Supplementary Information

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Supplemental field methods

Mice and shrews belonging to the genera *Apodemus* and Sorex were readily keyed to species from obvious external features. Two species within the genus *Microtus* are externally very similar - *M. arvalis* and *M. agrestis*. For *Microtus* voles, molar shape was examined under a dissecting microscope and used to key animals to species (Pucek 1981). For rodents, age was assessed using size and pelage characteristics as well as reproductive status (juveniles and sub-adults were non-reproductive). Adult females were recorded as reproductive if they were pregnant, and males if their testes were descended. Age and reproductive status could not be determined for shrews.

16S rRNA gene sequencing methods

Total genomic DNA was extracted from gut content samples using the MoBio PowerSoil extraction kit in tube format, according to manufacturer's instructions. Extraction batches were blocked by species and site to ensure each batch contained approximately equal numbers of samples from different species and sites, to minimise extraction batch effects on results. A single extraction control (H_20) was included per 48 samples. Bead-beating was carried out (10 min at 20 Hz) using a Qiagen TissueLyser. The V4 region of the bacterial 16S rRNA gene was amplified using primers 515F and 806R (Caporaso *et al.* 2011), with library preparations following a two-step (tailed-tag) approach with dual-indexing (D'Amore *et al.* 2016). Full primer sequences are given in Table S1 below. In a first PCR step, primers targeting the V4 region were used that incorporated a recognition sequence, allowing a second PCR reaction that added Illumina indices. Initially, a test PCR was performed to check sample amplification and a lack of contamination at the extraction and PCR stages. The following were combined in a 20µl reaction volume: 10µl KAPA 2x Mastermix (Kapa Biosystems, Wilmington, USA), 0.25µl of each primer at 10µM, 4.5µl PCR-grade water and 5µl undiluted DNA extraction. Cycling conditions were as follows: denaturation at 98°C for 2min, 35 cycles of 95°C for 20s, 65°C for 15s, 72°C for 30s, followed by a final extension at 72°C for 5min and a 4°C hold. Products were visualised on 2% agarose gels, and no amplification was observed in extraction or PCR controls. Following each successful test PCR, a first round PCR for use in sequencing was performed, using exactly the same conditions as described above but with fewer cycles (20 instead of 35). DNA extractions ranged in concentration from approx. 1 to 78ng/µl as measured using a Qubit® dsDNA HS Assay Kit, such that the amount of DNA template in first

round PCRs varied from approx. 5 to 390 ng per reaction. First round PCR products were purified using Agencourt Ampure XP beads (Beckman Coulter, Brea, USA), by adding 20µl beads at room temperature to samples, before being washed twice in 200µl 80% ethanol and eluted in 10µl H_20. For the second round PCR, the following were combined in a 20µl reaction volume: 10µl KAPA 2x Mastermix (KAPA Biosystems), 0.5µl of each primer at 10µM and 9µl of clean PCR product from the first round. Eight forward (i5) and twelve reverse (i7) barcoded primers were used, creating 96 unique combinations, with barcode sequences as described in the Illumina Nextera XT protocol. Second round PCR products were cleaned using Ampure XP beads as described above. Each second round PCR product (library) was analysed using a Fragment Analyzer (AATI, Ankeny, USA), and up to 96 libraries pooled per sequencing run, at equimolar concentration of fragments in the expected size range (300-600bp). Each pool was size selected using a Pippin Prep (Sage Science) prior to sequencing, to remove fragments outside that size range. DNA concentration within size selected pools was determined using a Qubit® dsDNA HS Assay Kit, while DNA quality and average fragment size was confirmed using the Fragment Analyzer (AATI). Subsequently, a quantitative real-time PCR (qPCR) assay was performed (Illumina® KAPA Library Quantification Kit, Kapa Biosystems, Wilmington, USA) to quantify the number of DNA fragments with both adaptor sequences on either end, and therefore able to successfully form clusters on a flowcell. Briefly, a 20 µl PCR reaction (run in triplicate for each pool) was prepared on ice with 12µl SYBR Green I Master Mix and 4µl diluted pooled DNA (1:1000 to 1:100,000 depending on the initial concentration). PCR cycling conditions were as follows: denaturation at 95°C for 5min, 35 cycles of 95°C for 30s, 60°C for 45s, melt curve analysis to 95°C (continuous) and cooling to 37°C (LightCycler® LC48011, Roche Diagnostics, Burgess Hill, UK). In preparation for sequencing, DNA in each pool was denatured for 5 minutes at room temperature using freshly diluted 0.1M sodium hydroxide (NaOH) and the reaction was subsequently terminated by the addition of the hybridization buffer (HT1) that comes with the Illumina MiSeq Kit. Following calculation of molarity using qPCR data, DNA was diluted to a loading concentration of 9pM using the HT1 buffer. Amplicon libraries were sequenced on an Illumina® MiSeq platform with version 2 chemistry using sequencing by synthesis (SBS) technology to generate 2 x 250 bp paired-end reads, with a 15% PhiX fragment library added to increase sample diversity. Four sequencing runs were used in total.

