Original investigation

Genetic variability of the cold-tolerant *Microtus oeconomus* subspecies left behind retreating glaciers

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ABSTRACT

The current range of the root vole (*Microtus oeconomus*) in Europe has been shaped by climate changes, including the last glaciation. In Central Europe there are two isolated subspecies: *M. oeconomus mehelyi* and *M. oeconomus arenicola* occurring in the Pannonian lowland and the Netherlands respectively. *M. oeconomus stimmingi* is present in the northern part of Central Europe in the continuous range of the species distribution. To establish the influence of the postglacial isolation on the subspecies performance, we sampled 192 individuals at seven sites from the three geographic regions. Individuals were genotyped at 14 microsatellite loci highly polymorphic for all subspecies studied. This is the first extensive research presenting the diversity of *M. oeconomus mehelyi* from Central Europe at the molecular level. Its genetic diversity (Ar = 6.6, SD = 0.4) as well as effective population size (LDNe = 170, SD = 62) were significantly lower than in the other two subspecies, their average Ar = 7.6, SD = 0.2 and LDNe = 504, SD = 134, suggesting *M. oeconomus mehelyi* is most threatened with extinction. None of the subspecies showed significant genetic bottleneck signatures. The differential analysis of the genetic structure of various subgroups resulting from clustering analysis was performed to reveal the genetic relationships among individuals within and between geographic regions. We found a closer relation of *M. oeconomus stimmingi* with each of the isolated subspecies than between them. Moreover, we detected a clear substructure in Pannonian *M. oeconomus* (two subgroups), that could result from more than one colonization episode.

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Introduction

The geographical extension of the glacial (Last Glacial Maximum – LGM 21 000 to 17 000 years BP) and postglacial ranges of the root vole *Microtus oeconomus* (Pallas, 1776) in Europe may be representative for a group of cold-tolerant rodents (Bilton et al., 1998). There is much evidence indicating the occurrence of root voles during the LGM far north from the traditionally recognized Mediterranean refugia (Huntley, 1988; Bennett et al., 1991). Due to its cold-tolerance, *M. oeconomus* was able to find suitable cli-

matic conditions during the LGM across Central Europe from the Atlantic coast eastwards up to the Russian plains (LGM climate simulation by Fløjgaard et al., 2009). Root vole fossils from this period have been registered as far north as southern Poland (Nadachowski, 1989; Kowalski, 2001; Sommer and Nadachowski, 2006). Phylogeographic studies based on mtDNA data also confirm its likely occurrence in Central Europe at the time of the last glacial advance (Brunhoff et al., 2003). The high haplotype diversity (mtDNA) of root vole in Poland (Dąbrowski et al., 2013; Jancewicz et al., 2015) corresponds well with the hypothesis that root vole could survive in the high-latitude refugia termed 'northern refugia' (Stewart and Lister, 2001). Similarly to root voles also other mammalian species like lemmings, bank voles (*Clethrionomys glareolus*, Schreber, 1780) and field voles (*Microtus agrestis*, Linnaeus, 1761) were

reported to be likely present north to the traditional Mediterranean refugia during the LGM (Jaarola and Searle, 2002; Fløjgaard et al., 2009). That is in concordance with scenarios reconstructing the post-glacial Scandinavia colonization from the south by bank voles (Wójcik et al., 2010), field voles (Herman et al., 2014) and common shrew (Lundqvist et al., 2011).

In the postglacial period, the root vole also expanded its range further northward, following the melting ice sheet. At the end of the Pleistocene, the root vole could likely reach its widest geographical distribution in Europe, but soon this began to contract due to the climate warming and the expansion of more temperate rodent species. Potential competitors of *M. oeconomus* recolonized Central Europe from their southern refugia (Tast, 1982; Chaline, 1987) including the closely related *M. agrestis* (Jaarola and Searle, 2002) and *M. arvalis* (Heckel et al., 2005). Moreover, the common vole colonized eastern Europe also from northern refugia (Stojak et al., 2015, 2016). In a consequence of those dynamic distribution changes and the retreat of the root vole from the southern and western parts of its range, some isolated populations of this species were left behind and survived until now. Currently, there are three root vole subspecies inhabiting Central and Western Europe which, according to mitochondrial DNA analysis (mtDNA), all belong to the Central European phylogroup (Brunhoff et al., 2003). The main European range of the root vole, represented in Central Europe by the subspecies *Microtus oeconomus stimmingi* (Nehring, 1899), extends eastwards from its western boundary along the Oder river, and northwards from its southern boundary in southern Poland (Sałata-Pilacińska, 1990; Van Apeldoorn, 1999). The two other subspecies are geographically isolated postglacial relics: the Pannonian root vole *Microtus oeconomus mehelyi* Éhik, 1928 present in Hungary, Slovakia and Austria, and the Dutch root vole *Microtus oeconomus arenicola* (Selys-Longchamps, 1841) inhabiting the Netherlands (Méhelyi, 1908; Tast, 1982). The Pannonian root vole marks the southernmost distribution of this Holarctic species in Europe (Fig. 1). These two subspecies are considered to be threatened with extinction and are listed in the Bern Convention and the Habitat Directive (Thissen et al., 2015).

