



## Research Article

## Reconstructing the phylogeny of Genus *Apodemus* (Rodentia: Muridae), providing the new mitogenome data

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### Abstract

The phylogenetic relationships between the subgenera and/or species of genus *Apodemus* are controversial and problematic. In this context, complete mitogenome analyses, along with Cytochrome-b and Cytochrome oxidase I data, were performed to elucidate the phylogeny of this genus. The complete mitochondrial genome characteristics of four species (*Apodemus mystacinus*, *Apodemus uralensis*, *Apodemus ponticus*, and *Apodemus witherbyi*) were reported for the first time, and mean genetic distance ( $d$ ) and Bayesian Inference (BI) trees were provided. Accordingly, *Sylvaemus*, *Karstomys*, *Apodemus*, and *Alsomys* subgenera were separated by high  $d$  values and hypothesised to be different genera. In terms of *Sylvaemus* species, mitogenome analyses gave more statistically reliable results, with *Syl. flavicollis*/*Syl. ponticus* being closer to *Syl. uralensis*, while *Syl. witherbyi* was closer to these species than *A. sylvaticus*. In addition, the results of the analyses support the idea that *Syl. ponticus* is a distinct species from *Syl. flavicollis*.

## Introduction

*Apodemus* Kaup, 1829, the most widespread rodent genus in the temperate zone of the Palearctic Region, is represented by more than 20 wood mouse species and is found in fields and broad-leaf forests (Corbet, 1978; Musser and Carleton, 2005). This genus began to diversify 8–10 MYA (Late Miocene/7.82–12.74 MYA), probably from Eastern or Central Asia, with an adaptive radiation event, and evolved into five subgenera with speciation triggered by climatic, geologic, and vegetation variations: *Sylvaemus* (*Apodemus sylvaticus* Linnaeus, 1758; *Apodemus flavicollis* Melchior, 1834; *Apodemus ponticus* Sviridenko, 1936; *Apodemus alpicola* Heinrich, 1952; and *Apodemus uralensis* Heinrich, 1952), *Apodemus* (*Apodemus peninsulae* Thomas, 1907; *Apodemus chevrieri* Milne-Edwards, 1868; *Apodemus agrarius* Pallas, 1771; *Apodemus speciosus* Temminck, 1845; *Apodemus draco* Barrett-Hamilton, 1900; *Apodemus ilex* Thomas, 1922; *Apodemus semotus* Thomas, 1908; and *Apodemus latronum* Thomas, 1911), *Karstomys* (*Apodemus mystacinus* Danford and Alston, 1877; *Apodemus epimelas* Nehring, 1902), *Argenteus* (*Apodemus argenteus* Temminck, 1844), and *Gurkha* (*Apodemus gurkha* Thomas, 1924) (Serizawa et al., 2000; Michaux et al., 2002; Suzuki et al., 2003, 2008; Liu et al., 2004). Recently, Mezhzherin and Tereshchenko (2023) recognised *Sylvaemus* as a separate genus and included the subgenus *Karstomys* in this genus; they also included species such as *A. argenteus*, *A. gurkha*, *A. speciosus*, and *A. draco* in the genus *Alsomys*.

As ideal species for biogeographic studies, especially for environmental changes such as habitat expansion and contraction (Serizawa et al., 2000), these wood mouse species have been systematically and phylogenetically studied by many researchers for many years. However, phylogenetic relationships, morphological distinctions between subspecies and species, and species boundaries are controversial, especially in *Sylvaemus*. One of the reasons for this situation is that the

morphological characteristics of these species are quite similar, which can lead to mistakes in species definitions (Hooper et al., 2007). Another reason is that studies often yield conflicting results, even when based on the same gene regions. For example, regarding *A. flavicollis*, *A. uralensis*, and *A. sylvaticus*, some authors suggested that *A. flavicollis* and *A. uralensis* are closer (Michaux et al., 2002; Bellinvia, 2004; Suzuki et al., 2008; Mezhzherin and Tereshchenko, 2023), some other authors offered that *A. flavicollis* and *A. sylvaticus* are closer (Liu et al., 2004; Hooper et al., 2007; Bugarski-Stanojević et al., 2011; Darvish et al., 2015), while Bellinvia et al. (1999) found that *A. uralensis* and *A. sylvaticus* are closer. Contrary to these authors, Filippucci et al. (2002) defined *A. flavicollis* and *A. hermonensis* (synonym: *A. witherbyi*) as the closest. As can be seen, there is confusion and uncertainty in the systematics of *Apodemus*. Therefore, to elucidate the phylogeny of this genus, more comprehensive methods are needed, rather than analyses focusing on single gene regions.

For many years, rodent systematics has been based primarily on morphology, followed by allozyme and molecular systematic studies evaluating mitochondrial and nuclear gene regions. Recently, studies analysing the complete mitochondrial DNA (approximately 16 kb in length, with 37 gene regions) (Anderson et al., 1981) have been on the rise. mtDNA is considered to be an ideal tool due to the absence of recombination and repair mechanisms and introns, and its rapid rate of evolution (Clary and Wolstenholme, 1985; Wolstenholme, 1992; Nabholz et al., 2009; Fernández-Silva et al., 2003; Ballard and Whitlock, 2004; Aanen et al., 2014). Considering the genus *Apodemus*, the mitogenome characteristics of *A. latronum*, *A. peninsulae*, *A. agrarius*, and *A. draco* (Kim and Park, 2011, 2012; Oh et al., 2011; Yue et al., 2012; Jeon et al., 2016; Yue et al., 2016; Wu et al., 2022; Kim et al., 2023) were extracted. In this study, in addition to the complete mitogenomes of four species (*A. mystacinus*, *A. uralensis*, *A. witherbyi*, and *A. ponticus*), phylogenetic analyses were performed to contribute to the systematics of wood mouse species. Besides, Cytochrome-b (CYTB) and Cyto-

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chrome oxidase I (COI) sequences were also analysed to compare the single gene and mitogenome results.

