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Population genetics and genotyping as tools for planning rat management programmes

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Abstract

Brown rats are a prolific synanthropic pest species, but attempts to control their populations have had limited success. Rat population dynamics, dispersal patterns, and resistance to rodenticides are important parameters to consider when planning a control programme. We used population genetics and genotyping to investigate how these parameters vary in contrasting landscapes, namely one urban and two rural municipalities from eastern France. A total of 355 wild brown rats from 5 to 6 sites per municipality were genotyped for 13 microsatellite loci and tested for mutations in the *Vkorc1* gene which confers resistance to some rodenticides. Results revealed a strong genetic structure of the sampled rat populations at both regional (between municipalities) and local (between sites within municipalities) levels. A pattern of isolation by distance was detected in the urban habitat and in one of the rural municipalities. GENECLASS and DAPC analyses identified 25 (7%) and 36 (10%) migrants, respectively. Migrations occurred mostly between sites within each municipality. We deduced that rat dispersal is driven by both natural small-scale movements of individuals and longer-distance (human-assisted) movements. Mutation Y139F on gene *Vkorc1* was significantly more prevalent in rural (frequency 0.26–0.96) than in urban sites (0.00–0.11), likely due to differences in selection pressures. Indeed, pest control is irregular and uncoordinated in rural areas, whereas it is better structured and strategically organised in cities. We conclude that simultaneous pest control actions between nearby farms in rural habitats are highly recommended in order to increase rat control success while limiting the spread of resistance to rodenticides.

Keywords *Rattus norvegicus* · Population genetics · Dispersal · Assignment · *Vkorc1* · Anticoagulant resistance · Control programme

Key message

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- We compared the genetic structure, population connectivity, and frequency of anticoagulant resistance (mutation in the *Vkorc1* gene) in rat populations from rural and urban municipalities in eastern France.
- Rat populations revealed a strong genetic structure although small and long-distance dispersals were evidenced.
- Frequency of mutation Y139F was significantly higher in rural habitats.
- In order to limit the frequency of resistance to rodenticides in rural areas, our results suggest that control programmes should be coordinated between neighbouring farms within each municipality.

Introduction

The brown rat, *Rattus norvegicus* (Berkenhout, 1769), is one of the most important and common pest species worldwide. It has significant adverse effects on agricultural productivity, ecosystems (i.e. on native species and habitats), and public health (Capizzi et al. 2014). Rat control is extremely costly, and since the 1950s, it has mostly relied on the use of antivitamin K (AVK) rodenticides, i.e. anticoagulants (Hayes and Gaines 1950). Anticoagulant rodenticides target the vitamin K epoxide reductase (VKOR) enzyme, preventing the production of functional clotting factors, thus inhibiting coagulation (Tie and Stafford 2008). Because anticoagulants are relatively safe for humans (an antidote, vitamin K1, exists) and are easy to use, rodents were largely managed through chemical intervention, with much less emphasis on mechanical and environmental measures. In 1958, however, it was discovered that brown rats were becoming resistant to first-generation anticoagulant rodenticides (FGARs) (Boyle 1960). Therefore, more potent second-generation anticoagulant rodenticides (SGARs) (e.g. bromadiolone, difenacoum, flocoumafen, difethialone, and brodifacoum) were developed in the 1970s/1980s. Nevertheless, both primary and secondary poisoning of non-target species (due to increased persistence of these more effective compounds within the body) were described (Hughes et al. 2013; Langford et al. 2013) as well as possible evidence of rodent resistance (Prescott et al. 2011).

Resistance to antivitamin K rodenticides is attributed to single-nucleotide polymorphisms (SNPs) in the vitamin K epoxide reductase complex subunit 1 (*Vkorc1*) gene (Pelz et al. 2005; Rost et al. 2004). In France, the Y139F mutation (tyrosine to phenylalanine amino acid at VKORC1 position 139) is the most widely distributed resistance allele in brown rat populations (Grandemange et al. 2010). The prevalence of resistance alleles in rodent populations is likely due to selection pressure caused by the intensity and frequency of anticoagulant use in rat control programmes (Bishop et al. 1977; Greaves et al. 1977), while mutant alleles are most probably spread by natural movements of rats and by anthropogenic displacement of individuals via terrestrial or shipping routes (Pelz et al. 2005). Genetic resistance and ecological considerations, combined with the paucity of alternative methods to control rodent populations, highlight the need for a better understanding of the interplay between rat population dynamics, rat dispersal, and the distribution patterns of resistance to AVKs.

