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Short Communication

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mtDNA evidence for a local northern latitude Pleistocene refugium for the root vole (*Microtus oeconomus*, Arvicolinae, Rodentia) from Eastern Poland

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Abstract

We analysed the genetic structure of 33 populations of the root vole (tundra vole, *Microtus oeconomus*, Pallas, 1776) inhabiting their typical habitats, located at different distances from the southern boundary of the species' range (52°14′–53°56′ N) in eastern Poland. We determined its phylogeographic pattern as well as the possible occurrence of a small, local high-latitude refugium of this species in southern Poland, previously suggested in palaeontological studies. 908 bp of cytochrome *b* sequences were analysed from 439 root voles, and 21 mtDNA cytb haplotypes belonging to the Central European (CE) phylogroup were found. Haplotype diversity in the examined populations varied between 0 and 0.872 (mean: 0.425 ± 0.332), while nucleotide diversity ranged between 0 and 0.62% (mean: 0.235% ± 0.217). Within the CE phylogroup of *M. oeconomus*, we identified with high bootstrap support a newly separated group of *M. oeconomus* that evolved from CE, denoted CE-PL S. This group is located in the southern and central part of eastern Poland and most likely diverged from phylogroup CE in a small, cryptic refugium situated in southern Poland, in the Kraków-Częstochowa Upland and/or the Holy Cross Mountains during the LGM and Younger Dryas.

Key words: Cytochrome *b* gene – high-latitude glacial refugium – *Microtus oeconomus* – phylogeography

Introduction

During the Pleistocene, the northern hemisphere was affected by rhythmic climatic changes resulting in subsequent coolings and warmings, associated with respective development and retreat of ice sheets. The appearance of ice sheets during cool periods caused the retreat of plants and animals from inhabited areas. The areas were recolonized after the disappearance of glacial cover during the warm periods separating the glaciations.

When Northern Europe was covered by an expanding ice sheet, temperate species found advantageous conditions in the southern part of continent, on the Iberian, Apennine and Balkan Peninsulas (Willett 1950; Bennett et al. 1991). Therefore, these regions were recognized as glacial refugia (Frenzel and Troll 1952; Taberlet et al. 1998; Hewitt 1999) and sources of postglacial migration of species to Central and Northern Europe. However, the role of Mediterranean refugia in the postglacial history of species is still unclear, mostly due to recent molecular studies in which palaeontological data were verified and completed (Bilton et al. 1998; Stewart and Lister 2001). Such a comprehensive approach deeply changed the state of knowledge and understanding of the distribution and function of particular refugia in postglacial colonization of Europe. According to Bilton et al. (1998), they served as zones of endemism rather than refugia, while species of the temperate zone survived the glaciation at other sites of favourable climate, located to the north of regions as earlier suggested by Taberlet et al. (1998) and Hewitt (1999). One such European area, dominated by climatic conditions advantageous for survival and serving as the origin of postglacial colonization of Central and Northern Europe, covered the Carpathians (e.g. Bilton et al. 1998). This region includes many sites with sediments of the last glaciation bearing pollen records of numerous tree species that are contemporarily observed in Northern and Central Europe (Willis et al. 2000), as well as remains of numerous vertebrate species (Kowalski 2001; Pazonyi 2004; Sommer and Nadachowski 2006). Its existence was confirmed by means of molecular methods on present-day populations of various plant and animal species (e.g. Bilton et al. 1998; Jaarola and Searle 2002; Kotlík et al. 2006; Wójcik et al. 2010; Fijarczyk et al. 2011).

The extensive Carpathian refugium is likely to be accompanied by other refugia located in Eastern and Central Europe or on the border between Europe and Asia: in the Crimea, north of the Alps, north of the Caucasus Mountains and north of the Ural Mountains, as well as on the Russian Plain (e.g. Markova 1984; Hewitt 1999; Schmitt 2007; Svenning et al. 2008; Markova 2011; Bilton et al. 1998). Furthermore, the proposed colonization routes of Northern and Central Europe became complicated by findings of numerous vertebrate remains providing fossil evidence for the likely presence of small, local Northern and Central European refugia located at higher latitudes than the Carpathian or southern refugia of the Iberian, Apennine and Balkan Peninsulas (e.g. Cruzan and Templeton 2000; Schmitt 2007). These small local refugia were referred by Stewart and Lister (2001) as cryptic northern refugia. They may have existed in any place that contained even small areas where climatic and habitat conditions were suitable enough to enable survival during glaciations. They may have been situated even very close to the ice-sheet limit (e.g. in southern and central Poland; Madeyska 1981; Nadachowski 1989) or formed nunataks, that is, enclaves elevated above the ice sheet (in northern Scandinavia; Fedorov and Stenseth 2001). According to Cruzan and Templeton (2000); Stewart and Lister (2001); Pearson (2006); Schmitt (2007), these small, local, high-latitude refugia could have been much more important for postglacial colonization than the southern ones. Small cryptic refugia may have become the source of migration that gave origin to new populations of cold-adapted species therefore greatly intensifying the process of colonization after ice-sheet retreat (Pearson 2006).

Abundant fossil and molecular records confirm the survival of numerous plant, invertebrate and vertebrate species in high-lati-