At present, the main potential threat to the survival of the isolated root vole subspecies populations is the loss of their genetic variability due to the degradation and fragmentation of suitable habitats. Habitats preferred by the root vole, wetlands in river valleys, humid meadows and bog alder forests, are drained, converted to croplands and separated by natural and artificial barriers. Therefore the isolated postglacial relic subspecies consist of scattered, isolated local populations. This makes the subspecies highly vulnerable to extinction through loss of genetic diversity (La Haye et al., 2004; Rácz et al., 2005; Van den Brink et al., 2011). For an appropriate conservation management of these subspecies it is important to estimate the degree of isolation of these local populations. It is important to know the effective population size and the minimal level of genetic diversity within each population for future survival. In populations of the Dutch subspecies, studied by Van de Zande et al. (2000), the genetic variability at the microsatellite level did not seem to be impoverished, although a previous study on allozymes indicated a low level of genetic variability (Leijs et al., 1999).

As far as the Pannonian root vole is concerned, little is known about its overall genetic condition and the present genetic variability within the local populations. It would be interesting to learn whether the populations remaining in geographical isolation since the LGM are doomed to extinction or form taxonomic groups that are adapted to the local environmental conditions, which are atypical for the cold-preferring species. The latter seems probable in view of the morphological distinctiveness of *M.o. mehelyi* found by Baláž and Fraňová (2013).

In this study, we present various measures of genetic variability of the two postglacial relic subspecies (*M.o. mehelyi* and *arenicola*) and compare them with *M.o. stimmingi* from the main European range of the species. For this purpose we collected 192 DNA samples from populations of the three subspecies and selected a set of 14 microsatellite markers which proved to be polymorphic in all these subspecies. For the Pannonian root vole we collected samples from multiple local populations in order to assess its internal genetic structure. The specific aims of our analyses were the following:

1. To assess the level of genetic variability in the two isolated subspecies of *M.o. mehelyi* and *M.o. arenicola* and compare it with *M.o. stimmingi* which inhabits the continuous range of the species.
2. To determine the overall genetic structure of the three *M. oeconomus* subspecies and the internal genetic structure of the Pannonian root vole, with special emphasis on the chances for the future existence of this glacial relic.
3. To evaluate how the genetic structure of the vole populations found in this study fits into the phylogeographical structure of *M. oeconomus* assessed by other authors with use of mitochondrial DNA markers (mtDNA).

Material and methods

Study areas and field sampling effort

For this study, samples of *M.o. mehelyi* were collected in the field, in sites located in Slovakia (SK, n = 84 individuals), Austria (AT, n = 24) and Hungary (HU, n = 24) between 2010 and 2014. In Slovakia, the samples were collected on three different sites: near Pataš (47°52′N 17°39′E; SK1, n = 25), Veľké Kosihy (47°46′N 17°53′E; SK2, n = 33) and Číčov (47°46′N 17°46′E; SK3, 26). In Austria the sampled population was located on the south-eastern bank of Lake Neusiedler (47°50′N 16°45′E). In Hungary, samples were collected in the Szigetkös area near Lipót (47 52′N 17 28′E; Fig. 1). Voles were captured with use of the live traps in optimal root vole habitats, i.e. wetlands covered mostly with reed *Phragmites* sp. and sedges *Carex* sp.

Additionally, for the inter-subspecies comparison, the genetic material of *M.o. arenicola* individuals was obtained from the Netherlands (NL, n = 34) and that of *M.o. stimmingi* from Poland (PL, n = 26) (Fig. 1). The material from Poland was collected in the natural sedgeland situated in the river valley in Bialowieża Primeval Forest (52°4′ N, 23°5′ E) in 2004–2005 (for more information see Pilot et al., 2010). Samples from the Netherlands were collected in the province of South Holland (52°9′ N, 4°47′ E) in 2011. All sampling sites are situated in the protected areas.

Ethic statement

Samples used in this study were obtained by collecting hair samples (Hungary), ear-punching (Slovakia, Austria and the Netherlands) or toe clipping (Poland) of individuals that were live trapped and then released. Appropriate permissions were obtained for all applied procedures (for ethical approval codes see Supplementary information).

Laboratory analysis

DNA was extracted with the use of a QiaAmp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. Isolated DNA was stored at −20 °C for the short term and at −80 °C for the long term. Out of 20, we have chosen 14 highly polymorphic microsatellite loci (Moe3, Moe5, Moe6 (Van de Zande et al., 2000); MsMM3, MsMM5, MsMM7 (Ishibashi et al., 1999); Mar049, Mar063, Mar076, Mar080
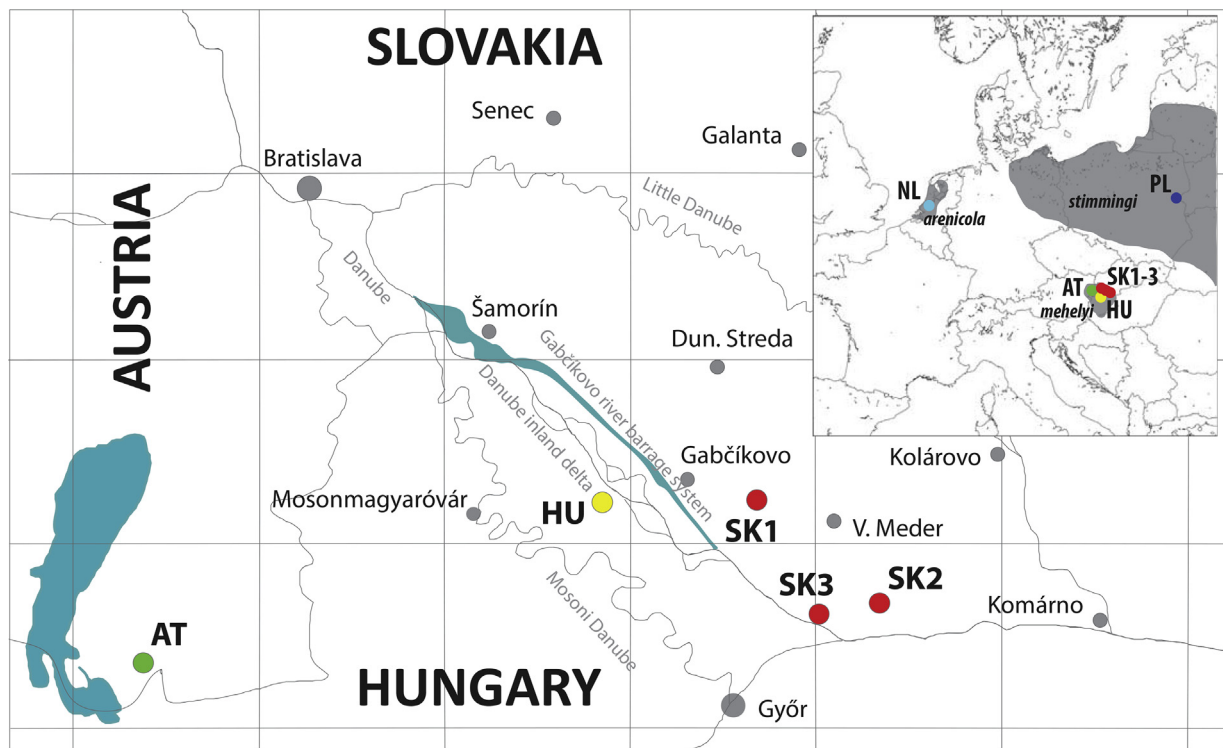
Fig. 1. Sampling sites in the Pannonia lowland. Top right panel: Distribution of the three subspecies of *M. oeconomus* in Europe; sampling sites indicated.