## Materials and methods

### Sampling

One specimen each of *A. mystacinus*, *A. uralensis*, *A. witherbyi*, and *A. ponticus* specimens for mitogenome analyses was collected under the permissions (#2722/01; 2302/01; R/057-21) issued by the Georgian Ministry of Environmental Protection and Agriculture. The field research and initial morphological analysis of the species were conducted by the Animal Research Group at the Institute of Zoology, Iliia State University (Georgia). The specimens were identified based on morphological characteristics and COI region analysis, as described by Maglakelidze et al. (2024). In addition, sequences of other *Apodemus* species (*A. flavicollis*, *A. sylvaticus*, *A. alpicola*, *A. agrarius*, *A. chevrieri*, *A. chejuensis*, *A. peninsulae*, *A. gurkha*, *A. draco*, *A. latronum*) and *Mus musculus* and *Arvicanthis rufinus* (outgroups) stored in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and BoldSystems (<https://boldsystems.org/>) (Tab. 1.) were also included in the analyses.

### Laboratory Studies

DNA extraction kits were utilized to isolate the genomic DNA using muscle tissues of the samples. The NEBNext Ultra DNA Library Prep Kit was utilized for subsequent library preparation; paired-end sequencing was optimized with a precise 150-base pair insert size configuration. For sequencing the high-throughput Illumina NovaSeq 6000 platform was employed; first, the quality of the reads obtained from the NGS reaction was evaluated. In order to evaluate the read quality and sequencing depth, summary statistical data were created. A set of high-quality reads in the “.fastq” or “.fq” format was also obtained with a quality filtering process. Following the stage above, the genetic composition, profile, and potential functionalities were clarified through three subsequent stages: quality control of NGS data and filtering, de novo genome assembly, and mitochondrial genome annotation. All of these processes were conducted by Genoks LifeScience Inc. (Turkiye).

### Genome Analyses

After the NGS sequencing reaction, the total number of reads, total sequence length, Phred Score (Q); Q20 and Q30 quality scores, and % GC contents were determined. Mitochondrial genome annotation was completed in MitoZ version 3.6 (Meng et al., 2019). For visualization of the assembled circular genome and its annotations, Circos v0.69-8 (Krzywinski et al., 2009) was used. Genetic diversity values (haplotype diversity (Hd), nucleotide diversity (Pi), number of mutations, and polymorphic sites) were calculated in DnaSP v6 (Rozas et al., 2017).

In Mega11 Software (Tamura et al., 2021), the sequences were aligned, and 16.882 (mitogenome), 347 (CYTB), and 488 (COI) base-pairs of raw data were generated, and the mean genetic distance ( $d$ ) values were also determined based on the  $p$ -distance parameter (Hamming, 1950). Bayesian Inference (BI) trees were constructed using mitogenome, CYTB, and COI data in MrBayes 3.2. Program (Ronquist et al., 2012) with 1.000.000 generations with 100 samples each, with a 25% burn-in. GTR+G (mitogenome and COI data, Tavaré, 1986 and HKY+G (CYTB data, Hasegawa et al., 1985) were identified as the best substitution models for BI trees in jModeltest 2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012).

Divergence times of the species were calculated in BEAST 1.7.5 Program (Drummond and Rambaut, 2007). The mammalian mtDNA divergence rate (2% per 1 my; Avise et al., 1998), and the *Mus/Arvicanthis* split (11.2 MYA-fossil data) (Fabre et al., 2013) were taken into consideration with a normal distribution as a calibration point. The “.xml” format file was prepared according to the Yule process of speciation” and “Lognormal relaxed clock” models in Beauti Program stored in BEAST 1.7.5 and it was analysed in five repetitions, each chain length being 20.000.000 which is sampled every 2.000 generations. The acquired five files were unified using LogCom-

biner, and a tree was established in TreeAnnotator with a burn-in of 5.000. Stationarity and convergence were checked in Tracer 1.5 Software (<http://beast.bio.ed.ac.uk/Tracer>), and it was ensured that all effective sample size (ESS) values were 200 or higher.

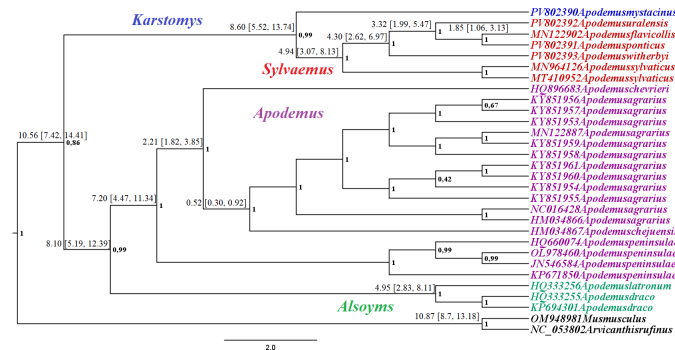
## Results

### Mitogenome Structures of the Studied Species

For the 4 species with complete mtDNA sequences, 37 gene regions comprising 13 protein-coding genes, 22 tRNA genes, and 2 rRNA genes were identified (Fig. S1-4). GC% values were calculated as 35.21% in the 16.422 base-pair *A. mystacinus* sequence, as 36.02% in the 16.462 base-pair *A. uralensis* sequence, as 35.46% in the 16449 base-pair *A. witherbyi* sequence, and as 36.31% in the 16311 base-pair *A. ponticus* sequence. Besides, while tRNA<sup>Pro</sup>, tRNA<sup>Glu</sup>, ND6, tRNA<sup>Ser</sup>, tRNA<sup>Tyr</sup>, tRNA<sup>Cys</sup>, tRNA<sup>Asn</sup>, tRNA<sup>Ala</sup>, and tRNA<sup>Gln</sup> are placed in the L strand, the other gene regions are located in the H strand (Fig. S1-4.). Considering all *Apodemus* sequences, 52398 polymorphic sites and 7007 mutations were accounted for with a total haplotype diversity (Hd) value of 0.992 and a nucleotide diversity (Pi) value of 0.107.