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tude refugia of various continents during the last glaciation (e.g. Fedorov and Stenseth 2001; Galbreath and Cook 2004; Brunhoff et al. 2006; Sommer and Nadachowski 2006; Provan and Bennett 2008; Fløjgaard et al. 2009; Markova 2011). In an area of Poland (the Kraków-Częstochowa Upland and the Pieniny Mountains, Fig. 1), Pleistocene sediments dated to the last glacial maximum (LGM) also comprise remains of several rodent species typical of various environments (Madeyska 1981; Nadachowski 1989; Nadachowski et al. 1993; Sommer and Nadachowski 2006). These findings included remains of the root vole (tundra vole, *Microtus oeconomus*) that coexisted during the LGM with, for example, lemmings (*Lemmus lemmus*), bank voles (*Myodes glareolus*) and narrow-headed voles (*Microtus gregalis*). This may indicate the existence of such a local refugium during the LGM in Poland. The root vole is a boreal species whose present range covers north-eastern Europe, Central and Northern Asia and the northern part of North America. The European range of this species includes Scandinavia, Poland, Lithuania, Latvia, Russia, Belarus, Ukraine, north-eastern regions of Germany and isolated populations in Hungary, Slovakia and the Netherlands (van Apeldoorn 1999). The western boundary of the range of *M. oeconomus* runs along the Oder river, while the southern boundary passes through southern Poland (Sałata-Piłacińska 1990). The root vole is a species that prefers humid meadows and bog alder forests and frequently inhabits wetlands in river valleys (Gliwicz and Jancewicz 2004). Studies on the phylogeography of the *M. oeconomus* have been conducted for several years (Brunhoff et al. 2003; Haring et al. 2011). Root voles from Central Europe and southern Scandinavia belong to the Central European phylogroup, which is one of four phylogroups presently distinguished for the species (Brunhoff et al. 2003). The authors used samples from only seven individuals at five Polish locations. Furthermore, one intensively studied population in eastern Poland (9 years, 169 individuals) appeared to include eight haplotypes, four of which were not previously recorded (Dąbrowski et al. 2013). This suggests high haplotype diversity in this part of Poland. Nevertheless, intensive phylogeographic analyses for *M. oeconomus* using molecular tools have still not been performed for a larger area of the country, especially where the species reaches its southern boundary, close to the areas it was recorded during the LGM (Madeyska 1981; Nadachowski 1989). In this study, we analysed the genetic structure of 33 root vole populations inhabiting eastern Poland, determined their phylogeographic pattern and investigated the possible occurrence of a small, local high-latitude refugium of *M. oeconomus* in southern Poland.

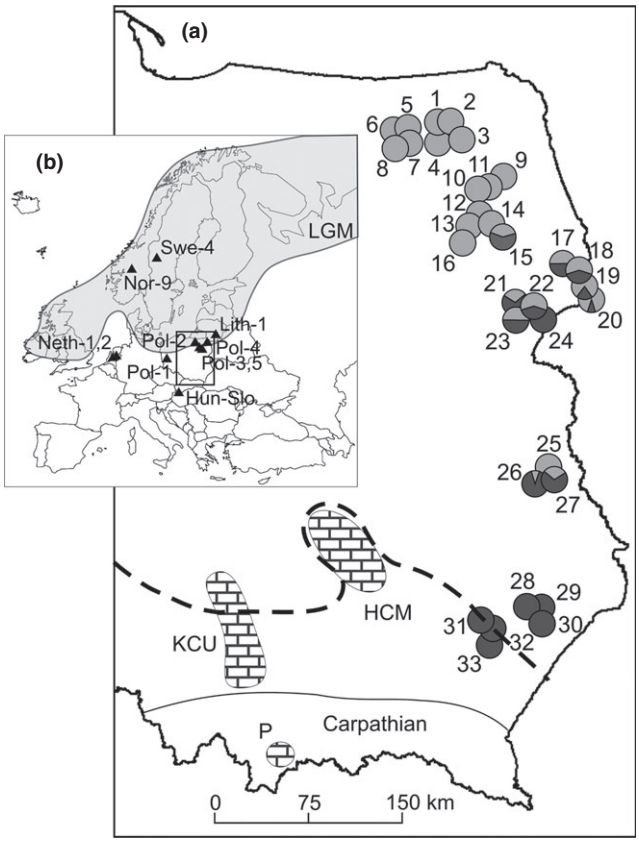

Fig. 1. (a) Location of analysed *Microtus oeconomus* populations and sampling sites in eastern Poland (1-33) and distribution of phylogenetic groups. The locations are described in Table 2. The frequency of haplotypes from the CE-PL S group is shown in dark grey. The map includes the southern boundary of the present-day range of species in East Poland (Sałata-Piłacińska 1990) (dashed line) and sites with remains of *M. oeconomus* preserved from the LGM (Madeyska 1981; Nadachowski 1989; Nadachowski et al. 1993): KCU – Kraków-Częstochowa Upland, HCM – Holy Cross Mountains, P – Pieniny Mountains. – Southern boundary of range of *M. oeconomus*. (b) Distribution of haplotypes from the CE group of *M. oeconomus* from previously published results, which was used to construct a network (black triangles) and ice sheet during the LGM (Marks 2002).

Materials and methods

Animal trapping

Intensive live trapping was carried out over the years 2008–2010 in eastern Poland (52°14′–53°56′ N), on 33 root vole populations inhabiting their optimal habitats located at different distances (0–400 km in a straight line) from the southern boundary of the root vole's range (Fig. 1, Table 1). The catch–mark–release (CMR) method, standardly applied in small rodent trapping, was used to collect tissue (ear punch samples) samples for genetic studies from 439 individuals (7–23 from each population). For each individual, its sex, body mass, approximate age and reproductive status were recorded. After measurements and sampling, all animals were released at the site of trapping. Analysis of variance (ANOVA) (NIR test – least significant differences test) was used to analyse body masses of adult male voles. Females were excluded from body mass analysis to remove possible confounding effects of their reproductive cycle. Young and adolescent individuals were also excluded. STATISTICA 10 (StatSoft Inc. 2012) was used to perform statistical analyses.

DNA extraction and sequencing

Genomic DNA was extracted from root vole tissue samples with the Genomic Mini Kit (A&A Biotechnology, Gdynia, Poland. The 908 bp fragment of the cytochrome *b* gene (mitochondrial DNA) was amplified in two separate PCRs using two primer pairs designed with FastPCR 5.4 (http://primerdigital.com/fastpcr.html) and based on root vole cytochrome *b* sequences available in GenBank. The primer sequences were as follows: Mo1-cytbF 5′-CATGATGAAACTTCGGCTCC-3′, Mo1-cytbR 5′-GTCTCCGAGAATATCTGGGA-3′ and Mo2-cytbF 5′-AGACAAAGC CACCCTCACAC-3′, Mo2-cytbR 5′-GAGGCTGTTTGTCCGATGAT-3. These two PCR products resulted in two overlapping cytochrome *b* fragments: from 86 to 756 nucleotide, 670 bp long and from 510 to 1064 nucleotide, 554 bp long of the cytochrome *b*, respectively.

