Bmac0316	Ramsay et al., 2000	6	101–135	6H	(AC)19
EBmac0415	Ramsay et al., 2000	1	269	2H	(AC)17
GMS003	Struss and Plieske, 1996	3	130–152	2H	(GT)15
HVM20	Liu et al., 1996	1	121	1H	(GA)19
HVM40	Liu et al., 1996	2	138, null	4H	(GA)6, (GT)4, and (GA)7
cpSSR3	Jakob and Blattner, 2010	4	348, 352, 355, 363	CP	Poly A/T
cpSSR5	Jakob and Blattner, 2010	1	101	CP	Poly A/T
cpSSR6Fi	Jakob and Blattner, 2010	3	99, 100, 101	CP	Poly T/A
cpSSR6Ri	Jakob and Blattner, 2010	2	116, 117	CP	Poly T/A

TABLE 3. Chloroplast intergenic spacers screened for mutations within *Hordeum murinum* complex.

Locus	Primers	Length (nt)
rps12-rpl20	Shaw et al., 2005	621
rps16-trnK	Shaw et al., 2007	644
trnV-ndhC	Shaw et al., 2007	882
petA-psbJ	Shaw et al., 2007	807/835
rps16-trnQ	Shaw et al., 2007	748/758
trnL-rpl32	Shaw et al., 2007	749
atpI-atpH	Shaw et al., 2007	585/588/595
ndhA (intron)	Shaw et al., 2007	1097/1142

by bootstrapping data for 1,000 replications. The median-joining network of haplotypes was calculated by Network 4.6.1.1. software (http://www.fluxus-engineering.com/).

RESULTS

NUCLEAR MICROSATELLITE GENOTYPING

From among six analyzed nuSSR loci, three appeared to be monomorphic (AF022725A – allele 115; EBmac0415 – allele 269; HVM20 – allele 122). At locus HVM40, besides the allele 138, also the null allele was detected. The replicated experiment confirmed the occurrence of the null allele in the same accessions. For this reason locus HVM40 was treated as a diagnostic feature in phylogenetic analyses. Within the remaining two loci, altogether nine alleles were detected: GMS003 – 3, Bmac0316 – 6. Within samples of tetraploid *murinum* and *leporinum* no more than two different alleles were found in each of the polymorphic loci, whereas the hexaploid *leporinum* accession presented three different alleles at locus Bmac0316. The occurrence of polymorphic nuSSR alleles revealed eleven genotypes. The genotypes were marked as GT A – GT L (Tab. 4).

The neighbor-joining dendrogram based on a simple matching method revealed three clades (Fig. 1). The most distinct clade (bootstrap 84)

TABLE 4. Genotypes within *Hordeum murinum* complex on the basis of three nuSSR loci.

Genotype		GMS003			Bmac0316						HVM40	
Symbol	No. of plants	130	144	153	101	121	125	129	133	135	138	null
GT A	1	0	1	0	0	0	0	1	0	0	0	1
GT B	6	0	1	0	0	0	0	0	1	0	0	1
GT C	1	0	1	0	0	1	0	0	0	0	0	1
GT D	1	0	1	1	0	1	1	0	0	0	1	0
GT E	5	0	1	1	0	1	0	0	0	0	1	0
GT F	143	0	1	1	0	1	0	0	1	0	1	0
GT G	46	0	1	1	0	1	0	0	0	1	1	0
GT H	3	0	1	1	1	1	0	0	1	0	1	0
GT J	231	1	1	0	0	1	0	0	1	0	1	0
GT K	26	1	1	0	0	1	0	0	0	0	1	0
GT L	10	1	1	0	0	1	0	0	0	1	1	0