Bioinformatics using the DADA2 pipeline

Processing through the DADA2 v1.4 pipeline followed the online tutorial (https://benjjneb.github.io/dada2/tutorial_1_4.html). First, sequence quality was examined across the length of sequence reads to inform the choice of read trimming parameters. Four samples from each sequencing run were inspected and trimming parameters chosen to

exclude sequence regions after which sequence quality dramatically declined. Forward reads were truncated at 230bp, and reverse reads at 150bp. In the same step, primer sequences were removed from the 5' end of all reads using the trimLeft parameter to remove 30bp. 30bp was chosen as initial analysis using cutadapt v1.13 (Martin 2011) on a sample of fastq files showed trimming this length would remove primer sequence from the vast majority of reads. Otherwise standard filtering parameters were used: maxN=0, truncQ=2, rm.phix=TRUE and maxEE=2. Between 78 and 98% of reads per sample (mean 92.5%) survived trimming and filtering. Amplicon sequence variants (ASVs) were then inferred for each sequencing run separately. Forward and reverse read error rates for each possible nucleotide transition were learnt using a subset of 2 million reads. After inference of ASVs and dereplication, paired reads were merged and putative chimeras removed. Sequences of extreme length were also filtered out, retaining only those with approximately the expected length (between 236 and 239bp). Overall, by the end of the DADA2 pipeline between 36% and 97% raw sequence reads per sample were retained, with a mean of 89%. Taxonomy was assigned to ASVs using the 13.8 release of the Greengenes database clustered at 97% identity, formatted for DADA2 (gg_13_8_train_set_97.fa.gz).

Table S1: Primer sequences used to amplify the V4 region of 16S rRNA. In round 1 primers, the primer sequence from Caporaso et al. 2011 are marked with red text, and the recognition sequence to allow the second round PCR is marked in blue. In the second round PCR that uses barcoded primers, two example barcodes (Illumina indices) are shown, marked with bold underlined text. Italic text indicates Illumina adapter sequences.

PCR round	Primer name	Primer sequence
1	515F	5' ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA 3'
1	806R	5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT 3'
2	N501F (example barcoded primer)	5' *AATGATACGGCGACCACCGAGATCTACAC***TAGATCGC**ACACTCTTTCCCTACACGACGCTC 3'
2	N701R (example barcoded primer)	5' *CAAGCAGAAGACGGCATACGAGAT***TCGCCTTA**GTGACTGGAGTTCAGACGTGTGCTC 3'

Table S2: **Numbers of animals captured at each site for which the gut microbiota was characterised.** Animals from the three host genera in which multiple species were sampled across multiple sites are shaded.

	Genus	Species	Site				
			BG	CC	LM	PL	WF
Mice	*Apodemus*	*A. agrarius*	5	6	3	2	11
		A. flavicollis	6	5	6	5	8
	Micromys	*M. minutus*	4	0	1	0	6
Voles	*Microtus*	*M. arvalis*	2	6	7	21	3
		M. agrestis	6	6	7	3	7
		M. oeconomus	5	4	1	0	8
	Myodes	*M. glareolus*	2	2	6	7	9
Shrews	*Sorex*	*S. araneus*	6	6	6	12	4
		S. minutus	3	2	3	0	2
	Neomys	*N. fodiens*	1	0	0	0	0

Table S3: Mean pairwise differences in microbiota Bray-Curtis dissimilarity or Jaccard distance among congeneric animals according to whether they belong to the same species or were caught at the same site. 'Diff' indicates the mean difference in dissimilarity metric between animals belonging to the two groups (Group2 – Group1). W values indicate the observed test statistic from a two-sample Wilcoxon test, and p-values were derived from Monte Carlo permutation tests with 10,000 permutations.

Metric	Group 1	Group 2	*Apodemus*			*Microtus*			*Sorex*		
			Diff	W	p	Diff	W	p	Diff	W	p
Bray-Curtis	Same species	Different species	0.180	621710	<0.0001	0.093	2639438	<0.0001	0.187	185638	<0.0001
	Same site	Different site	0.029	239436	0.0001	0.028	1086905	<0.0001	0.008	59922	0.532
	Conspecific, same site	Conspecific, different site	0.045	68410	<0.0001	0.027	163237	<0.0001	0.014	24659	0.2784
	Heterospecific, same site	Heterospecific, different site	0.021	67115	<0.0001	0.013	382820	<0.0001	0.012	8694	0.268
	Conspecific, different site	Heterospecific, same site	0.154	4425	<0.0001	0.076	60090	<0.0001	0.175	3308	<0.0001
	Conspecific, same site	Heterospecific, same site	0.199	26429	<0.0001	0.103	77728	<0.0001	0.189	4537	<0.0001
Jaccard	Same species	Different species	0.179	621930	<0.0001	0.095	2619826	<0.0001	0.197	182612	<0.0001
	Same site	Different site	0.032	242388	<0.0001	0.031	1098297	<0.0001	0.005	59379	0.6544
	Conspecific, same site	Conspecific, different site	0.048	70671	<0.0001	0.032	167061	<0.0001	0.013	24674	0.275
	Heterospecific, same site	Heterospecific, different site	0.023	67394	<0.0001	0.015	386454	<0.0001	0.008	8481	0.4306
	Conspecific, different site	Heterospecific, same site	0.151	4520	<0.0001	0.076	62773	<0.0001	0.188	3570	<0.0001
	Conspecific, same site	Heterospecific, same site	0.199	26529	<0.0001	0.107	78150	<0.0001	0.201	4444	<0.0001

Table S4: Results of PERMANOVA analyses on Bray-Curtis dissimilarity scores among samples within each host genus. ‡ indicates where dispersion tests (using the function *betadisper*) indicated significant differences in dispersion across host species or capture sites, with stars indicating the significance level of this test (*p<0.05, **p<0.01, ***p<0.001). Where groups differ in dispersion, PERMANOVA results should be interpreted with more caution as significance may be influenced by differences in group dispersion rather than means (Anderson 2001).