The PCRs were carried out in the GeneAmp PCR System 9700 DNA thermocycler (Applied Biosystems, a part of Thermo Fisher Scientific, Waltham, Massachusetts, USA), using the Multiplex PCR Master Mix kit (Qiagen, Hilden, Germany). The reaction mixture was prepared in accordance with the manufacturer's recommendations. PCR was performed as follows: initial denaturation at 95°C for 15 min, followed by 30–35 cycles including three steps: denaturation at 94°C for

Table 1. Geographic information and the GenBank accession numbers of *Microtus oeconomus* mitochondrial cytb gene haplotypes and source of information. The data are divided into four phylogroups according to Brunhoff et al. (2003). Haplotypes correspond to those shown in Fig. 2.

I. p.	Phylogroup	Country	Haplotype	Identical haplotype	GenBank accession number	Source
	Central European					
1		Poland	PL-1		KP684101	This study
2			PL-2		KP684102	This study
3			PL-3	Pol-3	KP684103, AY220010	This study, Brunhoff et al. (2003)
4			PL-4		KP684104	This study
5			PL-5		KP684105	This study
6			PL-6		KP684106	This study
7			PL-7		KP684107	This study
8			PL-8		KP684108	This study
9			PL-9		KP684109	This study
10			PL-10		KP684110	This study
11			PL-11		KP684111	This study
12			PL-12		KP684112	This study
13			PL-13	Pol-5	KP684113, AY220013	This study, Brunhoff et al. (2003)
14			PL-14		KP684114	This study
15			PL-15		KP684115	This study
16			PL-16		KP684116	This study
17			PL-17		KP684117	This study
18			PL-18		KP684118	This study
19			PL-19		KP684119	This study
20			PL-20		KP684120	This study
21			PL-21		KP684121	This study
22			Pol-1		AY220008	Brunhoff et al. (2003)
23			Pol-2		AY220009	Brunhoff et al. (2003)
24			Pol-4		AY220012	Brunhoff et al. (2003)
25		Lithuania	Lith-1		AY220011	Brunhoff et al. (2003)
26		Hungary	Hun-Slo		AY220014	Brunhoff et al. (2003)
27		Netherlands	Neth-1		AY220006	Brunhoff et al. (2003)
28			Neth-2		AY220007	Brunhoff et al. (2003)
29		Norway	Nor-9		AY220005	Brunhoff et al. (2003)
30		Sweden	Swe-4		AY220003	Brunhoff et al. (2003)
	North European					
31		Belarus	Bel-1		AY219998	Brunhoff et al. (2003)
32		Norway	Nor-2		AY219982	Brunhoff et al. (2003)
33			Nor-4		AY219984	Brunhoff et al. (2003)
34			Nor-5		AY219985	Brunhoff et al. (2003)
35			Nor-6		AY219987	Brunhoff et al. (2003)
36			Nor-7		AY219988	Brunhoff et al. (2003)
37			Nor-10		DQ452134	Brunhoff et al. (2006)
38			Nor-11		DQ452135	Brunhoff et al. (2006)
39			Nor-12		DQ452136	Brunhoff et al. (2006)
40			Nor-13		DQ452137	Brunhoff et al. (2006)
41			Nor-15		DQ452139	Brunhoff et al. (2006)
42			Nor-16		DQ452140	Brunhoff et al. (2006)
43			Nor-18		DQ452142	Brunhoff et al. (2006)
44		Finland	Fin-1		AY219986	Brunhoff et al. (2003)
45			Fin-2		AY219990	Brunhoff et al. (2003)
46			Fin-3		AY219991	Brunhoff et al. (2003)
47			Fin-4		AY219992	Brunhoff et al. (2003)
48			Fin-5		AY219993	Brunhoff et al. (2003)
49			Fin-6		AY219997	Brunhoff et al. (2003)
50			Fin-Swe		AY219989	Brunhoff et al. (2003)
51		Sweden	Swe-1		AY219994	Brunhoff et al. (2003)
52			Swe-2		AY219995	Brunhoff et al. (2003)
53			Swe-3		AY219996	Brunhoff et al. (2003)
54		Russia	Rus-1		AY219999	Brunhoff et al. (2003)
55			Rus-2		AY220000	Brunhoff et al. (2003)
56			Rus-3		AY220001	Brunhoff et al. (2003)
57			Rus-4		AY220002	Brunhoff et al. (2003)
	Central Asian					
58		Russia	Rus-5		AY220015	Brunhoff et al. (2003)
59			Rus-6		AY220016	Brunhoff et al. (2003)
60			Rus-7		AY220017	Brunhoff et al. (2003)
61			Rus-8		AY220018	Brunhoff et al. (2003)
62			Rus-9		AY220019	Brunhoff et al. (2003)
63			Rus-10		AY220020	Brunhoff et al. (2003)
64			Magadan1		AY305210	Galbreath and Cook (2004)
65			Magadan2		AY305209	Galbreath and Cook (2004)
	Beringian					

Table 1. (continued)

I. p.	Phylogroup	Country	Haplotype	Identical haplotype	GenBank accession number	Source
66		Russia	Rus-11		AY220021	Brunhoff et al. (2003)
67			Rus-12		AY220022	Brunhoff et al. (2003)
68			Bolshaya		AY305188	Galbreath and Cook (2004)
69			Kamchatka		AY305183	Galbreath and Cook (2004)

30 s, annealing at 57°C for 90 s and elongation at 72°C for 60 s. The cycles were followed by final elongation of PCR products at 60°C for 30 min. The PCR products were purified with ExoI and SAP enzymes (Fermentas), in a mixture prepared according to the producer's recommendations. The purified DNA strands were sequenced in both directions with the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit 3.1 (BDT 3.1; Applied Biosystems), in accordance with the manufacturer's recommendations and using the same primers (one of them each time) as for PCR. Sequencing products were purified of unincorporated ddNTPs with the ExTerminator kit (A&A Biotechnology). Sequencing products were separated by capillary electrophoresis and analysed in the ABI PRISM 3130 automated DNA sequencer (Applied Biosystems). DNA sequences were aligned to each other (the size of the overlapping region was 246 nucleotides) and 50 cytochrome *b* gene complete sequences downloaded from GenBank (Table 1) with the BIOEDIT v 7.0.4 software (Hall 1999).