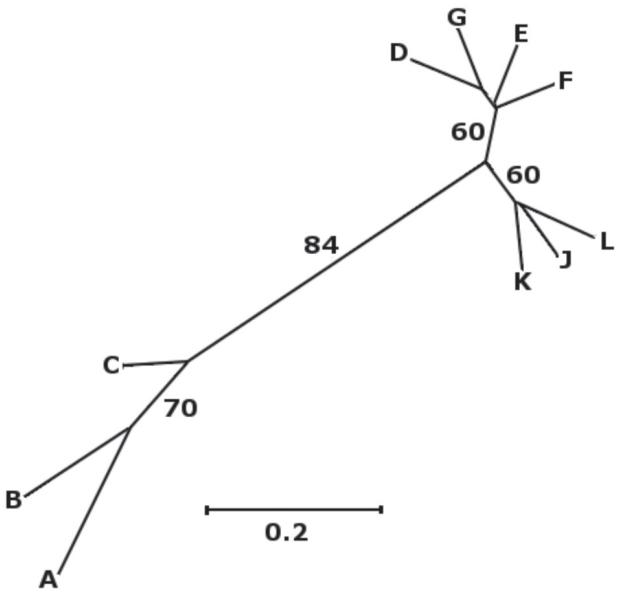

Fig. 1. Neighbor-joining dendrogram based on simple matching method for nuSSR genotypes of *Hordeum murinum* L. Bootstrap values > 50 are indicated. For genotype description see Table 4.

consists of *glaucum* from the Iberian Peninsula (genotypes A, B, C). The next significant clade consists of frequent F and G genotypes and rare D and E. This clade covers European *murinum*, *leporinum* accessions as well as one *glaucum* locality (Tunisia). The third clade includes both tetraploids: *murinum* and *leporinum* and consists of the most frequent genotype J and rare genotypes K and L. Therefore, in respect of the analyzed nuSSR markers, there are no differences between *murinum* and *leporinum*, while most of *glaucum* accessions are clearly distinguishable (see also Tab. 5). The detected nuSSR genotypes differ slightly in the geographical distribution. The most abundant genotype J is widespread across the Mediterranean and Temperate Europe except for Romania, Bulgaria and Ukraine, where it was found just in few locations. Moreover, two genotypes closely related to J (K and L) appear in the same area. The second common genotype F, together with closely related G, occupies the area from the Black Sea coast westwards to Germany. Only a limited number of genotypes F reach the north-western France. In Central Europe, the ranges of genotypes F, G and J partly overlap. The range of A, B and C genotypes, which are strictly tied to *glaucum*, is restricted to the Iberian Peninsula (Fig. 2).

NUCLEOTIDE POLYMORPHISM IN CHLOROPLAST GENOME

Five out of eight of the analyzed loci appeared to carry substitutions, insertions-deletions (indels) and inversion (Tab. 6), namely: *ndhA* (intron), *petA-psbJ*, *rps16-trnQ*, *rps16-trnK* and *trnL-rpl32*. At 835 bp long sequences of *petA-psbJ* one transition

TABLE 5. cpSSR allels, nuSSR allels and RFLP haplotype frequencies within *Hordeum murinum* complex.

			glaucum	leporinum	murinum
	locus	**allele**	**11**	**116**	**343**
nuclear microsatellites	**GMS003**	**130**	0.000	0.397	0.255
		144	0.864	0.500	0.500
		153	0.136	0.103	0.245
	Bmac0316	**121**	0.318	0.599	0.509
		125	0.000	0.000	0.001
		129	0.091	0.000	0.000
		133	0.591	0.345	0.427
		135	0.000	0.056	0.063
	HVM 40	**null**	0.727	0.000	0.000
		138	0.273	1.000	1.000
chloroplast microsatellites	**cpSSR6Fi**	**99**	0.364	0.009	0.000
		100	0.636	0.138	0.571
		101	0.000	0.853	0.429
	cpSSR6Ri	**116**	0.636	1.000	1.000
		117	0.364	0.000	0.000
	cpSSR3	**348**	0.000	0.853	0.429
		352	0.000	0.147	0.571
		355	0.636	0.000	0.000
		363	0.364	0.000	0.000
RFLP markers		**I**	0.000	0.147	0.571
		II	0.000	0.853	0.429
		III	1.000	0.000	0.000