(Walser and Heckel, 2008), Ma54, Ma78 (Gauffre et al., 2007); AV14, AV15 (Stewart et al., 1998)) that were amplified in three multiplex sets using a Qiagen Multiplex PCR Kit and following the manufacturer's protocol. The PCR products detection was established with use of ABI 3130 Genetic Analyzer (Applied Biosystems) followed by alleles length detection with use of GeneMarkerV1.80 (for details see Supplementary information).

Genotyping errors estimation

To estimate the rate of genotyping errors, for a set of randomly chosen individuals (n = 20) genotyping of 14 loci was repeated. The error rate per allele, was found to be low (1.8%, following Hoffman and Amos, 2005) and the mean PCR success rate was over 99% The mean frequency of allelic dropout was 3.2% across samples and 3.7% across loci (the most often on Mar080; by Gimlet v133 (Valiére, 2002)). False alleles occurred with a frequency of 1.5% across samples and 1.3% across loci (the most often on MM7). We performed Fst, Dest, Gst(Hed) with and without Mar80. As this locus had little influence on the overall result, we did not exclude it from further analyses.

For the purpose of the null allele detection analysis we used several different methods because any single method may give misleading results (Dąbrowski et al., 2014, 2015). All computations were performed for the whole data set and for each sampling site separately. The low accuracy of null allele detection and high inconsistency among results of the analyses by different methods showed that the detected putative null alleles were false positive, resulting probably from nonequilibrium populations (Dąbrowski et al., 2015) (for details see Supplementary information).

Kinship analysis

In a set of analyses, we wanted to include only unrelated individuals. Therefore we used Cervus, Kingroup (Konovalov et al., 2004) and Gimlet to detect kinship relations among individuals. All pairs

of relatives at the level of parent-offspring or full-siblings returned consistently by the programs were checked against the field data: time and place of capture, age and sexual maturity. Finally, we accepted 19 pairs of individuals as relatives. After excluding one individual of each pair (1 from SK2, 2 from SK3, 3 from SK1, AT, PL, NL and 4 from HU), we obtained a set of 173 unrelated individuals.

Genetic variability assessment

For individuals from each of the seven sampling sites as well as for local populations that clustered together and inhabited the same geographic region (hereafter regional populations), we computed a number of genetic parameters: the number of alleles (*Na*), the observed (*Ho*) and expected (*He*) heterozygosity using Gimlet; the number of private alleles (No. Private Alleles) and probability of identity (*PI*) in GenAlex 6 (Peakall and Smouse, 2006); the allelic richness (*Ar*) and deviations from Hardy-Weinberg equilibrium (HWE) using Fstat (Goudet, 1995); the coefficient of inbreeding (F_{IS}) using Genepop (Rousset, 2008). The effective population size (*Ne*) was computed using the linkage disequilibrium method implemented in NeEstimator V2.1 (Do et al., 2014) and in LDNe program (Waples and Do, 2008) after omitting alleles with frequency lower than 2% (P_{crit} = 0.02) and 5% (P_{crit} = 0.05). To verify hypotheses concerning the differences of variables for specific sampling sites or clusters we used parametric (Anova) or nonparametric (Kruskal-Wallis) statistical tests, depending on data normality. Chi-squared test was used for analysis of a categoric data. Pairwise comparisons of PL individuals with the individuals from remaining six sampling sites were performed using the Wilcoxon signed test with the Bonferroni correction.

Finally, to detect signals of genetic bottleneck we used the one-tailed Wilcoxon test (Luikart et al., 1998) implemented in Bottleneck 1.2.02 program (Cornuet and Luikart, 1996). We chose the Stepwise Mutation Model (SMM) and Two-Phase Model (TPM) with 90% of SMM as it is recommended for microsatellites using 10.000

interactions (Di Rienzo et al., 1994). A mode shift in allele frequencies was also assessed.