Phylogenetic analysis

Phylogenetic relationships among mtDNA cytochrome *b* haplotypes (908 bp fragment, from 138 to 1046 nucleotide of the gene, e.g. obtained alignment without primers) were illustrated in a neighbour-joining (NJ) as well as maximum likelihood (ML) trees of haplotypes constructed in the MEGA v.5.05 software (Tamura et al. 2011). The Tamura and Nei + G model of sequence evolution was used because it was determined as the best-fit model by the Bayesian information criterion (BIC) and Akaike information criterion (AIC) tests in MEGA. One thousand bootstrap replicates were used to assess support for tree nodes.

We used 48 previously published root vole haplotypes (Brunhoff et al. 2003; Galbreath and Cook 2004) of the GenBank accession numbers listed in Table 1.

These sequences represent all known mtDNA phylogroups of the species (Central European, North European, Beringian and Central Asian, respectively). We also created a median-joining network with default settings for the mtDNA cytochrome *b* gene haplotypes using the program NETWORK v.4.6.1.0 (Bandelt et al. 1999).

The net pairwise divergence (d_A) among the identified mtDNA groups of haplotypes in our study was calculated using MEGA. We used BEAST v.1.7.2 (Drummond et al. 2012) to calculate the time of divergence from the most recent common ancestor (tMRCA) and 95% highest posterior density interval, HPD with different molecular clock rates: 0.75–1.30 × 10^{-7} substitutions per site per year (Brunhoff et al. 2003, 2006) and 4.572 × 10^{-7} substitutions per site per year (Herman et al. 2014).

ARLEQUIN 3.11 (Excoffier et al. 2005) was used to estimate the number of haplotypes (N_h), haplotype diversity (*h*) and nucleotide diversity (π, in %). Genetic differentiation between populations was assessed as pairwise F_{ST} and statistically tested in ARLEQUIN (1000 permutations).

Principal component analysis (PCA) was performed for mtDNA F_{ST} data in GENALEX v6.0 (Peakall and Smouse 2006). Analysis of molecular variance (AMOVA; Excoffier et al. 1992) using ARLEQUIN (with 10 000 permutations) was performed to assess structuring within the data, where sampling sites were grouped as a single population. We also applied the spatial AMOVA procedure using SAMOVA ver. 1.0 (Dupanloup et al. 2002). Significance of Φ-statistics was tested by 10 000 permutations for $K = 2$ to $K = 7$ partitions of sampling sites. Subsequently, we used SAMOVA results of grouping to calculate AMOVA with F_{ST} in ARLEQUIN.

STATISTICA 10 (StatSoft Inc. 2012) was used to calculate the correlation between latitude and number of haplotypes in each population.

Results

Analysis of mtDNA cytb

The 33 studied populations of *M. oeconomus* in eastern Poland (Fig. 1, Table 2, 439 individuals in total) included 21 mtDNA cytb haplotypes (KP684101–KP684121, Tables 1–3). Haplotype diversity (*h*) in populations varied between 0 and 0.872 (mean for 33 populations: 0.425 ± 0.332), while nucleotide diversity (π) ranged between 0 and 0.62% (mean: 0.235% ± 0.217%) (Table 4).

Twenty-one haplotypes detected in our study as well as 48 cytb distinct sequences obtained from GenBank (Table 1) provided a basis for developing a network of phylogenetic relationships between 69 *M. oeconomus* haplotypes (Fig. 2a), clearly divided into the four phylogroups defined by Brunhoff et al. (2003). All haplotypes from eastern Poland belong to the Central European (CE) phylogroup (Table 1 and Fig. 2a), in which Polish haplotypes cluster with those of Lithuania (Lith-1), Hungary/Slovakia (Hun-Slo), the Netherlands (Neth-1, Neth-2) and southern Scandinavia (Nor-9, Swe-4).

Among haplotypes recorded in eastern Poland within the CE phylogroup, a distinct haplotype group (CE- PL S) was identified and included PL7, PL9, PL15 and PL16 haplotypes (Fig. 2b). This group differs from haplotypes PL-3 or PL-1 by at least five substitutions. The distinctiveness of this group was also confirmed in the phylogenetic NJ tree with high bootstrap support (100%, Fig. 3). The topology of ML and Bayesian trees (not shown) and bootstrap values as well as Bayesian posterior probability values was very similar to those obtained in NJ tree (Fig. 3). The net divergence between CE and CE-PL S was 0.5% ± 0.02 (TN + G), while between CE and NE phylogroups the corresponding value was 1.9% ± 0.04. The estimated tMRCA for CE phylogroup and CE-PL S based on the 7.5% rate (75.6 ka BP, 95% HPD 47.8–106 ka BP) and 13% rate (44.3 ka BP, 95% HPD 29.6–61.4% HPD) correspond to the last glacial period. On the other hand, the tMRCA for CE and CE-PL S haplotype group was estimated at 12.9 ka BP (95% HPD 8.61–17.3 ka BP) using the molecular clock rate recently suggested by Herman et al. (2014). For CE and NE haplogroups, the tMRCA was 36.6 ka BP (95% HPD 26.2–48.2 ka BP). Considering the geographic distribution of included haplotypes, this distinct group (CE-PL S) is referred to as the South Polish group within the Central European phylogroup (CE) (Fig. 2b). It comprises only four haplotypes, detected in nearly 35% of examined individuals from 17 populations among the 33 studied (Tables 3 and 4). Within this group, haplotype PL-9 was the most frequent and most widespread haplotype in eastern Poland (Table 3). This haplotype was fixed in all six southern populations studied. Other haplotypes (PL-7, PL-15 and PL-16) in this group are rare or are singletons (Table 3). Among the 33 studied populations, seven possessed haplotypes only of the CE-PL S group and 16 did not include haplotypes of this group, while in 10 populations (30.3%), haplotypes from the CE-PL S group and from CE phylogroup were present

Table 2. Location of examined root vole (*Microtus oeconomus*) populations, number of haplotypes and the most frequent haplotypes