G/A, one transversion G/T and one indel of 27 bp were found; 644 bp long fragment of *rps16-trnK* carried one transversion G/T; *rps16-trnQ* fragment of 788 bp contained two transversions G/T and one indel of 10 bp; while the 749 bp long sequence of *trnL-rpl32* comprised one transversion G/C, one transversion A/C and inversion of 14 bp fragment (Tab. 6). Also poly(A) tracts of variable length (i.e., chloroplast microsatellite loci) were found at loci *atpI-atpH* and *petA-psbJ*. Moreover, a length variation in microsatellite locus with a repeating unit of 5 bp (TCTAT) within the *atpI-atpH* spacer was detected. The occurrence of the detected substitutions, indels and one inversion revealed three distinct cpDNA haplotypes at *H. murinum* (Tab. 6). Haplotypes were marked as HT I (plants N01, N17, P03, P08, P12), HT II (plants C06, N14, R04, U01) and HT III (plant H05). The nucleotide sequences of the intergenic spacers *atpI-atpH*, *ndhA*, *petA-psbJ*, *rps16-trnK*, *rps16-trnQ*, *trnL-rpl32* of HT I, HT II and HT III were deposited at the GenBank (Tab. 7).

WIDE-SCALE PCR-RFLP

From among the detected mutations, three that can be revealed with the commonly used restriction enzymes, were chosen for PCR-RFLP analyses: (1) G/C substitution at position 294 of *trnL-rpl32* that turns the restriction site TCGA into

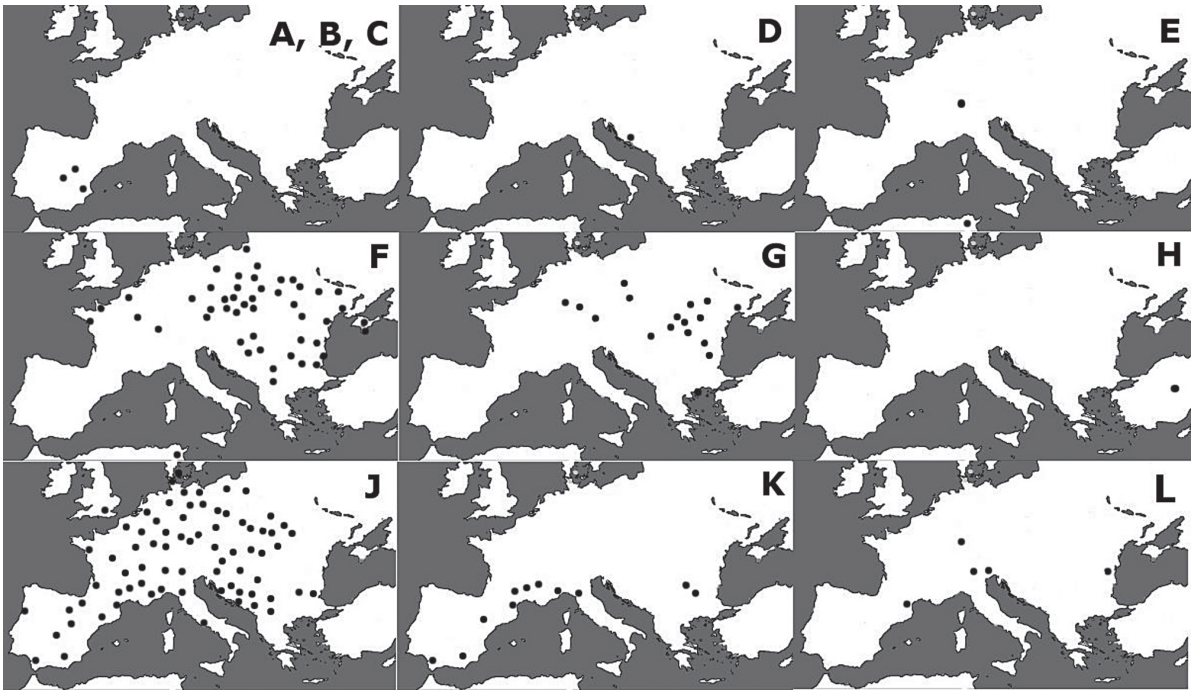

Fig. 2. Geographical distribution of eleven nuSSR genotypes of *Hordeum murinum* complex in studied localities.

TABLE 6. Summary of detected mutations and constitution of three haplotypes within *Hordeum murinum* complex.