Model	Term	*Apodemus*			*Microtus*			*Sorex*		
		F	p	R^2	F	p	R^2	F	p	R^2
Univariate	Host species	21.68	<0.001‡*	0.28	9.86	<0.001	0.19	7.07	<0.001‡**	0.14
	Site	1.78	0.015	0.12	2.9	<0.001‡***	0.13	1.26	0.041	0.11
Multivariate	Host species	22.99	<0.001	0.28	10.29	<0.001	0.19	7.43	<0.001	0.14
	Site	2.13	0.002	0.10	2.33	<0.001	0.09	1.42	0.002	0.11
	Age	1.54	0.038	0.04	1.28	0.057	0.02	n/a	n/a	n/a
	Sex_reprod	1.07	0.344	0.04	1.09	0.202	0.03	n/a	n/a	n/a
	Weight	1.38	0.148	0.02	0.96	0.457	0.01	0.83	0.600	0.02
	Read count	1.19	0.220	0.01	1.42	0.027	0.01	0.9	0.640	0.02
	Sequencing run	1.06	0.249	0.04	0.96	0.906	0.03	1.21	0.124	0.07
	Live caught	1.48	0.167	0.02	1.63	0.034	0.02	n/a	n/a	n/a
	Max dissection interval	1.13	0.266	0.01	1.57	0.061	0.01	1.08	0.450	0.02

Table S5: Results of PERMANOVAs testing for an effect of host species within each host genus, using four different dissimilarity/distance metrics. PERMANOVAs were run using the *adonis* function in R package vegan, using 10,000 permutations.

Dissimilarity metric	*Apodemus*			*Microtus*			*Sorex*		
	F	p	R^2	F	p	R^2	F	p	R^2
Jaccard	23.78	<0.001	0.30	11.04	<0.001	0.21	7.02	<0.001	0.14
Bray-Curtis	21.68	<0.001	0.28	9.86	<0.001	0.19	7.07	<0.001	0.14
Unweighted UniFrac	11.16	<0.001	0.17	5.68	<0.001	0.12	9.94	<0.001	0.19
Weighted UniFrac	5.24	0.002	0.09	4.09	0.001	0.09	8.4	<0.001	0.17

Table S6: Accuracy of random forest classifier (RFC) models at assigning samples within each host genus to the correct species, depending on the level of bacterial taxonomic resolution. All RFC models used 100,000 trees, and involve only the subset of ASVs that could be taxonomically classified to at least family level. Accuracy shown is based on each model's out-of-bag error rate.

Bacterial taxonomic resolution	*Apodemus*		*Microtus*		*Sorex*	
	N features	Accuracy (%)	N features	Accuracy (%)	N features	Accuracy (%)
ASV	676	100	1300	98.84	675	95.45
Family	54	98.25	53	72.09	69	93.18
Order	28	92.98	24	56.98	36	90.91
Class	20	92.98	18	59.3	25	86.36
Phylum	11	70.18	10	56.98	11	90.91

Table S7: Mean differences in diet Bray-Curtis dissimilarity among samples according to whether they belong to the same species or were caught at the same site. 'Diff' indicates the mean difference in pairwise Bray-Curtis dissimilarity between animals belonging to the two groups (Group2 – Group1). W values indicate the observed test statistic from a two-sample Wilcoxon test, and p-values were derived from Monte Carlo permutation tests with 10,000 permutations.

Group 1	Group 2	*Apodemus*			*Microtus*			*Sorex*		
		Diff	W	p	Diff	W	p	Diff	W	p
Same species	Different species	0.009	283414	0.3024	0.105	1480382	<0.0001	0.162	1017856	<0.0001
Same site	Different site	0.042	202565	0.0012	0.069	999960	<0.0001	0.038	56174	0.0688
Conspecific, same site	Conspecific, different site	0.041	47442	0.0694	0.068	154380	0.0106	0.021	22537	0.3440
Heterospecific, same site	Heterospecific, different site	0.044	53943	0.0124	0.041	341447	0.1144	0.038	7086	0.2498
Conspecific, different site	Heterospecific, same site	-0.033	50856	0.0892	0.052	144363	0.1344	0.126	8052	0.0132
Conspecific, same site	Heterospecific, same site	0.008	12385	0.9002	0.121	65070	0.0004	0.147	3348	0.0050

Figure S1: **Location and habitat of sampling sites**. (A) A map showing the location and (B) photographs of habitat at the five sampling sites, which were all within 25km of each other close to the city of Vilnius, Lithuania. Sites were the edge of a bog (BG), a re-growing clear-cut (CC), a natural meadow (LM), a site with short grass pastureland and a young tree plantation (PL), and a site between a wheat field and a pond (WF). GPS coordinates of sites are as follows, **BG:** 55° 56' N, 25° 17' E; **CC:** 55° 55' N, 25° 19' E; **LM:** 55° 1' N, 25° 21' E; **PL**: 55° 1' N, 25° 19' E; **WF**: 54° 55' N, 25° 14' E)