Location in Fig. 1		Geographic coordinates		*N*	Haplotypes	N_h	
		Latitude N	Longitude E			CE	CE-PL S
1	Szóstak	53° 56′ 28.63″	22° 11′ 16.44″	12	PL-1, PL-2, PL-3, PL-4	4 (PL-2)	–
2	Zawady Ełckie	53° 55′ 59.71″	22° 14′ 19.09″	12	PL-2, PL-4, PL-5	3 (PL-5)	–
3	Miluki	53° 52′ 35.54″	22° 22′ 02.18″	12	PL-3, PL-4	2 (PL-3)	–
4	Skomack Wielki	53° 51′ 34.27″	22° 04′ 57.85″	16	PL-3, PL-6	2 (PL-3)	–
5	Miłki	53° 55′ 59.12″	21° 51′ 06.87″	20	PL-3	1 (PL-3)	–
6	Paprotki	53° 55′ 05.65″	21° 47′ 48.45″	12	PL-1, PL-3	2 (PL-3)	–
7	Nietlice	53° 52′ 86.41″	21° 50′ 50.57″	12	PL-1, PL-3	2 (PL-1)	–
8	Dziubiele	53° 49′ 03.27″	21° 45′ 59.94″	13	PL-1, PL-3	2 (PL-3)	–
9	Dolistowo	53° 33′ 23.78″	22° 54′ 54.53″	13	PL-1, PL-4, PL-11, PL-13	4 (PL-1)	–
10	Osowiec	53° 29′ 11.67″	22° 37′ 52.46″	13	PL-1, PL-3, PL-4, PL-11, PL-13, PL-18	6 (PL-1)	–
11	Dawidowizna	53° 29′ 53.89″	22° 45′ 39.61″	11	PL-1, PL-4, PL-5, PL-11, PL-12, PL-13	6 (PL-4)	–
12	Wielka Luka	53° 17′ 12.93″	22° 36′ 36.67″	13	PL-3, PL-11, PL-12, PL-13, PL-19, PL-20, PL-21	7 (PL-13)	–
13	Kleszcze	53° 13′ 21.61″	22° 31′ 34.33″	12	Pl-1, PL-3, PL-4, PL-11, Pl-12	5 (PL-4)	–
14	Tykocin	53° 12′ 47.14″	22° 46′ 32.64″	12	PL-1, PL-3, PL-4, PL-13	4 (PL-4,13)	–
15	Rzędziany	53° 08′ 30.46″	22° 52′ 09.06″	7	PL-1, PL-12, PL-16	2 (PL-12)	1 (*PL-16*)
16	Grądy Woniecko	53° 08′ 23.81″	22° 22′ 52.35″	14	PL-1, PL-5	2 (PL-1)	–
17	Kordon	52° 55′ 02.24″	23° 40′ 06.92″	12	PL-4, PL-9, PL-12, PL-13	3 (PL-4)	1 (*PL-9*)
18	Podlewkowie	52° 52′ 26.93″	23° 42′ 41.06″	13	PL-4, PL-9, PL-11, PL-12	3 (PL-11)	1 (*PL-9*)
19	Reski	52° 42′ 27.15″	23° 50′ 03.48″	9	PL-1, PL-4, PL-9, PL-10, PL-11	4 (*PL-4*)	1 (PL-9)
20	Carska-most	52° 41′ 24.55″	23° 52′ 43.04″	16	PL-1, PL-4, PL-9	2 (*PL-4*)	1 (PL-9)
21	Dubno	52° 37′ 49.99″	23° 04′ 33.07″	13	PL-9, PL-11, PL-12, PL-15	2 (PL-12)	2 (*PL-9*)
22	Mołoczki	52° 36′ 26.26″	23° 09′ 29.94″	20	PL-4, PL-9, PL-12, PL-14	3 (*PL-12*)	1 (PL-9)
23	Wandalin	52° 35′ 48.74″	23° 04′ 11.21″	22	PL-4, PL-9, PL-12, PL-16, PL-17	3 (*PL-4*)	2 (PL-9)
24	Kleszczele	52° 33′ 47.86″	23° 19′ 43.57″	11	PL-9	–	1 (PL-9)
25	Bagno Bubnów	51° 22′ 30.00″	23° 17′ 24.50″	6	PL-1	1 (PL-1)	–
26	Garbatówka	51° 21′ 72.80″	23° 06′ 47.92″	15	PL-7, PL-8	1 (PL-8)	1 (*PL-7*)
27	Wojciechów	51° 21′ 39.33″	23° 20′ 15.46″	12	PL-1, PL-7, PL-8	1 (PL-1)	2 (*PL-7*)
28	Pisklaki	50° 24′ 43.88″	22° 55′ 14.70″	11	PL-9	–	1 (PL-9)
29	Łukowa	50° 22′ 32.92″	22° 58′ 07.47″	9	PL-9	–	1 (PL-9)
30	Zamch	50° 19′ 28.69″	23° 02′ 39.35″	10	PL-9	–	1 (PL-9)
31	Kulno	50° 20′ 03.52″	22° 26′ 22.45″	9	PL-9	–	1 (PL-9)
32	Tarnawiec	50° 17′ 09.69″	22° 28′ 10.19″	12	PL-9	–	1 (PL-9)
33	Leżajsk	50° 14′ 58.60″	22° 27′ 15.00″	23	PL-9	–	1 (PL-9)
	1–33			439		17 (PL-3)	4 (PL-9)

The most frequent haplotype in each group is given in parentheses. In admixed population, the most frequent haplotype is italicized.
CE-PL S, distinct South Polish group; CE, other haplotypes from Central European phylogroup; *N*, number of individuals; N_h, number of haplotypes