Locus	**ndhA**			**petA-psbJ**			**rps 16-trnK**	**rps16-trnQ**			**rpl32-trnL**		
Nucleotide position	300	645	713	105	411	627	506	84	351	546	214–227	294	453
H. murinum HT I	G	–	–	–	G	G	T	G	G	–	GAATCCTCATATTT	G	C
H. murinum HT II	A	–	–	**	G	G	T	T	T	–	AAATATGAGGATTC	C	C
H. murinum HT III	G	T	*	**	A	T	C	T	T	***	AAATATGAGGATTC	C	A
Hordeum vulgare[1]	G	–	*	**	G	G	C	T	T	–	GAATCCTCATATTT	C	C
Triticum aestivum[2]	C	–	*	**	G	G	C	T	T	–	AAATATGAGGATTC	C	C
Aegilops cylindrica[3]	C	–	*	**	G	G	C	T	T	–	GAATCCTCATATTC	C	C

* GCAGAATTGCTCATGAACTAATACYCMAMTCTTTCCATTTATTA
** GATGTCTGGAATTCSTTATTTATATCY
*** TTTTAAGTGA
GenBank acc. no.: [1]KC912689.1, [2]KC912694.1, [3]KF534489.1

TABLE 7. GenBank accession numbers of *Hordeum murinum* complex chloroplast nucleotide sequences characteristic of the three detected haplotypes.

haplotype (sample)	**rpl32-trnL**	**rps16-trnQ**	**rps16-trnK**	**petA-psbJ**	**atpI-atpH**	**ndhA**
HT I (P12)	KJ437159	KJ437161	KP793194	KJ437163	KP793198	KP793200
HT II (C6)	KJ437158	KJ437160	KP793195	KJ437162	KP793196	KP793199
HT III (H5)	KP793203	KP793204	KP793193	KP793202	KP793197	KP793201

TGGA (digestion with TaqI); (2) T/C substitution at position 506 of *rps16-trnK* that turns the restriction site CCGG into CTGG (digestion with HpaII); (3) deletion of 27 nucleotides at *petA-psbJ* that removes the restriction site GAATTC (digestion with EcoRI). These three PCR-RFLP markers were used to analyze the genetic variation among 473 plants representing 121 locations. Clear and readable band profiles were obtained for all samples. No other restriction sites were revealed and no other polymorphisms were found, as it had been expected on the basis of nucleotide sequences. Therefore, PCR-RFLP markers confirmed the presence of three haplotypes detected by DNA sequencing. Plants of HT I share the deletion of 27 bp at *petA-psbJ*, have G at position 294 of *trnL-rpl32* and T at position 506 of *rps16-trnK*. Plants of HT II have no deletion at *petA-psbJ*, have C at position 294 of *trnL-rpl32* and T at position 506 of *rps16-trnK*. HT III is characterized by the presence of C at position 506 of *rps16-trnK*, while the analyzed mutations within *petA-psbJ* and *trnL-rpl32* are identical to HT II. From among 473 analyzed plants, 213 represented HT I, 249 – HT II and 11 – HT III.

CHLOROPLAST MICROSATELLITE VARIATION

One out of the four analyzed loci appeared to be monomorphic (cpSSR5 – allele 101). Within the remaining three loci nine polymorphic alleles were detected altogether: cpSSR3 – 4, cpSSR6Fi – 3, cpSSR6Ri – 2 (Tab. 2). The results of cpSSR analyses are strongly correlated with the results of PCR-RFLP genotyping: HT I plants share the alleles cpSSR3-351 and cpSSR6Fi-100 (with the exception of one plant carrying allele cpSSR6Fi-99, subhaplotype HT Ib), all HT II plants share the alleles cpSSR3-347 and cpSSR6Fi-101. Allele cpSSR6Ri-116 is common for both: HT I and HT II haplotypes (Tab. 8). The cpSSR variation detected among HT III plants indicates three subhaplotypes: HT IIIa (cpSSR3-354, cpSSR6Fi-99, cpSSR6Ri-117), HT IIIb (cpSSR3-354, cpSSR6Fi-100, cpSSR6Ri-116), HT IIIc (cpSSR3-362, cpSSR6Fi-100, cpSSR6Ri-116) (Fig. 3).