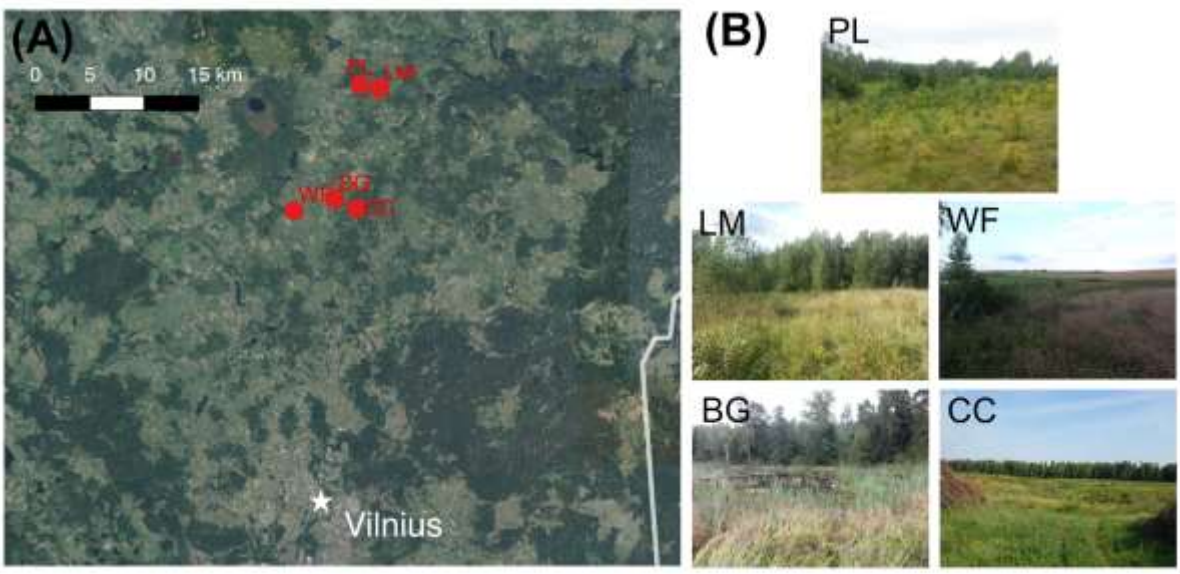


Figure S2: Diet of animals by site and species according to stomach contents composition. Each panel is a site, numbers on each bar indicate the number of samples used to calculate mean dietary profile, and host species are coded as: AA *Apodemus agrarius*, AF *Apodemus flavicollis*, MA *Microtus arvalis*, MT *Microtus agrestis*, MO *Microtus oeconomus*, SA *Sorex araneus* and SM *Sorex minutus*. All data are plotted, including 15 animals for which microbiota data was not available.

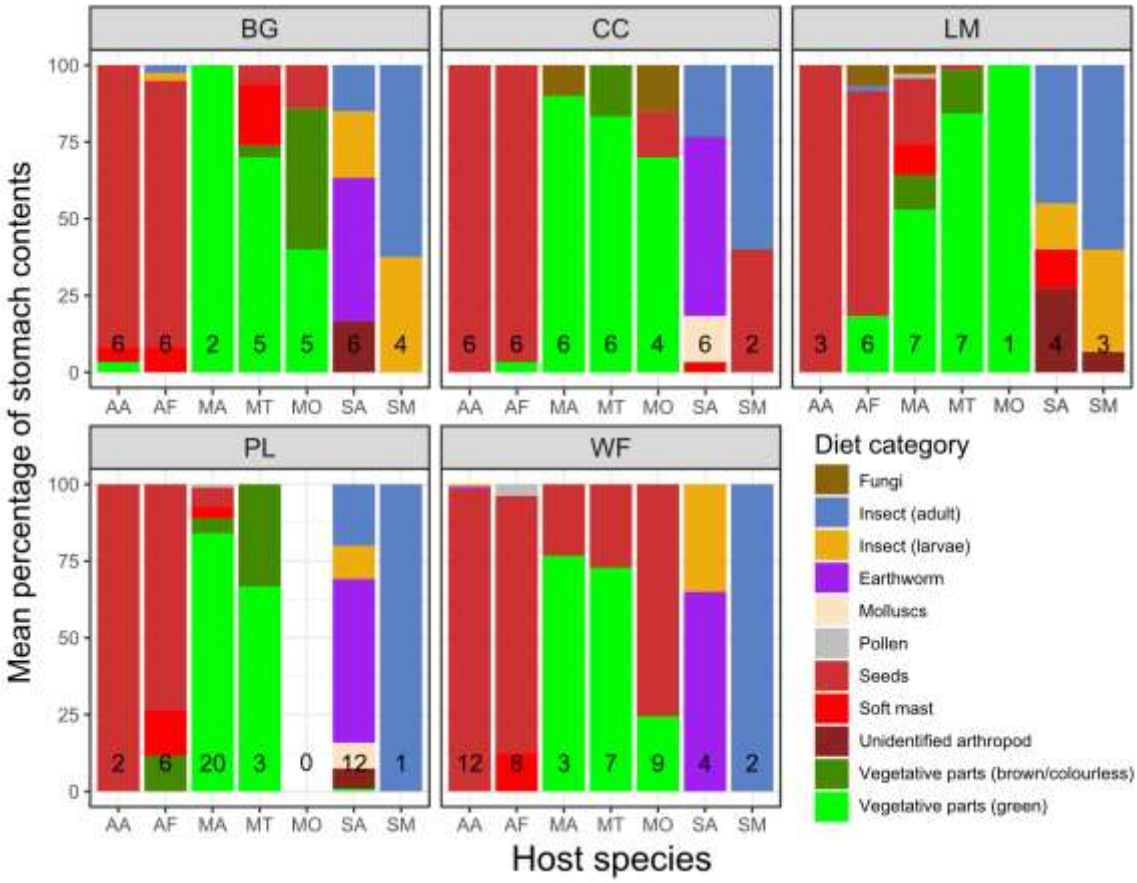


Figure S3: **Sample completeness curves**. Curves are shown for each sample after taxonomic filtering (removing sequences assigned to chloroplast, mitochrondria or not assigned a phylum) but prior to abundance filtering (removing sequences of low prevalence/abundance), and were generated in the R package iNEXT. 50 bootstrap replicates were used per sample.