Table 3. Details of haplotypes out of 439 individuals from 33 populations

Haplotypes nos	Number of populations	Number of individuals	Haplotype frequency	Haplotype group
PL-1	15	67	0.153	CE
PL-2	2	8	0.0182	CE
PL-3	11	74	0.169	CE
PL-4	8	59	0.134	CE
PL-5	3	10	0.0228	CE
PL-6	1	2	0.00456	CE
PL-7	1	22	0.0501	CE-PL S
PL-8	1	2	0.00456	CE
PL-9	14	122	0.278	CE-PL S
PL-10	1	1	0.00228	CE
PL-11	8	18	0.041	CE
PL-12	9	26	0.0592	CE
PL-13	6	12	0.0273	CE
PL-14	1	2	0.00456	CE
PL-15	1	1	0.00228	CE-PL S
PL-16	2	7	0.0159	CE-PL S
PL-17	1	1	0.00228	CE
PL-18	1	2	0.00456	CE
PL-19	1	1	0.00228	CE
PL-20	1	1	0.00228	CE
PL-21	1	1	0.00228	CE
	26	287	0.6537	CE
	17	152	0.3462	CE-PL S

CE-PL S, distinct South Polish group; CE, the Central European phylogroup

Table 4. Numbers of populations, numbers of haplotypes, haplotype diversity (*h*) and nucleotide diversity values (π, in %) for groups of *M. oeconomus* populations

	Groups of populations (populations nos)		
	CE (1–14, 16, 25)	CE-PL S only (24, 28–33)	Admixed (15, 17–23, 26, 27)
Number of populations	16	7	10
Number of haplotypes	13	1	13
h (SD)	0.5 (0.31)	0	0.603 (0.217)
min–max	0–0.872		0.133–0.861
π (SD)	0.188 (0.144)	0	0.473 (0.151)
min–max	0.043–0.37		0.146–0.62

SD, Standard deviation; CE-PL S, populations with haplotypes from distinct South Polish group only; CE, populations with other haplotypes from Central European phylogroup; admixed, populations with haplotypes from distinct CE-PL S group and other haplotypes from CE phylogroup

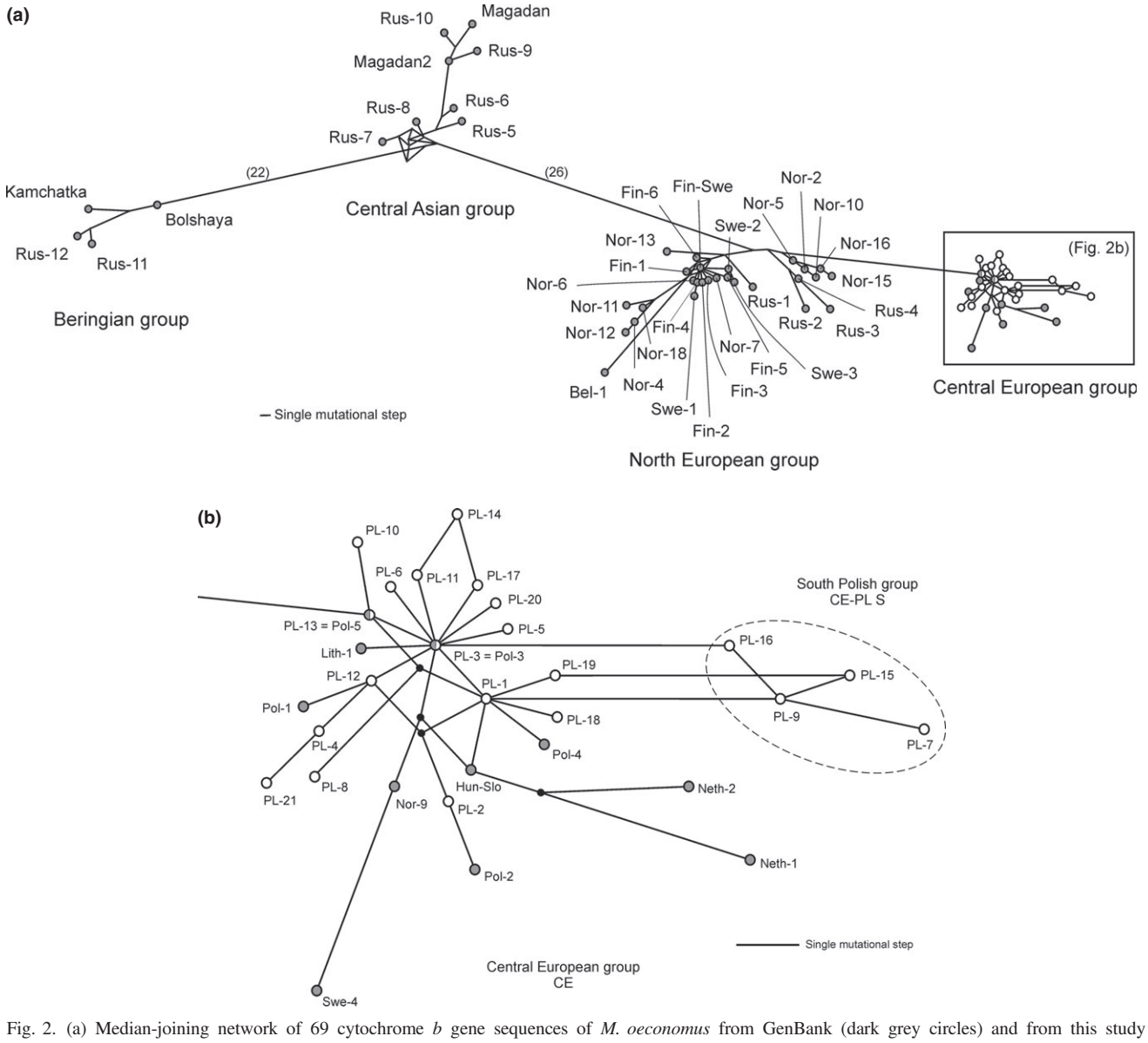

Fig. 2. (a) Median-joining network of 69 cytochrome *b* gene sequences of *M. oeconomus* from GenBank (dark grey circles) and from this study (white circles). Black circles – hypothetical sequences. Haplotypes correspond to Table 1. Numbers in parentheses denote mutational steps between lineages. (b) Network of cytochrome *b* gene sequences of Central European phylogroup only

(Tables 2 and 4, Fig. 1). As expected, these admixed 10 populations show the highest haplotype diversity (*h*) and nucleotide diversity (π) (Table 4).