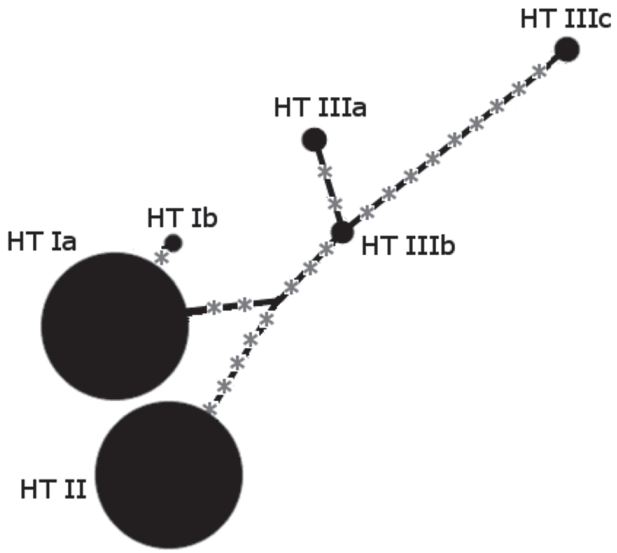

Fig. 3. Median-joining network of haplotypes based on cpSSR and PCR-RFLP data. Asterisks indicate mutations.

Generally, the samples from the same locality were uniform in terms of haplotype composition (104 localities, 86%), i.e., all the plants originating from the same locality share the same haplotype. The exceptions were 15 localities that consisted of plants HT I and HT II, 1 composed of HT I and HT III and 1 composed of all of the haplotypes. The haplotypes differ in their geographical distribution. The northern part of the sampled area (Belgium, Denmark, northern Germany, Poland, the Netherlands) is

TABLE 8. Numbers of plants sharing the same cpSSR allels within the described haplotypes of *Hordeum murinum* complex.

locus/allele	cpSSR3				cpSSR6Fi			cpSSR6Ri	
haplotype	**347**	**351**	**354**	**362**	**99**	**100**	**101**	**116**	**117**
HT Ia	0	212	0	0	0	212	0	212	0
HT Ib	0	1	0	0	1	0	0	1	0
HT II	249	0	0	0	0	0	249	249	0
HT IIIa	0	0	4	0	4	0	0	0	4
HT IIIb	0	0	3	0	0	3	0	3	0
HT IIIc	0	0	0	4	0	4	0	4	0

Fig. 4. Geographical distribution of three cpDNA haplotypes of *Hordeum murinum* complex in studied localities.

occupied only by HT I plants, while the southern part (Austria, Bosnia and Herzegovina, Bulgaria, Czech Republic, France, southern Germany, Hungary, Italy, Macedonia, Montenegro, Romania, Serbia, Slovakia, Spain, Ukraine) is populated by both haplotypes but with the predominance of HT II. The range of HT III in Europe is restricted to the Iberian Peninsula. Outside Europe HT III was detected in plants from Tunisia (Fig. 4).

Haplotype III is characteristic of *glaucum*, as it was not detected in any other subspecies. Haplotype I and II are characteristic of both tetraploids, however in *murinum* I and II are present in close proportions (I – 57% vs. II – 43%), while in *leporinum* II dominates over I (I – 15% vs. II – 85%) (Tab. 6).

DISCUSSION

Within European *H. murinum* representatives three genetic pools can be distinguished on the basis of variation within nuclear microsatellites loci. The most distinct genetic pool (GT A-B-C) consists of *glaucum* from the Iberian Peninsula, the remaining two (GT D-E-F-G and GT J-K-L) are mixtures of European *leporinum* and *murinum*. The genetic homogeneity of *leporinum* and *murinum* within the *H. murinum* complex was previously reported by numerous authors (De Bustos et al., 1998; Amirouche and Misset, 2003; Ourari et al., 2011; Cuadrado et al., 2013). This homogeneity is usually explained as a result of the recent divergence of these taxa (Jakob and Blattner, 2010). The novelty of the study is the detection of a slight tendency in distribution of genotypes. Although there are no distinct geographic boundaries between nuSSR genotypes, it is evident that the genetic pool GT D-E-F-G predominates in the area stretching from the Black Sea westwards to the Baltic Sea, while the genetic pool GT J-K-L covers the Mediterranean Basin and western Europe eastwards to the Balkans and Ukraine. The contact zone of these two lineages covers the area of Central Europe. As *H. murinum* is a cleistogamous, zoochoric taxon, it is assumed that the only way for the gene flow to occur is transportation of seeds. Thus the geographic pattern of genotypes should reflect its migration.