Genetic structure analysis

The genetic distance (F_{ST}) between groups was computed in Fstat with the Bonferroni correction. F_{ST} was originally developed to measure the genetic distance of biallelic markers (Wright, 1969) and therefore using GenAlex we also calculated the standardized G'_{ST}, for multiple alleles (Nei, 1973; Hedrick, 2005) and D_{ST} which measures the fraction of allelic variation among populations (Jost, 2008). The association of geographic and genetic distance (*Fst*, G'_{ST} and D_{ST}) between pairs of sampling sites or regional populations was verified using a Mantel test (Mantel, 1967). Moreover, for the purpose of the exploratory data analysis, we prepared the neighbour joining tree of genetic distances (D_{ST}) between the studied populations. For this purpose the online program Poptreew (Takezaki et al., 2014) was used. To compute the significance of differences of *Fst* values obtained in this study with other authors, we compared means and 95% confidence intervals (CI). Whenever the intervals did not overlap we assumed the mean values to differ significantly.

To assess the number of genetic clusters, we used Structure 2.3.4 (Pritchard et al., 2000) with the admixture model with correlated allele frequencies and no prior population information. There were 50 independent runs for the number of groups (K) between 1 and 15 with 100.000 burn-in period and 10^6 replicates. The most likely number of clusters was estimated using the ΔK approach (Evanno et al., 2005) implemented in Clumpak (Kopelman et al., 2015), which was also used to visualize the obtained results. Structure was remarked to bias results of evolutionary distant groups (Kalinowski, 2010) and was found to has a lower ability to assign individuals to populations of origin than classical assignment (Waples and Gaggiotti, 2006) as well as was found to be sensitive to patterns of isolation-by-distance (IBD) (Meirmans, 2012). Therefore we decided to use an alternative algorithm that integrates both the spatial prior and the likelihood of genetic data implemented in Baps (Corander et al., 2008; Cheng et al., 2013) applying 10.000 interaction. Calculations were performed on complete data (n = 192) and on unrelated individuals only (n = 173), and found no significant differences. Besides the bayesian clustering, we performed multivariate analyses which is less prone for bias due to the occurrence of linkage disequilibrium (Kaeuffer et al., 2007), number of loci (Berry et al., 2004) or dispersal rates among populations (Waples and Gaggiotti, 2006). For that purpose we performed a Discriminant Analysis of Principal Components (DAPC) (Jombart et al., 2010) implemented in Adegenet package (Jombart, 2008) for the R software (R Development Core Team, 2011). According to find.clusters analysis results, DAPC was run with various numbers of pre-defined clusters (K = 6, K = 4, K = 3)(for details see Supplementary information and Fig. S1-S3) as well as for the original seven populations.

Results

Genetic variability within sampling sites

The overall genetic variability was higher in the northern sampling sites (PL, NL) than in the southern (Pannonian region), except for AT (Table 1). The mean number of alleles ranged from 7.643 to 10.786 and the allelic richness from 5.925 to 7.831. The heterozygosity deficit was detected in all sampling sites and in consequence, positive F_{IS} values were obtained. When means of genetic variability were compared among all sampling sites (Kruskal-Wallis test) as well as in the pairwise comparison (Wilcoxon signed test) between PL and other sites, we found SK1 to be the least diverse. All Pannon-
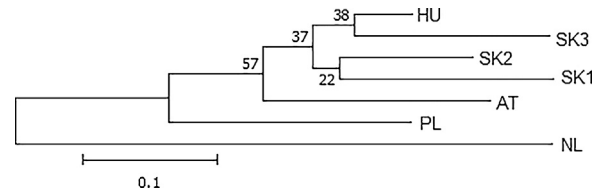

Fig. 2. Neighbour joining tree of genetic distances (D_{ST}) for all seven localities.

ian sampling sites except AT had significantly lower values than PL for at least two genetic parameters (Table 1). Unexpectedly, the genetic variability of NL was at the same level as for PL. From the Pannonian area AT had the highest genetic diversity which did not differ significantly from PL neither NL. Individuals from all sampling sites showed significant deviations from HWE toward heterozygote deficit. None of the genetic parameters was correlated with the number of individuals, even before the Bonferroni correction was applied.

The signs of genetic bottleneck were only detected for PL under the TPM model, but were not supported by SMM or the mode-shift test. The highest effective population size (*Ne*) was obtained for PL and was almost three to over six times higher than in the other sites, when alleles with a frequency lower than 2% were excluded (Table 1). When alleles with a frequency lower than 5% were excluded, the differences in *Ne* were not so great but remained statistically significant. The *Ne* for PL was similar to SK2, SK1 and NL, but still over three times higher than for SK3, HU and AT.

Genetic structure

The genetic distance computed between pairs of sampling sites was found to differ significantly between all of them regardless of the applied measure F_{ST}, Hendrick's G'_{ST} or D_{ST} (Table S1). All measures of genetic distance were significantly correlated with the geographic distance (Mantel test, $P_{FST} = 0.004$, $P_{G'ST} = 0.009$, $P_{DST} = 0.011$ respectively). In general HU exhibited low and similar genetic distance to all three Slovakian sampling sites as well as to AT. PL had the lowest genetic distance to HU at the first place and then to SK2. Moreover, the distance of PL to SK1 was at the same high level as between AT and SK1. At the same time PL distance to SK2 was at the same level as between AT and SK2. Finally, NL presented a similar genetic distance to all other sites with an exception of PL were it was lower (Table S1). We did not find the association of genetic and geographic distance among Pannonian sampling sites (Mantel test, $P_{FST} = 0.263$, $P_{G'ST} = 0.166$, $P_{DST} = 0.159$ respectively). Neighbour joining tree of genetic distances among all seven localities confirmed close relationships between HU and SK3, and between SK1 and SK2. AT was placed as the outer group to other Pannonian sites, followed by PL and NL (Fig. 2).