### Phylogenetic Analyses of Mitogenome Data

The mean genetic distance values were estimated as 1.49–15.55% in the mitogenome analyses (Tab. 2). According to these results, *A. agrarius* and *A. chejuensis* are the closest species, whereas *A. sylvaticus* and *A. draco* are the furthest species. Bayesian Inference (BI) tree (Fig. 1) showed that *A. latronum* and *A. draco* are closer to each other, formed a separate lineage (pp: 0.99). *A. agrarius*, *A. chevrieri*, and *A. chejuensis* also built a separate lineage and were closer to the *A. peninsulae* samples (pp: 1.00). Furthermore, the specimens of *A. flavicollis* and *A. ponticus* are close taxa although they split, and *A. mystacinus* formed a different lineage (pp: 0.99). On the other hand, in the mitogenome tree, *A. sylvaticus* differed from the others (pp: 1.00), and *A. uralensis* and *A. witherbyi* are more closely related to *A. flavicollis* and *A. ponticus* (pp: 1.00).



**Figure 1** – Bayesian Inference (BI) tree derived from the mitogenome data. Posterior probability (pp) values are given with the numbers on the nodes, and divergence times and 95% HPD interval values are provided on the branches.

Moreover, 4 lineages belong to *Sylvaemus* (*A. sylvaticus*, *A. flavicollis*, *A. ponticus*, *A. uralensis*, and *A. witherbyi*), *Karstomys* (*A. mystacinus*), *Apodemus* (*A. peninsulae*, *A. chevrieri*, *A. chejuensis*, and *A. agrarius*), and *Alsomys* (*A. draco* and *A. latronum*) were identified in the BI tree (Fig. 1) (pp: 0.86–1.00) with the  $d$  values of 13.6–15.4% (Tab. 3). Divergence times of these lineages and species were dated back to 10.56 MYA as provided in Fig. 1 in detail.

### Phylogenetic Analyses of CYTB and COI Data

$d$  values were detected as 1.73% (*A. agrarius*–*A. chejuensis*)–18.23% (*A. sylvaticus*–*A. latronum*) for the CYTB marker and 2.01% (*A. agrarius*–*A. chejuensis*)–16.84% (*A. sylvaticus*–*A. chejuensis*) for the COI marker (Tab. S1-2).

In the Bayesian Inference (BI) trees of CYTB, and COI data (Fig. S5-S6), similar to mitogenome tree (Fig. 1), distinct clades of *Sylvaemus*, *Karstomys*, *Apodemus*, and *Alsomys* lineaged were observed. The

**Table 1** – Genbank and BoldSystems(\*) Accession Numbers of the sequences analysed in this study.

Gene	Species	Accession numbers	References
Mitogenome	<i>A. mystacinus</i>	PV802390	This study
	<i>A. ponticus</i>	PV802391	This study
	<i>A. uralensis</i>	PV802392	This study
	<i>A. witherbyi</i>	PV802393	This study
	<i>A. flavicollis</i>	MN122902	Margaryan, direct submission
	<i>A. sylvaticus</i>	MN964126	Nicolas et al. (2020)
		MT410952	Leerhoei, direct submission
	<i>A. agrarius</i>	HM034866	Oh et al., unpublished data
		KY851953-KY851961	Andersen et al. (2017)
		MN122887	Margaryan, direct submission
		NC016428	Kim and Park, direct submission
	<i>A. chejuensis</i>	HM034867	Oh et al., unpublished data
	<i>A. chevrieri</i>	HQ896683	Yue et al. (2012)
	<i>A. peninsulae</i>	HQ660074	Oh et al. (2011)
		JN546584, OL978460	Kim and Park (2011)
		KP671850	Jeon et al. (2016)
	<i>A. latronum</i>	HQ333256	Fan et al., unpublished data
	<i>A. draco</i>	HQ333255	Fan et al., unpublished data
		KP694301	Chen, direct submission
	<i>M. musculus</i>	OM948981	Sendon et al., unpublished data
<i>A. rufinus</i>	NC_053802	Mikula et al., direct submission	
CYTB	<i>A. mystacinus</i>	KY753943	Steppan and Schenk (2017)
		MW287352, MW287353	Mohammadi et al. (2020)
		OR573581-OR573585	Amir Afzali and López-Antoñanzas (2024)
	<i>A. ponticus</i>	DQ844687-DQ844692	Balakirev et al. (2007)
		FN433629-FN433634	Chelomina and Atopkin (2010)
		KR003103-KR003105	Darvish et al. (2015)
	<i>A. uralensis</i>	AB096837	Suzuki et al. (2003)
		AJ311154, AJ311155	Michaux et al. (2002)
		AY389021	Liu et al. (2004)
		MN454323-MN454325	Yu et al. (2020)
		OR573605-OR573607	Amir Afzali and López-Antoñanzas (2024)
	<i>A. witherbyi</i>	KR003093-KR003098	Darvish et al. (2015)
		KR003100-KR003102	
		KY753945	Steppan and Schenk (2017)
	<i>A. flavicollis</i>	AJ311150, AJ311151,	Michaux et al. (2002)
		AJ605627, AJ605653,	
		AJ605670, AJ605672-AJ605679,	
		AJ605681, AJ605682, AJ605684,	
		AJ605686-AJ605690	
	<i>A. sylvaticus</i>	AJ511967-AJ511972	Michaux et al. (2003)
	<i>A. alpicola</i>	AJ311152, AJ311153	Michaux et al. (2002)
		AB032854	Serizawa et al. (2000)
		AF159391	Martin et al. (2000)
	<i>A. agrarius</i>	AB096817	Suzuki et al. (2003)
		AB303225, AB303226	
		OM970159, OM970162,	Yalkovskaya et al. (2022)
		OM970163, OM970165	
	<i>A. chejuensis</i>	HM034937-HM034940	Oh et al., unpublished data
	<i>A. chevrieri</i>	HQ896706-HQ896709	Yue et al. (2012)
	<i>A. gurkha</i>	AB032852	Serizawa et al. (2000)
	KU214576	Karmacharya et al. (2016)	
<i>A. peninsulae</i>	AB073808-AB073811	Serizawa et al. (2002)	
<i>A. latronum</i>	GU982929-GU982932	Fan et al., unpublished data	
<i>A. draco</i>	AB096825	Suzuki et al. (2003)	
	KU239927-KU239929	Cheng, unpublished data	
<i>M. musculus</i>	AB205275	Terashima et al. (2006)	
<i>M. musculus</i>	AB125774	Suzuki et al. (2004)	