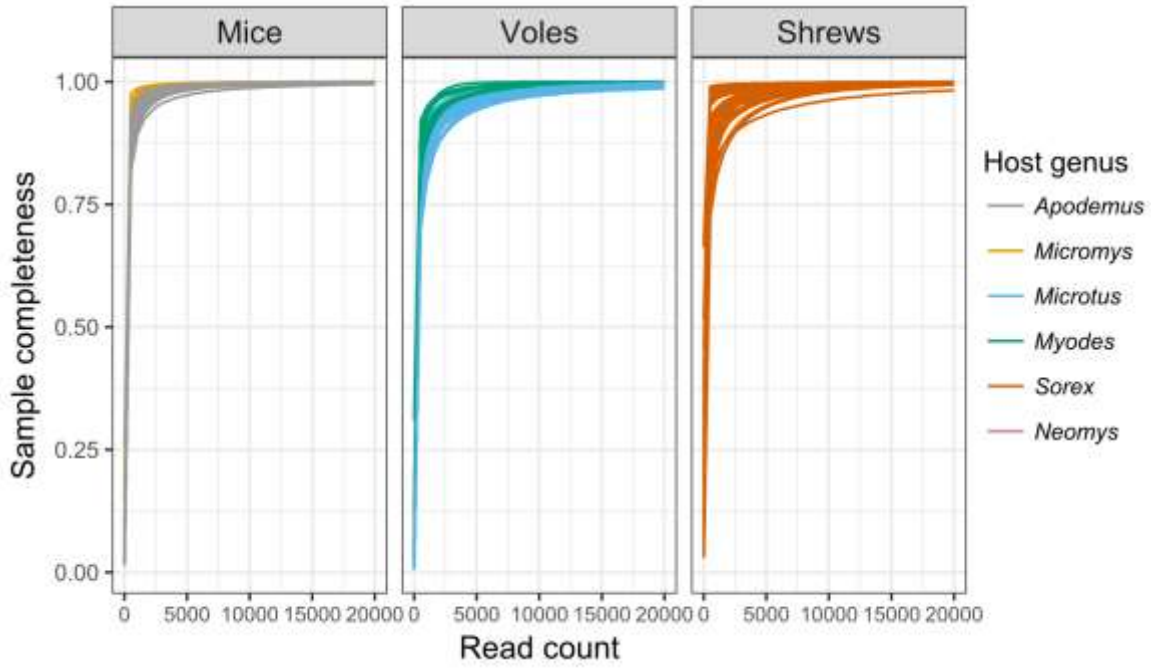


Figure S4: Principle coordinates (PCoA) plots based on Bray-Curtis dissimilarity according to sampling method. Plots indicate no obvious clustering according to capture method (lethal vs live-caught).

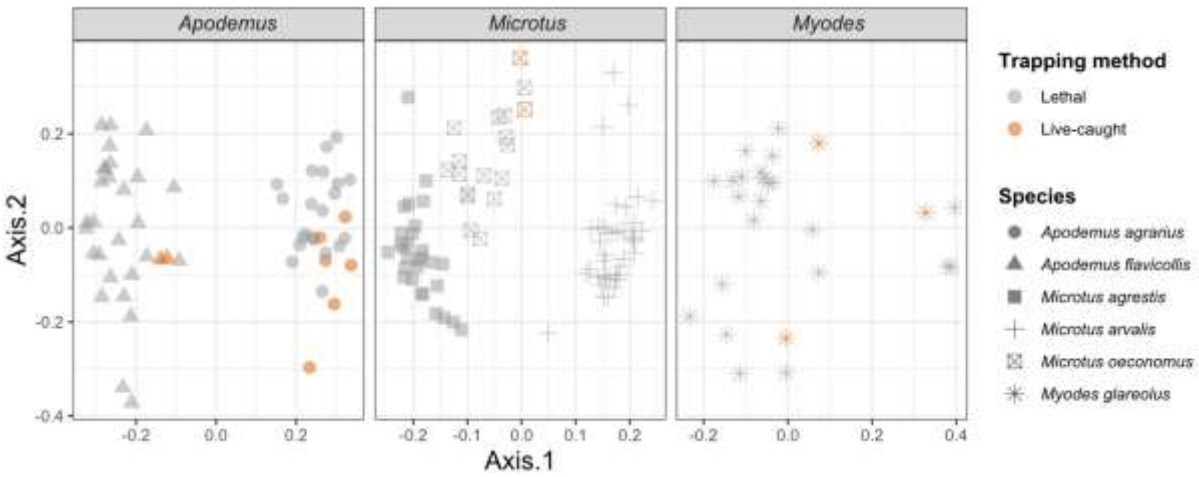


Figure S5: Principle coordinates (PCoA) plot based on Unweighted UniFrac distances, indicating clustering of samples by host family and genus.