In order to assess genetic similarities among the considered subspecies and among all sampled local populations of Pannonian subspecies, we looked for genetic clusters as revealed by several methods. Two programs based on the Bayesian approach returned contrary results. Structure analysis of both datasets (all individuals and with relatives excluded) identified the uppermost hierarchical level of structure K = 2 as the most likely (ΔK = 1935.260, ΔK = 618.722 respectively). The second uppermost hierarchical level of structure was K = 6 (ΔK = 137.118, ΔK = 121.774 respectively). Here each cluster represented one sampling site with the exception of the samples from HU, which clustered mostly with SK3, and partly with SK1 and AT. For details about assignment probabilities see Table S2A. Here we present the assignment for the dataset of non-kin individuals only (Fig. 3). The results for all individuals are presented in Fig. S4.

Table 1
Comparison of genetic variability in the root vole populations at all sampled sites.

Variable		SK1	SK2	SK3	HU	AT	PL	NL	F	P	Test
N		22	32	24	20	21	23	31	2.89	>0.05	Chi
Na		7.643	9.000	8.500	8.929	8.857	10.500	10.786	12.648	**0.049**	KW
Ar		5.925	6.564	6.368	7.134	6.833	7.831	7.353	13.951	**0.030**	KW
Ho		0.559	0.669	0.741	0.679	0.636	0.719	0.680	9.509	0.147	KW
He		0.742	0.778	0.769	0.771	0.764	0.798	0.814	16.376	**0.0119**	KW
F_{IS}		0.253	0.151	0.057	0.116	0.157	0.110	0.147	5.997	0.424	KW
HWE		HD	HD	HD	HD	HD	HD	HD			
No.Private Alleles		3	3	1	4	6	15	16			
TPM 90%		0.620	0.213	0.548	0.335	0.077	**0.002**	0.941			
SMM		0.866	0.821	0.924	0.787	0.404	0.134	0.999			
Ne (**LDNe**)	$P_{cri} = 0.02$	187.7	130.2	105.6	146.2	281.3	638.3	369.8	361.2	**P<0.005**	Chi
	$P_{cri} = 0.05$	209.2	148.4	40.0	58.4	61.0	159.7	161.0	126.4	**P<0.005**	Chi
Ne (**NeEstimator**)	$P_{cri} = 0.02$	121.4	128.1	105.6	74.0	79.7	491.8	172.6	277.5	**P<0.005**	Chi
	$P_{cri} = 0.05$	128.1	145.0	40.0	43.3	37.0	147.5	112.1	100.3	**P<0.005**	Chi

N – the number of samples, *Na* – the mean number of alleles, *Ar* – allelic richness, *Ho* – observed heterozygosity, *He* – expected heterozygosity, F_{IS} – inbreeding coefficient, HWE – test for Hardy-Weinberg equilibrium (HD heterozygote deficit); No.Private Alleles – the mean number of private alleles; *Ne* – effective population size calculated using a linkage disequilibrium method with two programs given in brackets on $P_{crit} = 0.05$ and $P_{crit} = 0.02$; TPM 90% – two-phase model with 90% of SMM; SMM – stepwise mutation model; Chi – chi squared tests; KW – Kruskal-Wallis test.
Statistically significant results are bolded. In grey: values significantly (P< 0.05) different from PL (pairwise comparison using Wilcoxon signed test).

Admixture analysis based on the spatial clustering model implemented in Baps confirmed six clusters (K = 6) to be the most likely genetic structure (Fig. S5). Individuals from different study sites formed a separate cluster with an exception of those from SK3 and HU which clustered together (SK3_HU). Finally, using DAPC, we assigned individuals to 3, 4 and 6 clusters (Fig. 4) as well as to original seven sampling sites. The subdivision into three clusters (Fig. S6A) brought together individuals from PL, most of AT and SK3, over half of HU and a few from SK1, SK2 into cluster 1. It was found that this cluster was placed in the middle of the minimum spanning tree linking the remaining two clusters (Fig. 4A). NL formed a separate cluster. Finally, the last cluster accommodated most of individuals from SK1, SK2 and the remaining Pannonian individuals. The subdivision into four clusters revealed a clear substructure among Pannonian individuals. The other two clusters grouped separately individuals from the PL and NL sites (Fig. 4B, S6B).

When analysing assignment to six clusters, we observed a similar pattern as this obtained using Structure (Fig. S6C). The minimum spanning tree (Fig. 4D) supported the position of PL individuals as a link connecting NL individuals with individuals from Pannonian region. The probabilities of being assigned to one of the six or four clusters are given in Table S2.

Regional genetic variability

Considering the clustering results, population of origin and geographical specificity we defined *Microtus* regional groups. We found K = 3 to reflect some of the genetic similarities between PL individuals and group of individuals from the Pannonian lowland. But in general K = 3 is a superset of K = 4. At the same time the analy-

sis for K = 6 would be too similar to single sampling sites already shown. That is why we considered that separation of regional groups according to K = 4 to be more informative than the other two clustering results.

On average the genetic variability for these four regional groups was higher (Table 2) than for the single sampling sites (Table 1). What is important, this increase applies also to parameters independent of the sample size. One can realize, that the genetic variability of the two Pannonian regional groups becomes much more similar to the levels observed in the NL and PL populations (Table 2) than it was observed among sampling sites (Table 1). Besides that, the general pattern of other genetic estimates i.e. heterozygosity deficit, F_{IS}, bottleneck signs, *Ne* values was found to be similar with the previous results obtained for single sample sites.