continues

mean genetic distance ( $d$ ) values between these lineages were defined as 13.3–15.3% (CYTB) and 12.5–15.6% (COI) (Tab. S3-S4). However, *A. peninsulae* (from *Apodemus* group) was located with *Al-*

*somys* group (pp: 0.93) in the CYTB tree and these two groups split with pp: 0.56. Also, *A. gurkha* is closer to *Apodemus* than *Alsomys* (pp: 0.95). Furthermore, the specimens of *A. flavicollis* and *A. ponti-*

Gene	Species	Accession numbers	References	
COI	<i>A. mystacinus</i>	SMGEO184, SMGEO185, SMGEO187-SMGEO189*	Maglakelidze et al. (2024)	
	<i>A. ponticus</i>	ABMEE039-ABMEE042, ABMEE128, ABMEE132, ABMEE139, ABMEE142, ABMEE144, ABMEE145, ABMEE150-ABMEE152, ABMEE153-ABMEE158, ABMEE160, ABMEE168*, SKMZM931, SKMZM943-SKMZM952, SKMZM968, SKMZM969, SKMZM971, SKMZM973, SKMZM974*, SMGEO284, SMGEO311-SMGEO313, SMGEO318, SMGEO320-SMGEO322*	Bogdanov, unpublished data  Bogdanov, unpublished data	
	<i>A. uralensis</i>	ABMEE032*, JF499304, JF499305, MK329620	Bogdanov, unpublished data Lissofsky, unpublished data Ge et al. (2019)	
	<i>A. witherbyi</i>	ABMEE033, ABMEE035-ABMEE037*, SMGEO065*	Bogdanov, unpublished data	
	<i>A. flavicollis</i>	JQ935786, JQ935787, KP869134, KP869151, KP869161, KP869163, KY754481, MZ661170, MZ895174, OQ706733-OQ706734	Maglakelidze et al. (2024) Panculescu-Gatej et al. (2014) Santos et al. (2015)	
	<i>A. sylvaticus</i>	MW478031, MZ661163	Schäffer et al. (2017) Wyler, unpublished data	
	<i>A. alpicola</i>	OQ706727- OQ706729, MZ661164	Deng et al., direct submission Ruedi et al. (2023) Mondino, unpublished data Wyler, unpublished data	
	<i>A. agrarius</i>	BKMAM005, BKMAM013*, CICA012*, FBMAM093*, JQ043486, JQ935784, MZ389785, SKMZM096, SKMZM301, SKMZM328, SKMZM329, SKMZM345 *	Ruedi et al. (2023) Wyler, unpublished data Chassovnikarova, direct submission Secansky, direct submission Feulner, direct submission Ma and Lu, direct submission Panculescu-Gatej et al. (2014) Gu, direct submission Bogdanov, unpublished data	
	<i>A. chejuensis</i>	MN756660, OL639150-OL639152	Kim, unpublished data Kim, unpublished data	
	<i>A. chevrieri</i>	MK329682, MK329683,	Ge et al. (2019)	
	MK329686, MK329688	<i>A. peninsulae</i>	MK329561, MK329607, MK329610, MK329669	Ge et al. (2019)
		<i>A. latronum</i>	MK329665, MK329676-Ge et al. (2019), MK329678	
		<i>A. draco</i>	KU239941-KU239944	Cheng, unpublished data
		<i>M. musculus</i>	MN228580	Bogdanov et al. (2020)
		<i>M. musculus</i>	MN228589	Bogdanov et al. (2020)

*cus* are not separated in the CYTB tree, but are close in the COI tree, whereas *A. mystacinus* differs from these species. In the CYTB tree, *A. uralensis* samples were separated from the others (pp: 0.51–0.96), and *A. witherbyi* and *A. alpicola* were closely positioned (pp: 0.96). In the COI tree, *A. witherbyi* was the most distant taxon (pp: 1.00), while *A. uralensis* was closest to *A. alpicola*, *A. flavicollis*, and *A. ponticus* (pp: 0.51–0.91).

## Discussion

### Mitogenomes of *Apodemus* Species

The complete mitochondrial DNA data, ranging from 16.311 to 16.462 base pairs, of four wood mouse species were presented for the first time. Base pair lengths vary because there are overlaps in some regions (the longest overlap between ATP6 and ATP8 is composed of 43 nucleotides), as well as the length of some gene regions varies between species by 1–141 base pairs (Fig. S1-4.). Also, GC% values were lower than AT% values (35.21–36.31 %). The evolutionary significance of this situation is that it leads to a high mutation rate in rodents (Triant and

**Table 2** – Calculated mean genetic distance (*d*) values (below the diagonal) between species with the standard errors (above the diagonal) employing mitogenome data\*.