ANOVA (NIR test) showed that for the distinct group that included only CE-PL S haplotypes (populations nos 28–33 and 24), the mean body mass of males was significantly lower than

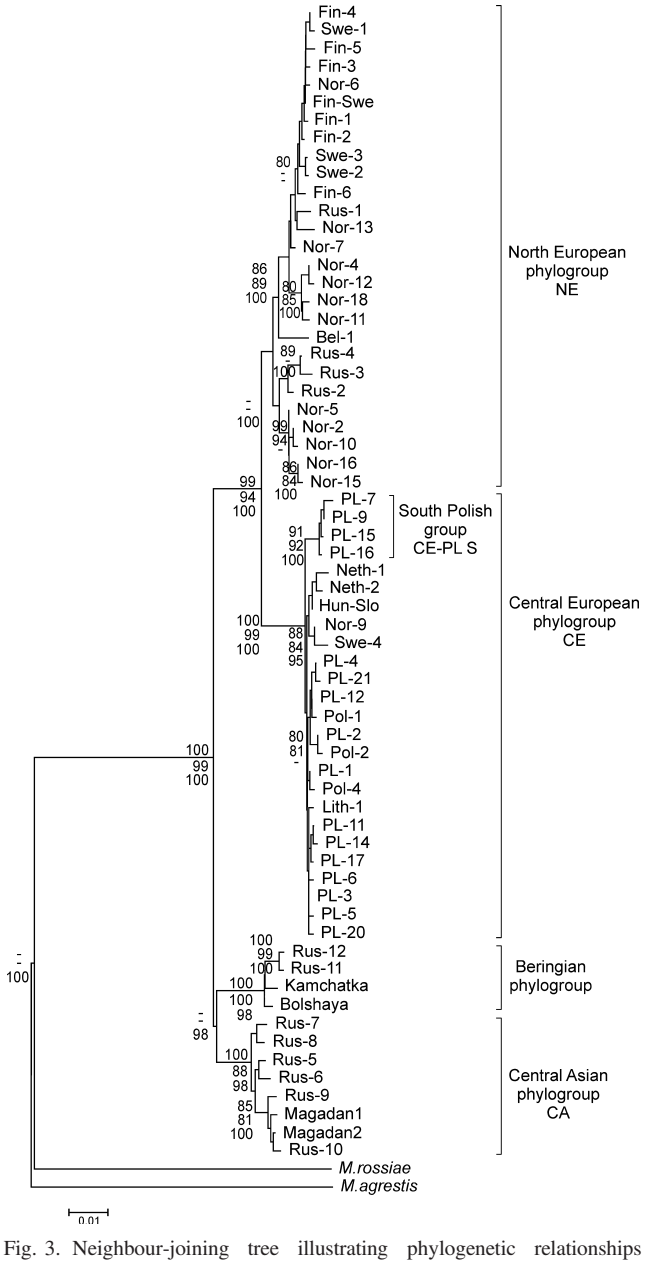

Fig. 3. Neighbour-joining tree illustrating phylogenetic relationships between cytochrome *b* haplotypes in the root vole *M. oeconomus*. Bootstrap supports for NJ and ML reconstructions and Bayesian posterior probability values are shown at nodes (at the top, middle and bottom, respectively). Haplotype designations are the same as in Table 1. Central European, North European, Central Asian, Beringian – phylogroups according to Brunhoff et al. (2003), South Polish group CE-PL S – phylogenetic group of *M. oeconomus* populations from southeastern Poland.

in the CE phylogroup from north-eastern Poland (populations nos 1–14 and 16) (Table 5).

Geographic distribution of haplotypes in eastern Poland

Haplotypes identified in this study display a specific pattern of distribution.

The CE-PL S group includes voles inhabiting southern and central-eastern Poland. The other investigated haplotypes from the CE phylogroup are recorded in populations of north-eastern and central-eastern Poland (Fig. 1, Table 2).

In populations including haplotypes from the distinct CE-PL S group and other haplotypes from the CE phylogroup, frequency of southern haplotypes (CE-PL S) varied between 13 and 69% (Fig. 1). Generally, in the zone of occurrence of southern haplotypes, their frequency decreased northwards, as they were replaced by CE haplotypes ($r = -0.85$, $p < 0.001$) (Fig. S1).

Geographical structuring among the studied Polish root vole populations was supported by the AMOVA results, where all sampling sites were treated as a single group of populations ($F_{ST} = 0.503$, $p < 0.001$; $\Phi_{ST} = 0.543$, $p < 0.001$). According to the SAMOVA results, differentiation of cytb among the studied root vole populations was best explained if two groups were considered: (1) populations nos 28–33 from southern Poland and population no. 24 and (2) all other populations from eastern Poland (nos 1–23 and 25–27). The percentage of variation was highest among groups of populations at 48.87%; among populations and within groups, it was 21.17%; and within populations, it was 21.96% ($\Phi_{CT} = 0.487$, $p < 0.001$; $\Phi_{SC} = 0.414$, $p < 0.001$; $\Phi_{ST} = 0.700$, $p < 0.001$, respectively). The first and second axes of the PCA (PC1 and PC2, Fig. S2) performed on the whole data set explained 44.77% and 22.84% of the total variability, respectively. The analysis showed that most north-eastern and central-eastern root vole populations in Poland formed one group, southern populations (nos 28–33) and population No. 24 were most divergent from all other populations with respect to PC1, and populations nos 17–18 and 21–23 were intermediate (Fig. S2).