Finally, the F_{ST} values between these groups ranged from 0.034 to 0.098 and differed significantly from each other (Bonferroni corrected *P* = 0.008). Although, Hendrick's G'_{ST} and D_{ST} reached higher values, they followed similar pattern as F_{ST} and also differ significantly between pairs of regions. The novelty here was that none of the measures of genetic distance correlated with the geographic distance (*P* > 0.05) (Table S3).

Discussion

Genetic variability and perspectives of relic Pannonian subspecies

Our analysis of the genetic variability of *M.o. mehelyi* carried out at the level of individual study sites has revealed a significant genetic depletion of alleles in the local populations, despite the fact that these populations resided in the habitats optimal for root voles
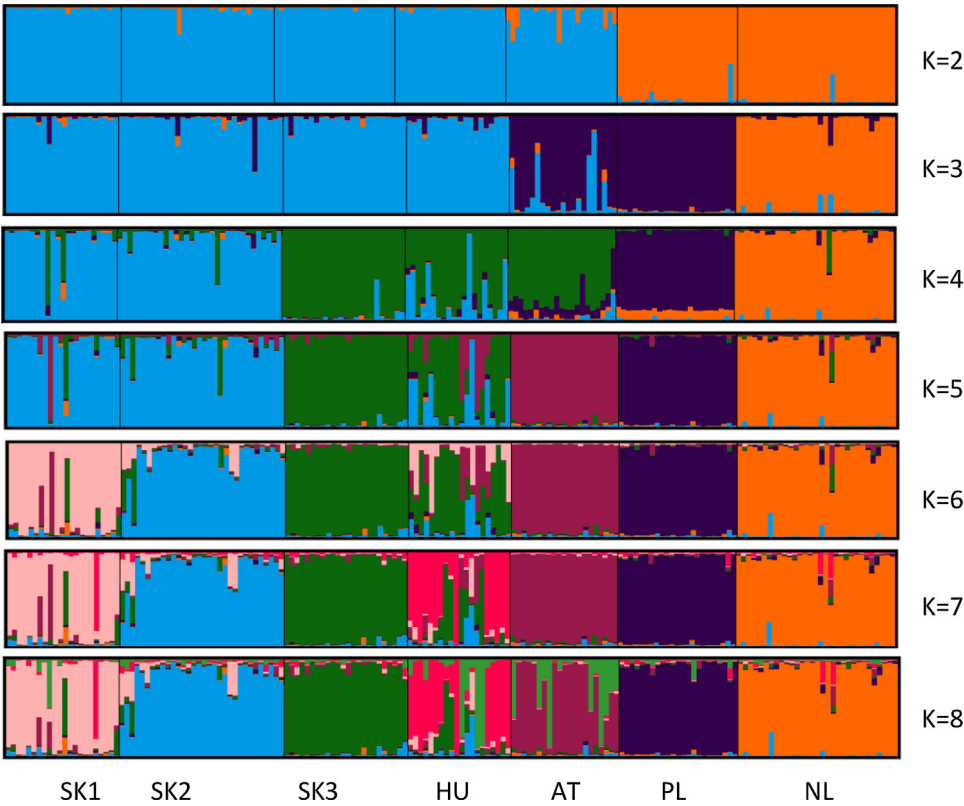
Fig. 3. Structure results based on non-kin individuals for a number of clusters K = 2-8. Each colour represents assignment probability of an individual to the cluster. Abbreviations of the original sampling sites explained below the frame.

Table 2
Comparison of genetic variability in four clusters identified in the genetic structure analysis.

		Cluster 1	Cluster 2	Cluster 3	Cluster 4	F	P	
N		23	31	65	54	13.77	**P < 0.005**	Chi
Na		8.072	8.009	12.500	10.429	4.922	0.178	KW
Ar		10.315	9.876	10.079	8.806	0.757	0.523	A
H_O		0.761	0.758	0.688	0.622	3.565	0.312	KW
H_E		0.857	0.818	0.813	0.794	6.220	0.101	KW
F_{IS}		0.110	0.147	0.140	0.214	1.478	0.231	A
HWE		HD	HD	HD	HD			
No.Private Alleles		15	16	16	6			
TPM 90%		**0.029**	0.805	0.620	0.729			
SMM		0.368	0.982	0.999	0.982			
Ne (**LDNe**)	$P_{crit} = 0.02$	638.3	369.8	104.8	125.6	181.640	**P < 0.005**	Chi
	$P_{crit} = 0.05$	159.7	161.0	70.6	86.9	29.66	**P < 0.005**	Chi
Ne (**NeEstimator**)	$P_{crit} = 0.02$	491.8	172.6	101.6	122.7	298.33	**P < 0.005**	Chi
	$P_{crit} = 0.05$	147.5	112.1	69.8	85.3	16.38	**P < 0.005**	Chi

Cluster 1 consists of samples from PL, cluster 2 of samples from NL, cluster 3 of samples from SK3_HU_AT, cluster 4 of samples from SK1_SK2 sampling site. Statistically significant results are bolded. Other symbols as in Table 1.

and therefore generally favourable for the maintenance of high genetic variability (Ehrich and Jorde, 2005; Vuorinen and Eskelinen, 2005; Pilot et al., 2010). In the 4 out of 6 variables (compared in Table 1) the significant differences between analyzed groups indicate a lower variability in the Pannonian root voles, especially SK1, SK2 and SK3, when compared with PL.