	FLA	PON	URA	WIT	SYL	MYS	PEN	AGR	CHEJ	CHEV	LAT	DRA
FLA		0.0018	0.0023	0.0023	0.0023	0.0028	0.0028	0.0028	0.0028	0.0029	0.0028	0.0028
PON	0.0544		0.0023	0.0023	0.0023	0.0027	0.0028	0.0028	0.0029	0.0029	0.0028	0.0028
URA	0.0878	0.0879		0.0024	0.0024	0.0028	0.0029	0.0028	0.0029	0.0029	0.0028	0.0028
WIT	0.0959	0.0961	0.1004		0.0023	0.0027	0.0027	0.0027	0.0028	0.0028	0.0028	0.0027
SYL	0.0942	0.0956	0.0996	0.0973		0.0027	0.0028	0.0028	0.0028	0.0028	0.0028	0.0028
MYS	0.1427	0.1420	0.1408	0.1380	0.1396		0.0028	0.0028	0.0028	0.0028	0.0028	0.0028
PEN	0.1514	0.1530	0.1532	0.1519	0.1492	0.1506		0.0026	0.0026	0.0026	0.0025	0.0025
AGR	0.1535	0.1545	0.1534	0.1510	0.1503	0.1505	0.1297		0.0009	0.0018	0.0026	0.0026
CHEJ	0.1533	0.1547	0.1542	0.1509	0.1508	0.1525	0.1303	0.0149		0.0018	0.0027	0.0026
CHEV	0.1540	0.1552	0.1546	0.1541	0.1522	0.1508	0.1308	0.0563	0.0573		0.0026	0.0026
LAT	0.1520	0.1528	0.1533	0.1510	0.1517	0.1511	0.1325	0.1347	0.1353	0.1350		0.0023
DRA	0.1550	0.1534	0.1543	0.1547	0.1555	0.1518	0.1355	0.1370	0.1368	0.1354	0.1075	

\*: FLA: *A. flavicollis*, PON: *A. ponticus*, URA: *A. uralensis*, WIT: *A. witherbyi*, SYL: *A. sylvaticus*, MYS: *A. mystacinus*, PEN: *A. peninsulae*, AGR: *A. agrarius*, CHEJ: *A. chejuensis*, CHEV: *A. chevrieri*, LAT: *A. latronum*, DRA: *A. draco*.

**Table 3** – Calculated mean genetic distance (*d*) values (below the diagonal) between subgenera with the standard errors (above the diagonal) employing mitogenome data.

<i>Sylvaemus</i>	<i>Karstomys</i>	<i>Apodemus</i>	<i>Alsoyms</i>	<i>M. musculus</i>	
<i>Sylvaemus</i>		0.002	0.002	0.002	0.003
<i>Karstomys</i>	0.140		0.003	0.003	0.003
<i>Apodemus</i>	0.152	0.151		0.002	0.003
<i>Alsoyms</i>	0.154	0.151	0.136		0.003
<i>M. musculus</i>	0.171	0.169	0.167	0.167	

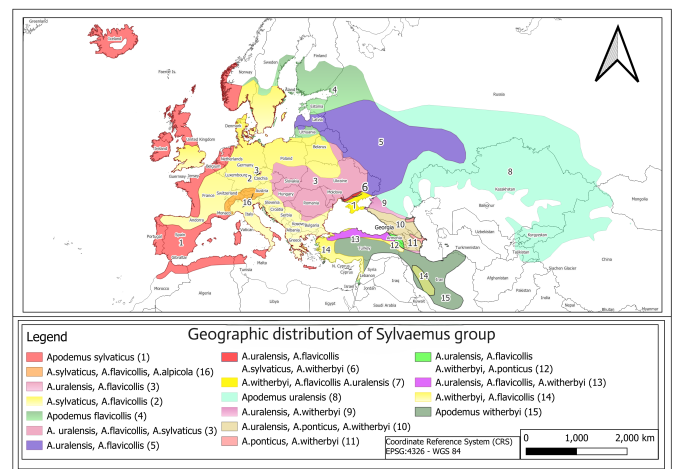
DeWoody, 2006); because AT and GC pairs were bonded with two and three hydrogen bonds, respectively, and the stability of the double helix structure of DNA was decreased due to the high AT content (Yakovchuk et al., 2006). Furthermore, as a typical feature of mammals, the 12S rRNA and the 16S rRNA genes were located between tRNAPhe and tRNA<sup>Leu</sup> genes and were separated by the tRNA<sup>Val</sup> gene (Wolstenholme, 1992; Ding et al., 2016).

Upon examining the studies in the literature, for *A. peninsulae*, Kim and Park (2011); Oh et al. (2011); Jeon et al. (2016), and Kim et al. (2023) reported 16.268, 16.266, 16.457, and 16.268 bp of mitogenome data, respectively; Oh et al. (2011) also detected similar gene overlaps. Kim and Park (2012) evaluated the complete mitogenome of *A. agrarius* (16.263 bp). Yue et al. (2012) studied the mitogenome of *A. chevrieri* (16.298), defining the overlapping of some genes. Yue et al. (2016) provided 16.288 base pairs of *A. latronum* mitogenome sequence and found that the ATP8 and ATP6, ATP6 and COX3, and ND4L and ND4 overlapped in reading frames. Wu et al. (2022) found high AT content for *Apodemus agrarius ningpoensis* (16.262 bp, 63.74 %) and *Apodemus draco draco* (16.222 bp, 64.12 %). Besides, they interpreted the presence of gene overlap and spacing as murid rodents need to continuously improve DNA utilization, and it is extremely beneficial to the evolutionary process.