Population genetics has become a well-established tool to infer population dynamics, gene flow, and movement pathways (Abdelkrim et al. 2005; Piertney et al. 2016;

Richardson et al. 2017). In this study, we investigated how these parameters vary in different landscape contexts, namely one urban and two rural municipalities from eastern France. We also examined the distribution of *Vkorc1* variants within the sampled populations. Our results highlight the importance of upstream genetic investigation in the planning of rat control programmes. Finally, we present our recommendations for improving the rat population management scheme of the studied region and more generally, we highlight some sociological and scientific limitations which could be better addressed in future rat control campaigns and associated research.

Materials and methods

Study sites and sampling

Trapping was conducted in two rural municipalities, Givros (GIV) and Saint-Romain-de-Popey (ROM), and one urban agglomeration, the city of Lyon (LYO), in eastern France (Fig. 1a). Pairwise distances (calculated using the *fossil* R package) between LYO, GIV, and ROM ranged from 18 to 34 km. Six sites (i.e. farms) per rural municipality (GIV1 to GIV6 and ROM1 to ROM6), selected on the basis of owner agreement and rat sighting reports, were investigated, while five urban sites in the city of Lyon (LYO1 to LYO5) were sampled. Animal trapping was conducted between 04/01/2010 and 28/03/2012.

Rats were trapped alive using Manufrance© live-traps (280 × 100 × 100 mm) baited with a mixture of peanut butter, oat flakes, and sardine oil. Traps were set at dusk and retrieved at dawn. Rats were euthanized by cervical dislocation, weighed, sexed, and a toe was collected and stored in individual tubes with 100% alcohol for subsequent DNA extraction. Body mass was used as a proxy for age (McGuire et al. 2006).

Animals were treated in accordance with European regulations and legislation governing the care and use of animals in research (Directive 86/609/EEC) (European Parliament 2010). The CBGP laboratory received approval (No. B34-169-003) from the Departmental Direction of Population Protection (DDPP, Hérault, France) for the sampling of rodents and the storage and use of their tissues. None of the species investigated in this study have protected status.

DNA extraction

Genomic DNA was extracted from toe samples using silica columns (Bio Basic Kit Inc, New York, USA) following manufacturer's instructions. DNA samples were stored at −20 °C.

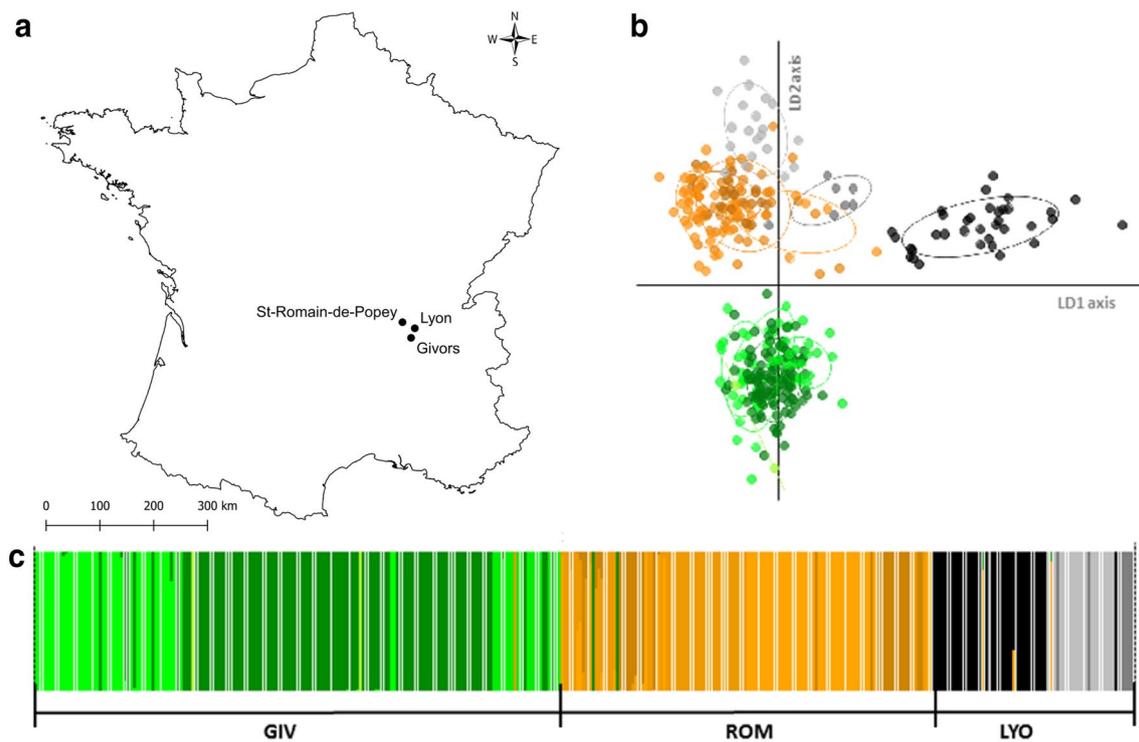


Fig. 1 Results of discriminant analysis of principal components of 355 brown rat genotypes collected in 17 different sites in the region of Lyon, eastern France. **a** Geographic location of the three studied municipalities in France. **b** Scatterplot of the genetic structure of the 355 sampled animals using 13 microsatellites, showing the individuals (points) and clusters (ellipses) in the first two axes of the DAPC

space (horizontal: axis 1, vertical: axis 2). **c** Individual membership assignment of the 355 sampled animals to the genetic clusters identified by DAPC using 13 microsatellite loci. Individuals are represented by vertical bars, colours correspond to different genetic clusters, and each individual's colour proportion indicates its membership to the corresponding cluster