However, at the regional level, when the two groups of *M. o. mehelyi* were compared with the PL of *M. o. stimmingi* and the NL of *M. o. arenicola* (Table 2), most differences in the genetic variability indices become non-significant, indicating a similar amount of genetic variability in each of the three subspecies (except for the *Ne* – effective population size). Although *Ne* is a key evolutionary genetic parameter, there is doubt about the expressiveness, when populations are not ideal i.e. there is no random mating (Fewster, 2016) and a small sample size is tested (Russell and Fewster, 2009).

In our case, despite sample size increase (due to combining individuals from several sampling sites) in the two Pannonian regional groups, *Ne* remained unchanged. These results lead us to the conclusion that (1) the overall genetic variability in the Pannonian subspecies is still relatively high and has not been significantly depleted despite the long- term postglacial isolation from the main range of the root vole, but (2) the local populations of the *M.o. mehelyi* now live in isolation from one another and the genetic variability has significantly eroded at each site.

Generally, genetic variability indicates the population's long-term potential for adaptability and persistence. When starting this study we expected that the variability of *M.o. mehelyi*, as a post-glacial relic, might be depleted due to (1) the insular character of its range precluding dispersal and (2) significant changes of Pannonian region in the last centuries that increased wetland fragmenta-
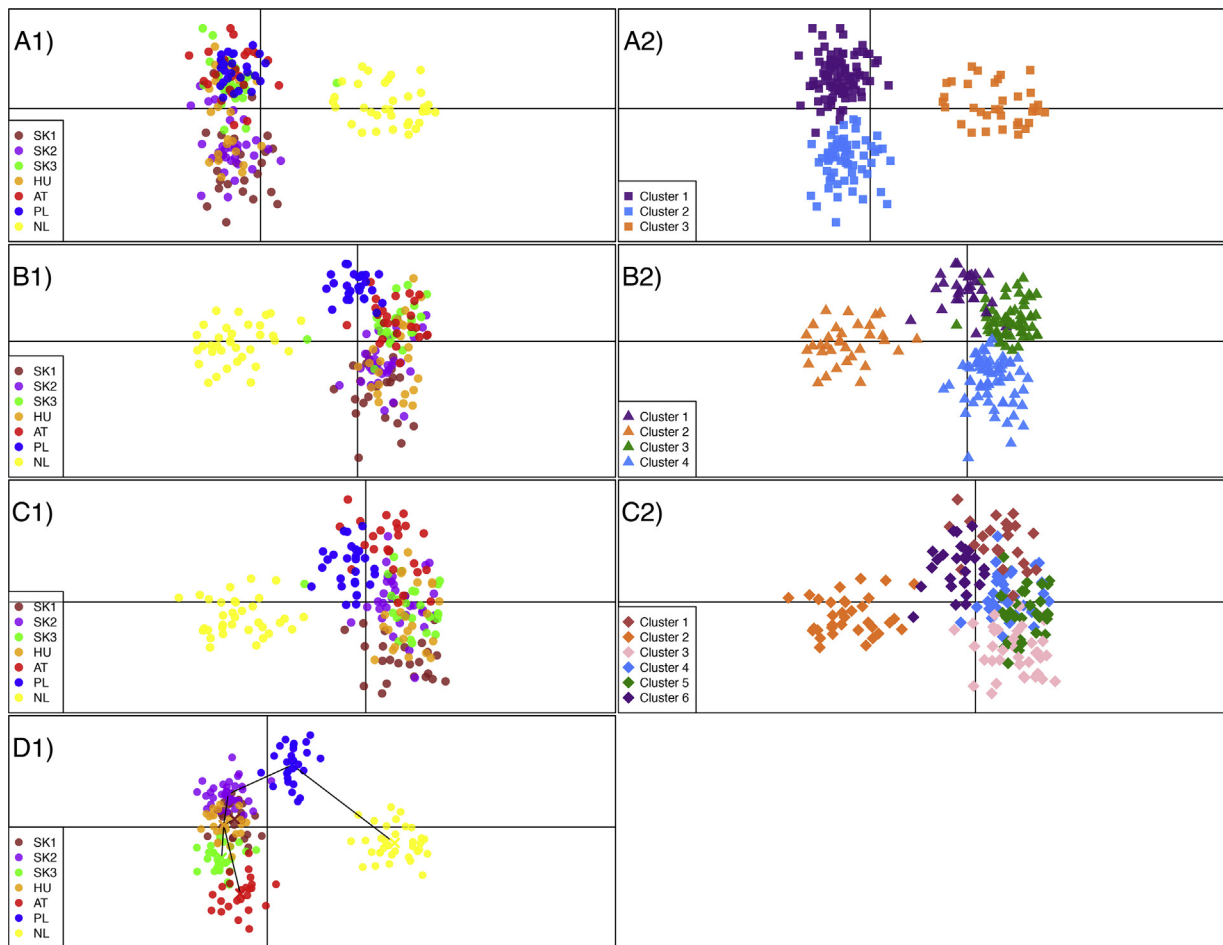
Fig. 4. Individuals assigned to respective clusters (right column) coming from sampling sites (left column), returned by Adegenet as the most likely genetic structure in case of: A) three, B) four or C) six clusters D) seven sampling sites distinguished. Cluster centres are connected by a minimum spanning tree based on the squared distances between clusters within the entire space.

tion and loss of connection between local populations. However, our results indicating the relatively high allelic richness for this subspecies have not supported these concerns indicated by a relatively high allelic richness for the subspecies. Additionally, other studies on morphological traits evidenced a high adaptability of root voles to local conditions (Bauer, 1953; Van Wijngaarden and Zimmermann, 1965; Baláž and Fraňová, 2013). Baláž and Fraňová found that *M.o. mehelyi* individuals from Slovakia were smaller and lighter, but had longer tails and hind feet than *M. o. stimmingi* individuals from higher latitudes (Poland). Therefore root voles exhibit patterns of adaptation to a warmer and colder climate respectively in accordance with Bergmann's rule (Meiri and Dayan, 2003).