Discussion

This study is the first performed for *M. oeconomus* using samples from over 30 populations inhabiting a relatively narrow geographical area between the Baltic Sea and the southern margin of the present-day species' continuous range (Table 1 and Fig. 1). 90% of detected haplotypes were described for the first time. Our haplotypes could be incorporated into the network of Eurasian phylogenetic relationships of the species studied (Fig. 2a). All detected haplotypes in Poland belong to the CE phylogroup, distinguished by Brunhoff et al. (2003) (Fig. 2b). All phylogenetic analyses indicated that within the Central European (CE) phylogroup, there is a distinct haplotype group (CE-PL S). This is clearly shown in the phylogenetic network (Fig. 2a), NJ tree (Fig. 3) and PCA (Fig. S2). Noteworthy, the ML and Bayesian trees exhibited topology similar to NJ tree and they also confirmed the distinctness of the Ce-PL S group (not shown). The estimated tMRCA for CE and CE-PL S based on the previously published molecular clock rate (Jaarola and Searle 2002; Brunhoff et al. 2003, 2006) correspond to the last glacial period. However, when we assumed higher molecular clock rate 4.572×10^{-7} substitutions per site per year (Herman et al. 2014), the origin of CE-PL S can be associated with the end of the LGM and/or Younger Dryas (12.9 ka BP 95% HPD 8.61–17.3 ka BP). Haplotypes of the distinct CE-PL S group were recorded exclusively in populations located in the southern and central part of the study area and were absent in populations of northern Poland (Fig. 1). The pattern of distribution of CE-PL S and other CE haplotypes (Fig. 1) and their relationships with other European haplotypes (Figs 2 and 3), as well as palaeontological and palaeoclimatic data, suggest a different origin of voles from these groups. This differentiation is also supported by ecological parameters of individuals, for example, body mass. Adult males from southern Poland that only possessed CE-PL S haplotypes are characterized by lower body mass in comparison with males from northern Poland without CE-PL S haplotypes (Table 5). However, the body mass can be also shaped by environmental and population factors. Our result is also in accordance with the Bergman's rule (Rensch 1938).

Table 5. Statistical analysis of body masses of adult male *M. oeconomus* from three groups of populations. Population names are described in Table 4.

Groups of population	No. of individuals	Body masses (g)			
		Min	Max	Median	Average (SD)
CE	109	19.0	56.0	40.0	39.482 (7.433)*
CE-PL S	39	20.0	55.0	36.0	35.846 (8.628)*
Admixed	54	20.0	58.0	36.0	37.037 (8.587)

*Statistically significant (P-value ≤ 0.05)
SD, Standard deviation

We suggest that *M. oeconomus* haplotypes of the distinct South Polish group, distinguished in this study, may have originated from a small, local refugium situated in Poland.

Constant occurrence of the root vole within the vertebrate group of this part of Poland throughout the last glaciation resulted from the dominant local climatic and environmental conditions tolerated by the species. During the Vistulian, the morphologically diversified Kraków-Częstochowa Upland formed a mosaic of microclimates and vegetation zones, providing life conditions advantageous for species with different ecological requirements, as long as they withstood the temperatures. Furthermore, reconstructions of glacial plant associations, based on palaeobotanical and palaeozoological analyses as well as vegetation distribution models developed with palaeoclimatic data, show that during the LGM (Upper Pleniglacial), Central Europe was dominated by conditions enabling survival of mammals from boreal temperate zones (e.g. Madeyska 1981; Svenning et al. 2008), including root voles (Fløjgaard et al. 2009). Within plant communities, woody tundra was dominant; meltwaters that were not drained from the permafrost enabled the development of wetlands (Madeyska 1981), the habitats most preferred by the root vole (Gliwicz and Jancewicz 2004). The rodent, which shows boreal and eurytopic features, could have survived in the foreground of the ice sheet and was therefore present in the small vertebrate fauna of southern and central Poland. According to Nadachowski (1989), *M. oeconomus* inhabited the Kraków-Częstochowa Upland until the Subatlantic. Occurrence of the root vole during the Vistulian was also recorded in other areas of Central Europe (however, at much greater distances from the ice-sheet limit in the LGM than in the Kraków-Częstochowa Upland and Holy Cross Mountains), for example, in the Gigny cave (Chaline et al. 1995).

The palaeobotanical data (Madeyska 1981; Starkel 1988; Ralska-Jasiewiczowa et al. 2004) and palaeozoological analyses (Madeyska 1981; Nadachowski 1989), supported by numerous simulations of changes in climate and distribution of trees and small mammals (Svenning et al. 2008; Fløjgaard et al. 2009), provide basis for the assumption that southern Poland served as a small, local refugium of *M. oeconomus* during LGM and/or the Younger Dryas and site of origin of some present-day root vole populations. This conclusion, also confirmed by the results of studies presented herein (SAMOVA, PCA), supports the concept of Bilton et al. (1998), Stewart and Lister (2001) and Pearson (2006) of high-latitude cryptic refugia. Populations of *M. oeconomus* originating from the South Polish refugium may presently inhabit southern Poland in areas adjacent to the southern European boundary of its present-day range (Sałata-Piłacińska 1990) and part of central Poland. It may be expected that these voles from southern Poland created the CE-PL S group distinguished in this current study. Such an assumption is also confirmed by the distinctness of the South Polish and Hungarian/Slovakian haplotypes in the phylogenetic network (Fig. 2b). The diagram also shows a relatively close relationship between haplotypes of north-eastern and central-eastern Poland, Lithuania and southern Norway and haplotypes of Hungary/Slovakia and the Netherlands, as they all belong to the CE lineage (Fig. 2b), indicating their common origin. The suggested origin of the distinct CE-PL S group of voles from a small, local refugium, located near the ice-sheet limit, is also supported by the fixation of one haplotype (PL-9) in the southernmost populations (nos 28–33; Fig. 1, Table 1), likely resulting from a genetic bottleneck and a population size limited by harsh climatic conditions of the LGM.

As haplotypes of the Central European phylogroup (CE) described for north-eastern and central-eastern Poland are closely related to haplotypes of voles from Slovakia, Hungary and the Netherlands.

Conclusions

1 In an area of Poland, a distinct haplotype group, CE-PL S, within the CE phylogroup of *M. oeconomus,* was identified with high bootstrap support. Voles of this group were found in southern and central Poland and 10 of 33 populations studied harboured individuals of both groups.
2 Results from phylogenetic studies confirmed the possible presence of a small, cryptic refugium in an area of Poland, the Kraków-Częstochowa Upland and the Holy Cross Mountains. Among all known refugia, the above-mentioned was located closest to the ice-sheet limit during the LGM and the Younger Dryas. Voles from this refugium most likely gave origin to populations of the distinct South Polish group (CE-PL S) within the Central European phylogroup (CE).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Relationship between latitude and frequency of haplotypes from the CE-PL S phylogroup in populations of *M. oeconomus* from eastern Poland

Fig. S2 Principal component analysis (PCA) performed for mtDNA F_{st} data between *M. oeconomus* populations. Population studied – Table 2, Fig. 1a