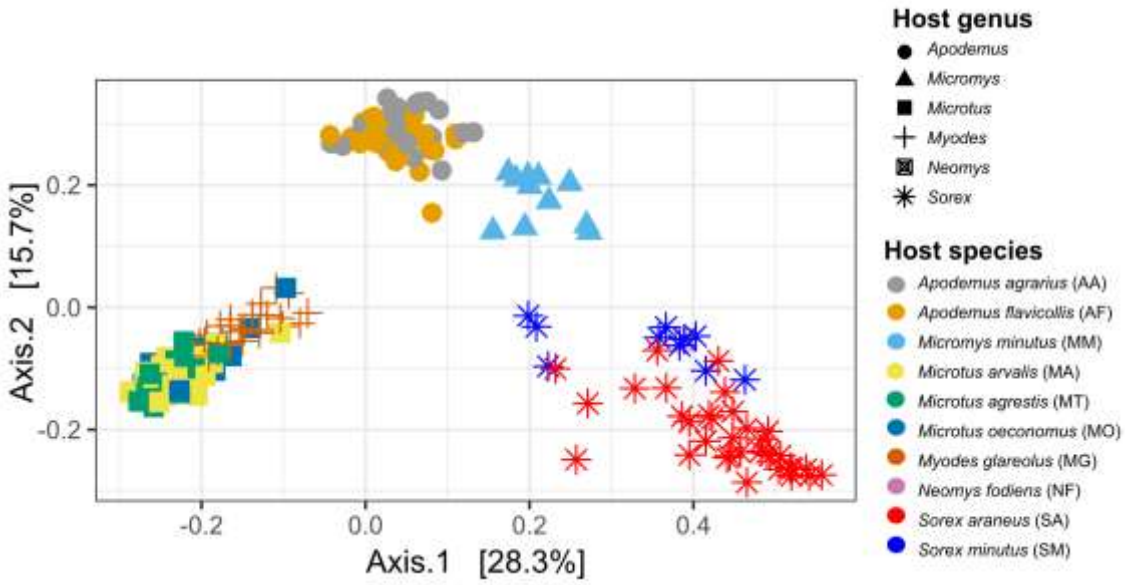


Figure S6: Hierarchical clustering plots for each host genus based on the Jaccard, Unweighted and Weighted UniFrac dissimilarity indices. Branches are coloured according to host species as in Figure 2, with left-hand bars also indicating species identity and right-hand bars indicating capture site.