### Phylogeny of Genus *Apodemus* Based on the Mitogenome Data

Regarding the results obtained in terms of subgenera, four groups appeared in the mitogenome tree (Fig. 1): *Sylvaemus* (*A. sylvaticus*, *A. flavicollis*, *A. ponticus*, *A. uralensis*, and *A. witherbyi*), *Karstomys* (*A. mystacinus*), *Apodemus* (*A. peninsulae*, *A. chevrieri*, *A. chejuensis*, and *A. agrarius*), and *Alsoyms* (*A. draco* and *A. latronum*). Distribution of the studied species were given in Fig. 2-5. The mean genetic distance (*d*) values between these groups were calculated as 13.6–15.4 %. Accordingly, *Sylvaemus* and *Karstomys*, which are the West Palearctic wood mice, are close groups, with genetic distance value of 14.0 %. These two groups have some morphological similarities (the number of roots on the upper molars and mammary glands) and according to the results of allozyme and CYTB data in the literature (Britton-Davidian

et al., 1991; Mezhzherin, 1997; Filippucci et al., 2002; Michaux et al., 2002; Mezhzherin and Tereshchenko, 2023), *Karstomys* genetic distance (CYTB gene region) values were reported by Martin et al. (2000) (16.0 %), Michaux et al. (2002) (8.8 %), Liu et al. (2004) (6.9 %; 6.8 % with other *Apodemus* species), and Mezhzherin and Tereshchenko (2023) (17.6 %). On the other hand, the fact that *A. mystacinus* (*Karstomys*) is distinctly different from other *Sylvaemus* species in terms of external morphological features (Rietschel and Storch, 1973; Storch, 1975) and the genetic distance values are quite high, seems to support the idea that *Karstomys* may be a separate genus rather than a subgenus of *Sylvaemus*.

**Figure 2** – Distribution areas of the studied *Sylvaemus* group species retrieved from IUCN (2025) data.

The 4 pairs of mammary glands are also a common morphological feature for the East Palearctic subgenera, *Alsoyms* and *Apodemus*, but the number of roots on the upper molars is different. Phylogenetic analyses also show that they are quite distinct in our and other studies, and their genetic distance levels are 13.6–15.2 % (17.8 % reported by Mezhzherin and Tereshchenko, 2023). In our study, the *d* values between the 4 groups were quite high and even close and/or high with the genus *Mus*. Considering the findings, we suggest that *Apodemus*, *Karstomys*, *Sylvaemus*, and *Alsoyms* should be considered as separate genera, especially due to the high levels of genetic distance. To summarise from a phylogenetic point of view, divergence of the ancestral form of “*Apodemus*” started back to the Late Miocene-Vallesian period in the south of the Eastern Palearctic (possibly southeastern China; Yue et al. 2012) and evolved to the modern *Sylvaemus*, *Alsoyms*, *Karstomys*, and *Apodemus* genera. Followed by the first split, the second divergence caused the separation and radiation of the East

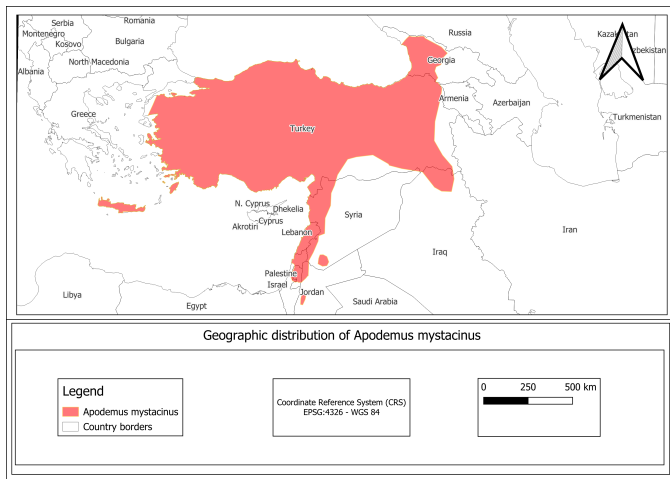


Figure 3 – Distribution areas of the studied *Karstomys* group species retrieved from IUCN (2025) data.

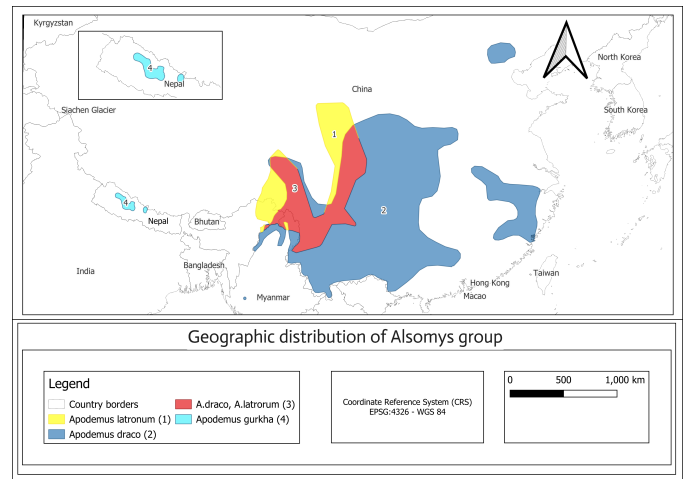


Figure 5 – Distribution areas of the studied *Alsomys* group species retrieved from IUCN (2025) data (Some authors have placed *A. gurkha* within *Apodemus*, but the most recent article (Mezhzherin and Tereshchenko 2023) considered it as *Alsomys gurkha*).