H. murinum is recognized as an archaeophyte of Mediterranean-Irano-Turanian origin (Zając 1979, 1987, 1988). Therefore, the obtained geographical pattern can be the testimony of its spread along with the spread of early agriculture. Asia Minor is thought to be the center of biodiversity of the *H. murinum* complex (Richards and Booth, 1976). Importantly, this particular area was the starting point for the expansion of agriculture and farming into Europe. At the turn of the 6th millennium B.C. farmers from Anatolia probably penetrated the Aegean islands, reached the Peloponnese, and then went further through the northern Balkans to the middle and lower Danube region. Separate Anatolian farming culture spread westwards along the Mediterranean coast to the Iberian Peninsula and the Atlantic coast (Bogucki, 1996; Price, 2000). Moreover, it is stated that the *H. murinum* complex has accompanied humans since the beginning of agriculture (Savard et al., 2006), so it can be assumed that it colonized South-eastern and Central Europe by migrating with Neolithic farmers from the Middle East (Zeder, 2008). Given the fact that *H. murinum* in Central Europe is restricted to anthropogenic habitats, it is obvious that its spread must be associated with human migration. Interestingly, the occurrence of genetic lineages within *H. murinum* coincides with the ways of migration of Neolithic agriculture (Bogucki, 1996). Distribution of lineage J-K-L corresponds to the Mediterranean pathway to Atlantic Europe, while lineage D-E-F-G spreads from the northern Balkans and the Black Sea along the Danube and Dniester valleys.

Additionally, the conspicuous pattern of geographical distribution of the haplotypes has been detected within the European range of the *H. murinum* complex. However, this pattern differs from the pattern of nuSSR markers. In the southern part of the analyzed area both haplotypes have been found with a predominance of HT II, whereas the northern part of the analyzed area

is colonized mainly by HT I. The contact zone between haplotypes I and II goes from the Black Sea through northern Romania, Hungary, Slovakia, Czech Republic to southern Germany (Fig. 4). In Central Europe, the reported geographical structure of the haplotypes corresponds to the Main European Watershed, namely: the occurrence of HT II is limited to the area of the Atlantic Ocean, the Black Sea and the Mediterranean Sea drainage basins [except for localities F12 (Nancy), C6 (Opava), C13 (Semtín) and U2 (Lviv) located near the Main European Watershed], whereas HT I is the only one that occurs in the North Sea and the Baltic Sea drainage basins. Nonetheless, HT I is also present in the Atlantic, the Black Sea and the Mediterranean drainage basins. The localities with both haplotypes have been found in the Moravian Gateway and the Pannonian Basin. The correlation between the watershed and the range of haplotypes is surprising as there are no known factors that can limit the spread of such a taxon as *H. murinum* on the line of the watershed. Therefore, the described correlation can be accidental and the observed pattern may be a result of subsequent founder effects during the migration of *H. murinum* towards north. On the other hand, haplotype HT I could be selected in temperate climate.

The relationship between the spread of agriculture in Europe and the geographical structure of haplotypes in the grass species has already been stated in the case of *Lolium perenne* L. (Balfourier et al., 2000). However, there are also grass species associated with humans that seem to migrate regardless of human influence. Within the ruderal species of *Aegilops geniculata* Roth, that represents the Triticeae tribe and shares similar reproductive biology with *H. murinum*, the ordered natural pattern of the two haplotypes was observed in the Mediterranean area and only a slight human impact on its spread was detected (Arrigo et al., 2010). Similarly, the phylogeographical structure of a forage grass, *Festuca pratensis* Huds., indicates that natural migration is of greater importance to its spread than human impact (Fjellheim et al., 2006). However, none of the above mentioned research, nor any other phylogeographical studies known by the author (e.g., Taberlet et al., 1998; Hewitt, 1999) have revealed a geographical structure similar to the obtained results. Therefore, the phenomenon of natural geographical structure of the haplotypes within a synanthropic plant that is non-native to the considered area, is difficult to explain. The genetic-geographic structure may be a result of different migration waves or the process of natural selection of the haplotypes. Similar results regarding N – S genetic cline was obtained in the xerothermic grass *Bromus erectus*, however it was attributed to long-distance migration from southern refugia during the Holocene (Sutkowska et al., 2013).