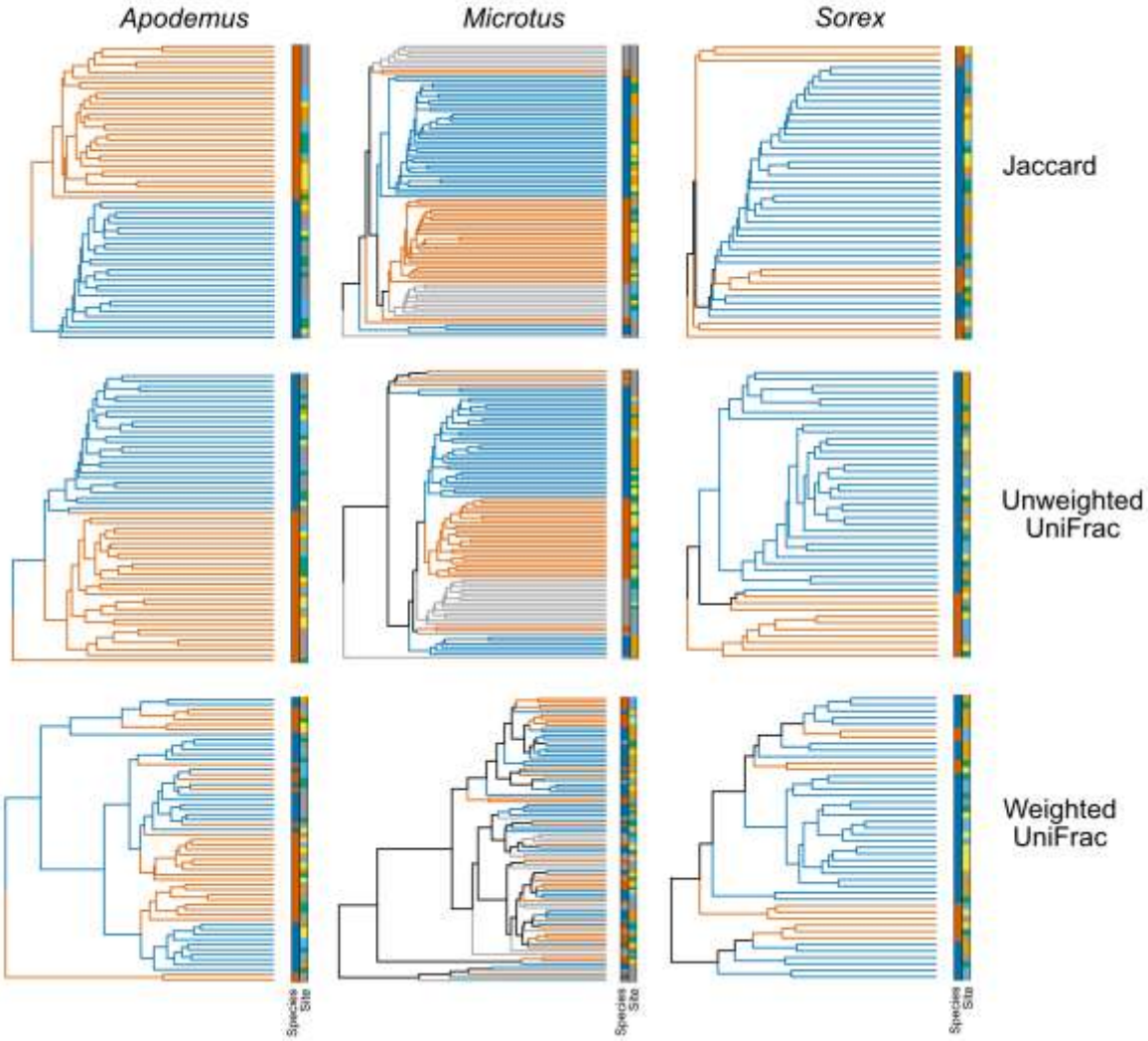


Figure S7: Heat maps illustrating how representation of ASVs important for indicating host species are distributed across bacterial families and orders. Only families or orders containing at least 10 ASVs are shown. Four indicators of SV importance are depicted: % species-specific: percentage of ASVs in each bacterial taxon that were only found in one host species within the genus; % in RFC top20: percentage of ASVs in each bacterial taxon that were in the Random Forest Classifier model top 20 most important variables; % in RFC top50: percentage of ASVs in each bacterial taxon that were in the Random Forest Classifier model top 50 most important variables; Mean RFC importance: mean importance value (Mean Decrease Gini) of ASVs belonging to a bacterial taxon in the RFC model. For comparability, values of all four indicators across the depicted bacterial taxa have been scaled between 0 and 1.

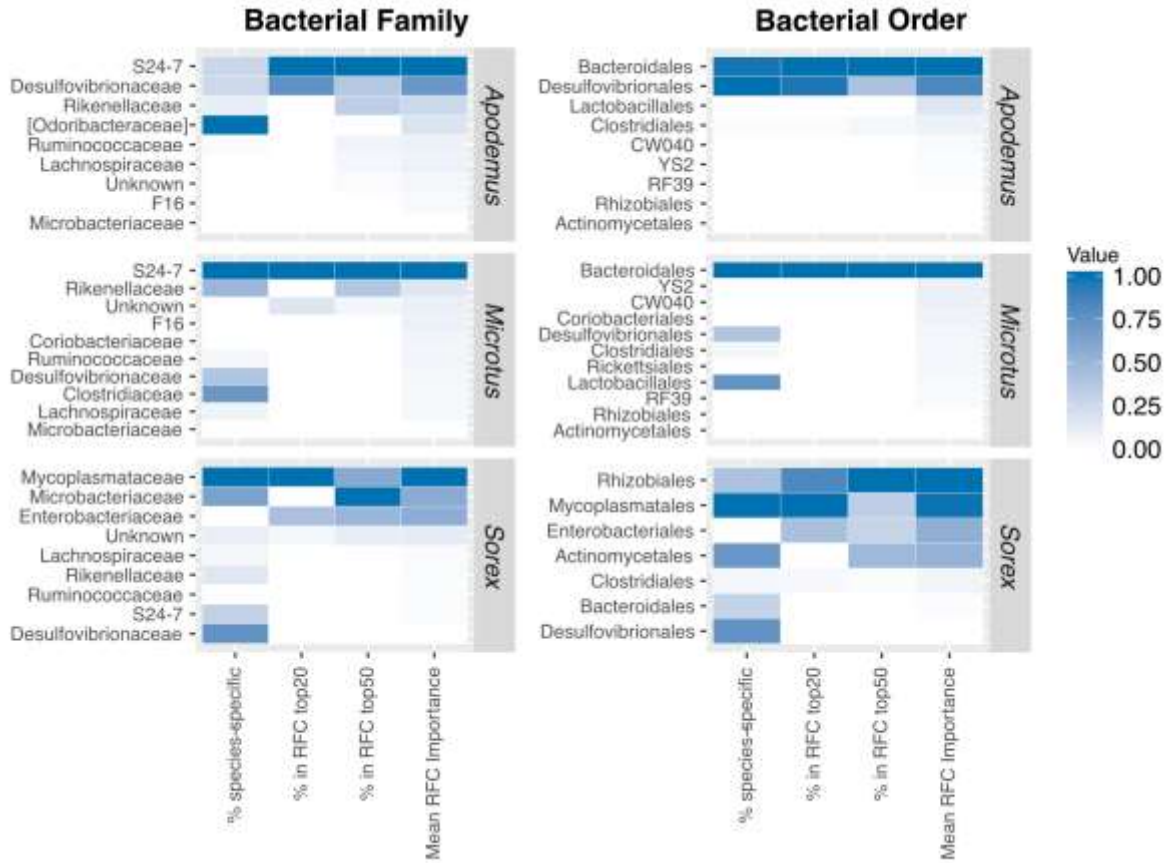


Figure S8: Phylogenetic distribution of host species-indicative members of the family S24-7. (A) ASVs that were the most informative for classifying either *Apodemus* or *Microtus* samples to host species in genus-level RFC models are indicated by coloured dots. The 20 most important ASVs are shown for each genus, with importance represented by the Mean Decrease Gini. (B) Distribution of ASVs according to apparent host specificity (the species each was found in) in the dataset. Through abundance filtering, each ASV was detected in at least 12 samples.