Our conclusion concerning adequate genetic variability of Pannonian subspecies maybe somewhat too optimistic, because it is based on the comparison of pooled genetic data from several *M.o. mehelyi* populations with the genetic variability of a single local population of *M.o. stimmingi* and of *M. o. arenicola*. This may require some further support. However, in the Dutch postglacial relic subspecies studied thoroughly by Van de Zande et al. (2000), the genetic variability at the microsatellite level also did not seem to be significantly impoverished.On the other hand, the depletion in genetic variability of the local Pannonian populations, manifested in a reduced number of alleles, allelic richness and expected heterozygosity, is typically caused by genetic drift or/and founder effect in absence of immigration, and evidently results from insular distribution of fragmented populations of *M.o. mehelyi*. Similar trends in genetic depletion were shown by Neuwald (2010) for

endangered fragmented populations of *M. californicus scirpensis* in comparison to the widely distributed subspecies of *M. c. sanctidiegi* demonstrating the vulnerability of fragmented populations with lowered genetic diversity to extinction (Lande, 1999). Even though the Pannonian populations exhibit a similar amount of genetic variability to local populations of *M.o. arenicola* and a somewhat higher amount than isolated populations of *M.o. finmarchicus* studied by Van de Zande et al. (2000), the loss of genetic variability is evident when compared with the population situated in the main range of the root vole (*M.o. stimmingi*), in vast optimal habitat open to immigrants (Pilot et al., 2010). In summary, it seems that limited migration has reduced the genetic variability of the aforementioned *Microtus oeconomus* subspecies except for *M.o. stimmingi*.

The cluster analysis (K = 6) which has grouped our five studied Pannonian populations into four different clusters, each site separately (except for the Hungarian one), clearly indicated their genetic distinctness as a result of the lack of connectivity which precludes free gene-flow among these populations. HU individuals were clustered with root voles from the other sites in various configurations, depending on the method (Structure, Adegenet or Baps – Fig. 3, 4, Fig. S5). This indicates some less restricted migration through the Hungarian site, confirmed by the low pairwise *Fst* values of HU with the other Pannonian sites. The HU sampling site is located in the rich network of the Danube branches (inland delta area – Fig. 1) with hydrophilous vegetations providing suitable migration routes for *M. oeconomus* individuals, similar to those present along ditches in the Netherlands (Mauritzen et al., 1999). These routes connect or

connected in the past extensive marshes in the north of Hungary along the Danube with the Neusiedlersee-Hanság area where the AT sampling site is located.

Even the main river bed of the Danube does not provide a significant barrier for migrants as regular floods probably support passive transport of individuals. On the other hand, the sites that are isolated by distance from the Danube river and the inland delta system, like SK1 and SK2, are surrounded by an agricultural landscape without suitable passages for root vole migrants. The neighbour joining tree (Fig. 2) well supports this view, grouping together HU and SK3, but not other SK populations.

It was documented that the advanced fragmentation of the Pannonian root vole habitats as well as the loss of suitable humid habitat has mainly occurred in the 20th century (Dudich et al., 1985; Pachinger, 1994, 2003; Stollmann and Ambros, 1998; Gubányi et al., 2009; Hulejová Sládkovičová et al., 2013). However, as we pointed out above, the overall genetic variability of *M.o. mehelyi* (observed heterozygosity and allelic richness) was significantly higher for the two Pannonian regional groups than for each of the sampling sites, and so its genetic variability has not been lost yet and can possibly be restored at the local populations' level. The most urgent measure for the efficient conservation of the subspecies is restoration and protection of migration routes through proper habitat management. This will prevent the formation of small isolated populations which are most sensitive to extinction (Lande, 1999). If habitat restoration is not possible or only in the long term, it is, from a conservation point of view, an option to start with genetically controlled reintroductions or supplementations of *M.o mehelyi* into suitable habitats (La Haye et al., 2017) to strengthen local populations and to restore genetic diversity at the local level.

Insight into the past and present distribution of the root vole from microsatellite DNA and mtDNA analyses

All sampling sites included in our study represent the Central European phylogroup according to an extensive mtDNA study of *M. oeconomus* (Brunhoff et al., 2003). That study reported that one haplotype (Hun-Slo) was shared by root voles from Hungary and Slovakia, and indicated its close genetic distance to haplotypes from Poland. In the paper by Dąbrowski et al. (2013) concerning mtDNA of voles from the PL sampling site, several new haplotypes were reported and a close relation of the haplotype Hun-Slo with other haplotypes from this and other Polish populations was confirmed. At the same time, haplotypes reported from the Netherlands (by Brunhoff et al., 2003) were placed as the most outer branches at the median-joining network, with their genetic origin derived from ancestors inhabiting the current territory of Poland (Dąbrowski et al., 2013; Jancewicz et al., 2015). This confirms the hypothesis of a longer connection between *M.o. mehelyi* and *M.o. stimmingi* than between any of the two subspecies with *M.o. arenicola* subspecies. This is in concordance with our minimum spanning tree (Fig. 4) where *M.o. stimmingi* is in the middle between the two relics.

In future studies it will be interesting to see if the differentiation of the Pannonian voles into two regional groups, as revealed in our study can be confirmed by the detailed mtDNA analysis.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.mambio.2017.11.007.

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Data Accessibility

Microtus oeconomus genotypes and GPS coordinates are available on Dryad, doi: https://doi.org/10.5061/dryad.56c9p.