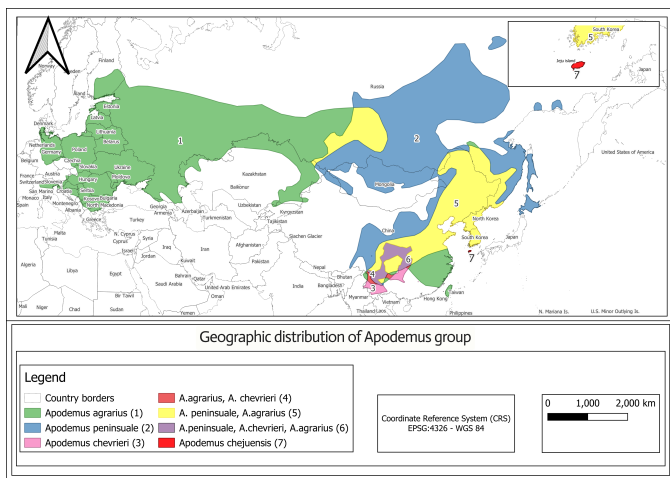


Figure 4 – Distribution areas of the studied *Apodemus* group species retrieved from IUCN (2025) data and www.inaturalist.org.

Palaearctic wood mice (*Apodemus* and *Alsomys*). After that, the West Palaearctic wood mice group (*Sylvaemus* and *Karstomys*) differentiated in the southwestern borders of the Western Palaearctic. The speciation following these events continued at the Pleistocene Era, and present species have evolved (Mezhzherin and Tereshchenko, 2023). Geographical barriers such as mountains and deserts, climatic changes caused by Quaternary glacial cycles, and changes in habitat structure with expansion and/or contraction of forests, and refugia have been highly influential in the differentiation of these species (Wu et al., 2001; Sakka et al., 2010). It should be emphasized that besides the mitogenome analyses, ecological, morphological, and other genetic (e.g., nuclear markers) analyses must be conducted in future studies in order to support the validity of these different genera.

The results also confirm the controversial status of *A. chejuensis* as a subspecies of *A. agrarius*, with a genetic distance of 1.49–2.01%. *A. a. chejuensis* was described by Jones and Johnson (1965) as a subspecies from Jeju Island in the southern Korean Peninsula and differs significantly from other subspecies, particularly *Apodemus agrarius coreae* (from mainland Korea), in its larger body size (Koh, 1986). There is no consensus on the species or subspecies status of *A. a. chejuensis*, which has been studied by various authors using methods such as morphology, hybridization, and mitochondrial DNA analyses. Koh (1991); Han et al. (1996); Oh and Mori (1998), and Koh et al. (2000) consider *A. a. chejuensis* to be a distinct species due to the reproductive isolation, morphological, and genetic differentiation. On the other hand, Sun et al. (2014) supported the subspecies status based on low genetic distance (1.83% of Jukes-Cantor distance), and Jo et al. (2016)

also supported it based on low fixation index values and, consequently, high gene flow, suggesting that reproductive isolation can not be taken into consideration. Oh (2012) found mtDNA distance values of 0.5–2.7% between *A. a. chejuensis* and *A. agrarius coreae*, and Oh et al. (2012) determined that they diverged recently (0.3 MYA, Late Pleistocene (CYTB)). Yoon (1997) offered that Jeju Island is a young area compared to the Korean Peninsula and approximately 2 Mya, volcanic activities caused the formation of this island and Jeju Island has been isolated for 12,000 years due to the increasing of sea level (Ohshima, 1990). About the phylogeographic history of *A. a. chejuensis*, two hypothesis have been postulated: Either ancestral *A. a. chejuensis* or *A. agrarius* (distinct from “*chejuensis*”) migrated from the mainland to Jeju Island and diversified there (Han et al., 1996; Koh et al., 2000; Yoon et al., 2004; Oh et al., 2012). According to CYTB results of Oh et al. (2012), ancestral *A. a. chejuensis* reached Jeju Island via the Yellow Sea basin route, and as indicated by high haplotype diversity and low nucleotide diversity values, rapid expansion occurred after a period of low effective population size. Han et al. (1996) and Koh et al. (2000) calculated the split time of insular *A. a. chejuensis* and mainland *A. a. coreae* as 1.2 MYA and 7000–500,000 years ago (during the last ice age), respectively. Over time, it is inevitable that mainland and island populations will diverge both morphologically and genetically (Johnson et al., 2000). *A. a. chejuensis* has formed a distinct lineage on Jeju Island, and speciation is ongoing. However, data in the literature and our study, particularly the low genetic distance values, strengthen the possibility of subspecies status.

*A. chevrieri*, endemic to southwestern China, was initially described by Allen (1940) as *A. agrarius*, but Corbet (1978) considered it as a subspecies of *A. agrarius* based on dorsal pelage and body size. However, based on certain morphological characteristics, serum protein, and DNA analyses, it has been accepted as a separate species, being the closest sibling species to *A. agrarius*. (Xia, 1984; Wang, 1985; Musser et al., 1996; Wang, 2003; Liu et al., 2004; Musser and Carleton, 2005; Ge et al., 2019). For example, the genetic distance between two species was reported as 8.2% (CYTB, Liu et al., 2004), 7.4% and 6.9% (CYTB and COI, Ge et al., 2019), while in our study this value is 5.63–8.08%, confirming the idea that *A. chevrieri* has formed a distinct species through allopatric speciation.