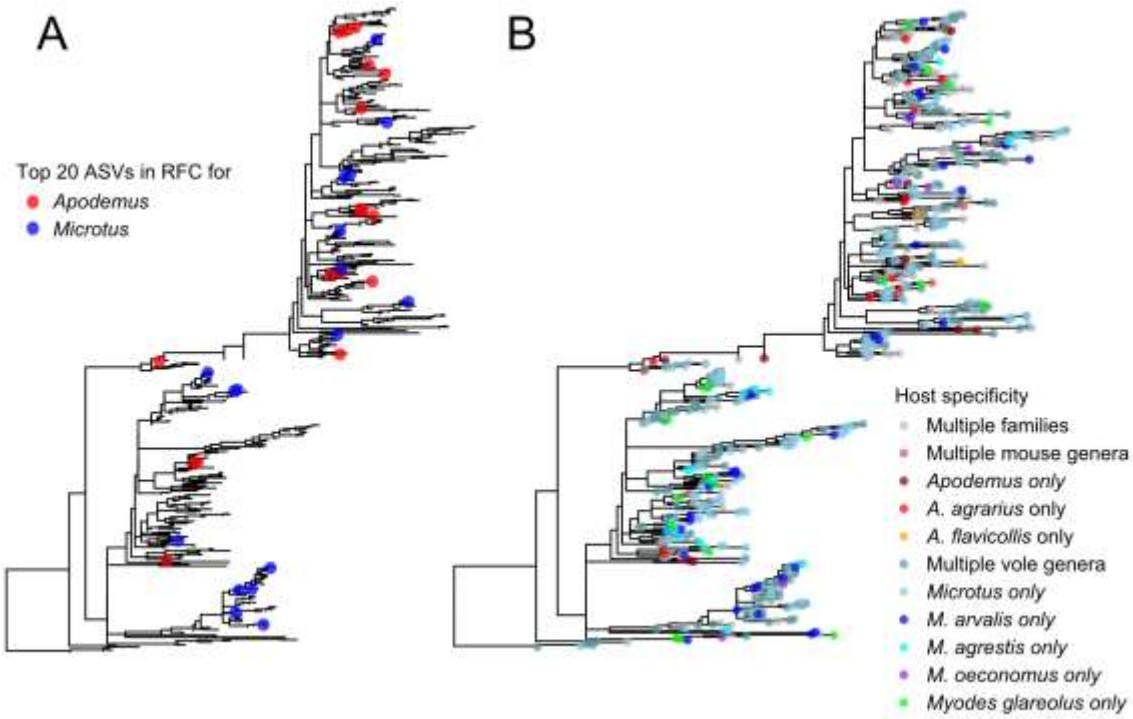
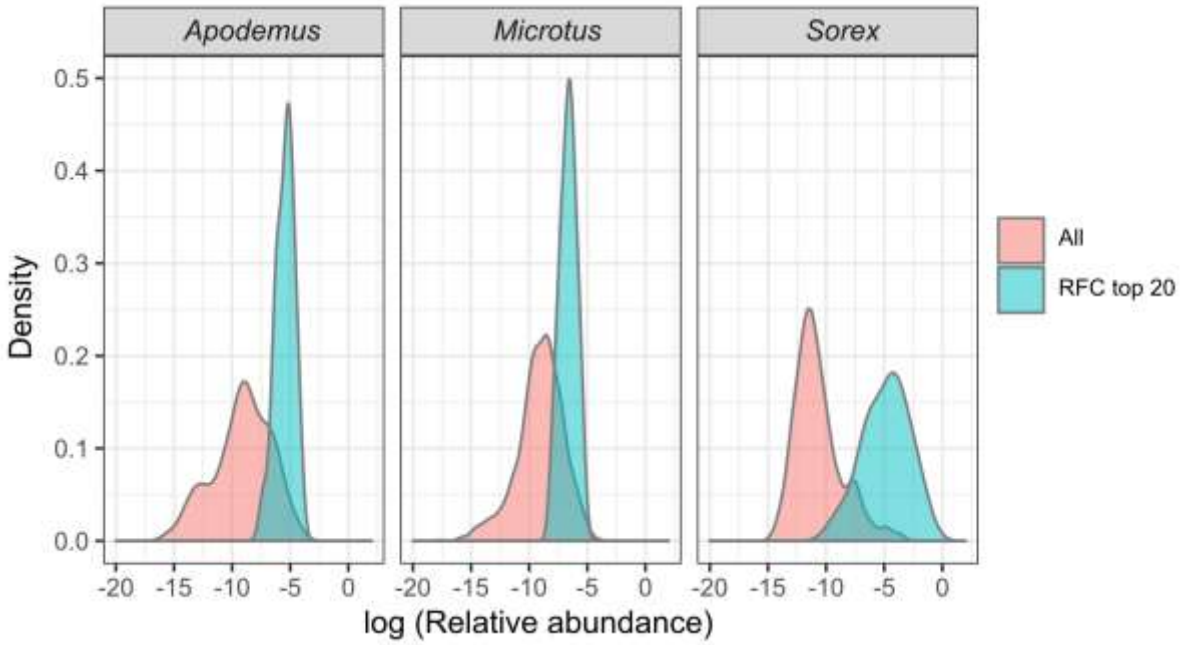


Figure S9: Density histograms showing the relative abundance of ASVs in the top 20 most informative ASVs for host species classification in RFCs, compared to all ASVs.



Supplementary Information References

Anderson, M.J. (2001). A new method for non parametric multivariate analysis of variance. *Austral Ecol.*, 26, 32–46.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., *et al.* (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc. Natl. Acad. Sci. U. S. A.*, 108 Suppl, 4516–22.

D'Amore, R., Ijaz, U.Z., Schirmer, M., Kenny, J.G., Gregory, R., Darby, A.C., *et al.* (2016). A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. *BMC Genomics*, 17, 55.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*.

Pucek, Z. (1981). *Keys to vertebrates of Poland, mammals.* PWN Polish Scientific Publishers, Warszawa.