Another interesting issue arising from the obtained results is the origin of haplotypes within tetraploids. The alignment of the sequences characteristic of each of the haplotypes detected within *H. murinum* revealed distinct differences between them. Previous cpDNA studies of *H. murinum* reported only a deep split between the diploid subspecies *glaucum* and the polyploids, but hardly any variation within the polyploid taxa (Doebley et al., 1992; Jakob and Blattner, 2010). Thus the observed distinctness of *glaucum* (manifested as HT III) was expected. However, this study is the first report on different haplotypes (HT I and HT II) within the polyploid representative of the *H. murinum* complex. Jakob and Blattner (2010) reported only slight variation in the length of cpSSR loci within *leporinum* and *murinum* group. The split between HT I and HT II is so deep that HT II is more similar to the related species than to HT I of the same species (Tab. 6). However, no significant differences in morphology were found between the plants of HT I and HT II (W. Bieniek, unpublished data). On the contrary, the BLAST algorithm found no other sequences more similar to HT I within the GenBank accessions than HT II of *H. murinum*.

Within *Hordeum* four basic genomes (designated as H, I, Xa and Xu) were defined on the basis of cytogenetic studies (von Bothmer et al., 1995). The *H. murinum* complex is a monophyletic group containing only the Xu genome and no other *Hordeum* taxa carry this genome (von Bothmer et al., 1995; Wang et al., 1996; Blattner, 2006). Previous cytogenetic (Rajhathy and Morrison, 1962; Taketa et al., 2000), karyological (Richards and Booth, 1976; Cuadrado et al., 2013) and molecular studies (Jakob and Blattner, 2010) indicated an alloploid origin of all polyploid forms within the *H. murinum* complex. One of the diploid ancestors of these polyploids is the progenitor of the contemporary subspecies *glaucum*. Other parental species of the polyploids remain unknown and are considered to be extinct diploid Xu-carrying species (Jakob and Blattner, 2010; Brassac et al., 2012; Cuadrado et al., 2013). Basing on the sequences of the chloroplast *trnL-trnF* locus, Jakob and Blattner (2010) found two distinct haplotypes within the *H. murinum* complex: one characteristic of diploid *glaucum* and the other characteristic of all tetra- and hexaploids. This finding led the authors to conclude that the extinct species was probably the maternal parent of the polyploids.

Jakob and Blattner (2010) analyzed, inter alia, two samples of subspecies *murinum* from the Nordic Gene Bank resources, namely NGB6543

(from Denmark) and NGB6526 (from Switzerland) and no sequence variation was found between them within the *trnL-trnF* locus. However, the same GenBank samples were analyzed in this study and it was revealed that the plants of NGB6543 are of HT I, whereas the plants of NGB6526 are of HT II. It follows that HT I and HT II are different forms (subhaplotypes) within the genetic type previously characterized on the basis of the *trnL-trnF* for polyploid representatives of the *H. murinum* complex (Jakob and Blattner, 2010). HT II seems to be evolutionary older and HT I has most likely derived from HT II, as other Triticeae species share the most mutations with HT II (especially the deletion at *petA-psbJ*) (Tab. 6).

Recent genetic analyses have revealed extensive extinction of populations of *Hordeum* in the Old World that possibly occurred during the Pleistocene and led to the loss of many intermediate chloroplast haplotypes (Blattner, 2006; Jakob and Blattner, 2006). Therefore, the revealed distinct haplotypes of subspecies *murinum* might be remnants of a pre-extinction diverse genetic pool. These haplotypes may have originated from the extinct diploid progenitors being a testimony to the multiple origin of the tetraploids within the *H. murinum* complex. On the other hand, the haplotypes could have diverged after the tetraploid formation. However, the extinction of some putative diploid progenitors and insufficient knowledge of the variability of cpDNA within subspecies *glaucum* hamper the indication of the actual origin of the haplotypes (Jakob and Blattner, 2010).

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