### Phylogenetic Positions of The *Sylvaemus* Species

The distribution areas of the species belonging to *Sylvaemus* were given in Fig. 2 (IUCN, 2025). The phylogenetic relationships of these species have been tried to be solved for years, but the results obtained by various authors with allozyme, mitochondrial DNA (CYTB, 12S rRNA, D-loop), and nuclear DNA (IRBP, RAG1, I7) analyses contradict each other. Michaux et al. (2002); Bellinvia (2004); Suzuki et al. (2008), and

Mezhzherin and Tereshchenko (2023) argued that *A. (Syl.) flavicollis* and *A. (Syl.) uralensis* are closer to each other than the remaining species. However, according to Liu et al. (2004); Hofer et al. (2007); Bugarski-Stanojević et al. (2011), and Darvish et al. (2015), *A. (Syl.) flavicollis* and *A. (Syl.) sylvaticus* are closely-related species. In contrast, Bellinvia et al. (1999) offered that *A. (Syl.) uralensis* and *A. (Syl.) sylvaticus* are the closest species. Filippucci et al. (2002) underlined the proximity of *A. (Syl.) flavicollis* and *A. (Syl.) hermonensis* (synonym: *A. (Syl.) witherbyi*). These discrepancies may be due to the high rate of saturation in *Sylvaemus*, as suggested by Bellinvia et al. (1999) and Bellinvia (2004), or may be because analysing a single gene region yields limited data, which cannot clearly show the relationship between species, especially those that have evolved rapidly. Therefore, the use of complete mitochondrial DNA will help to solve this problem. In our study, as shown in Fig. 1, the BI tree constructed using the mitogenome yielded higher posterior probability (pp) values than the CYTB and COI trees constructed using much shorter sequences (Fig. S5-S6). According to the mitogenome data (Fig. 1), *A. (Syl.) flavicollis* and *A. (Syl.) ponticus* were positioned more closely with *A. (Syl.) uralensis*, followed by *A. (Syl.) witherbyi* and *A. (Syl.) sylvaticus*, respectively (pp: 1.00). This result is in agreement with Michaux et al. (2002); Bellinvia (2004); Suzuki et al. (2008); Bogdanov et al. (2012), and Mezhzherin and Tereshchenko (2023) that *A. (Syl.) flavicollis* and *A. (Syl.) uralensis* are close, but in the first 3 studies (*A. (Syl.) witherbyi* was not analysed in the last study), *A. (Syl.) sylvaticus* is closer to these two species than *A. witherbyi*. In our study, the mean genetic distances were 5.44–10.04 % (mitogenome data), and these *d* values are quite high (>8 %) except for *A. (Syl.) flavicollis* and *A. (Syl.) ponticus*. Based on the mitogenome data, it can be concluded that *A. (Syl.) sylvaticus* first diverged and spread in the west (Europe) during a Quaternary glacial period, probably undergoing a postglacial recolonisation and rapid expansion from refugia in the Iberian Peninsula and Italo-Balkan Region. *A. (Syl.) witherbyi*, formerly recognised as a subspecies of *A. (Syl.) sylvaticus*, may also have diverged and spread to Russia, the Caucasus, and the Near East, with limited distribution in the west (Greece and Turkiye). Similarly, *A. (Syl.) uralensis*, formerly considered as a subspecies of *A. (Syl.) sylvaticus* by Corbet (1978); Ellerman and Morrison-Scott (1951), and Pavlinov and Rossolimo (1987), may have diverged at a later stage, and its sister species, *A. (Syl.) alpicola*, was found allopatrically in Central Europe, while *A. (Syl.) uralensis* may have spread from Eastern Europe to Turkiye, Russia, and Caucasus. While these aforementioned species have a narrower distribution and habitat preferences, *A. (Syl.) flavicollis* is the most widely distributed and most common wood mouse species (Mitchell-Jones et al., 1999). *A. (Syl.) ponticus*, which is allopatrically distributed and morphologically similar to *A. (Syl.) flavicollis*, was suggested by Bellinvia (2004) to be a subspecies of *A. (Syl.) flavicollis*. Balakirev et al. (2007); Suzuki et al. (2008); Bogdanov et al. (2012), and Mezhzherin and Tereshchenko (2023) emphasised the relativity of these two species. Darvish et al. (2015) argued that their divergence time was 2.6 MYA, and that the Praetiglian Cold Stage (Ruddiman and Raymo, 1988) led to the formation of steppe habitats instead of forests (Fauquette et al., 1998, 1999), so that ancestral populations were reduced and restricted. Thus, *A. (Syl.) flavicollis* remained in refugia in the southern Balkans, and *A. (Syl.) ponticus* in the eastern Mediterranean. We calculated the divergence time as 1.85 million years ago, which is a much closer time frame according to Darvish's results. According to Bradley and Baker (2001), genetic distance values among either conspecific populations or valid species can range from 2 % to 11 %, in general. The average intraspecific genetic distances are 1.5 % (0.0–4.7 %) for rodents (Baker and Bradley, 2006). On the other hand, the two species have a low genetic distance (2.68 %) (Tab. S1) and are not separated in the CYTB tree (Fig. S5). Likewise, Balakirev et al. (2007) noted that in CYTB analyses, two species with a 1.77 % distance came together in trees with low bootstrap values. The CYTB marker may not always strongly reveal phylogenetic relationships between closely related species as it can be observed in the tree which could not discriminate even subgenera (Fig. S5). Additionally, in our study, the *A. (Syl.) ponticus* sequence

(from GenBank) mixed with *A. (Syl.) flavicollis* was collected from Iran and may have been misidentified due to the morphological similarity between the two species. Therefore, *A. (Syl.) ponticus* is most likely a separate species, but further mitogenome analyses with a larger sample size would be more useful. ☞

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## Supplemental information

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

- Tab. S1** Calculated mean genetic distance (d) values (below the diagonal) between species with the standard errors (above the diagonal) employing CYTB data.
- Tab. S2** Calculated mean genetic distance (d) values (below the diagonal) between species with the standard errors (above the diagonal) employing COI data.
- Tab. S3** Calculated mean genetic distance (d) values (below the diagonal) between subgenera with the standard errors (above the diagonal) employing CYTB data.
- Tab. S4** Calculated mean genetic distance (d) values (below the diagonal) between subgenera with the standard errors (above the diagonal) employing COI data.

**Figure S1**  
**Figure S2**  
**Figure S3**  
**Figure S4**  
**Figure S5**  
**Figure S6**