Microsatellite typing

We selected 13 unlinked (i.e. located on different chromosomes) microsatellite loci from the Rat Genome Database (<http://rgd.mcw.edu/>) (Table S1). Two further microsatellite loci, hereafter named *Vka* and *Vkc* (Table S1), were chosen for their physical proximity (9000 and 41,000 base pairs, respectively) to the locus *Vkorc1*. Because *Vka* and *Vkc* loci are physically linked to *Vkorc1*, they were expected to provide information on Y139F mutation flow between studied sites.

Microsatellite amplifications were performed in a 10 μ l reaction volume containing 1 μ l DNA, 5 μ l 2X Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), and 0.2 or 0.4 μ M of each primer (Table S1). The microsatellite cycling protocol was: 95 $^{\circ}$ C for 15 min followed by 40 cycles at 94 $^{\circ}$ C for 30 s, 57 $^{\circ}$ C for 90 s, 72 $^{\circ}$ C for 60 s, and a final extension step of 60 $^{\circ}$ C for 10 min. Genotyping was carried out using an ABI3130 automated DNA sequencer (Applied Biosystems, Waltham, USA). Alleles were scored using GeneMapperTM software (Applied Biosystems, Waltham, USA). Fifteen per cent of the samples,

chosen randomly, were genotyped twice, and repeatability was 100% for all loci.

Vkorc1 genotyping and sequencing

DNA samples were screened for the Y139F mutation using an allele-specific qPCR. A 91 base pair-segment was amplified using an Mx3000P qPCR System (Stratagene, Agilent Technologies, Massy, France). The reverse primer (5'-TCA GGGCTTTTGGACCTTGTG-3') matched both the non-mutated *Vkorc1* sequence and the Y139F mutated allele, whereas we used either a forward primer (F_{wt}) specific for the non-mutated wild-type *Vkorc1* sequence (5'-CATTGT TTGCATCACCACTA-3') or a primer (F_{Y139F}) specific for the Y139F mutated allele (5'-CATTGTTTGCATCAC CACCTT-3'). Y139F genotyping could not be performed in 14 rats (GIV1 $n=1$, GIV3 $n=4$, GIV5 $n=1$, GIV6 $n=2$, ROM1 $n=2$, ROM3 $n=1$, ROM4 $n=1$, ROM5 $n=2$), leaving a genotyped sample size of 341.

To determine whether other *Vkorc1* mutations existed in Y139F PCR-negative samples, *Vkorc1* exons 1, 2, and 3

were PCR amplified as previously described (Grandemange et al. 2010), and then sequenced (Biofidal, France).

Statistical analysis

General statistics and mapping

The proportion test was used to compare allelic frequencies between urban and rural landscape. The Wilcoxon rank-sum test was used to compare A_r , H_o , H_e , and distance of migration between urban and rural habitat. Wilcoxon rank-sum test was also used to compare F_{ST} statistics between population pairs located within a municipality ($F_{STintra}$) and population pairs from different municipalities ($F_{STextra}$). Statistical analyses were performed using R v.3.2.2 (R Development Core Team 2015), and the level of significance was set to 0.05. Base maps “BD ORTHO® 5 m”, freely available on the website of the National Institute of Geographic and Forestry Information (IGN, <http://professionnels.ign.fr/bdort-ho-5m#tab-3>), were used in QGIS v.2.16.2 (QGIS Development Team 2016).

Genetic diversity

At each site we calculated the frequency of the Y139F mutation. At each site the observed (H_o) and expected (H_e) heterozygosities were calculated at loci *Vkorc1*, *Vka*, *Vkc*, and for the 13 microsatellite loci, using unbiased estimates (Nei 1978) implemented in GENETIX v.4.05 (Belkhir et al. 2004). Allelic association patterns between the Y139F mutation and the two linked microsatellite markers, *Vka* and *Vkc*, were investigated by comparing resistant and susceptible homozygous genotypes. At each site, deviation from Hardy–Weinberg equilibrium (HWE) was tested for each investigated locus and the 13 microsatellite loci using the exact test procedure implemented in GENEPOP v.4.2 (Raymond and Rousset 1995). Linkage disequilibrium (LD) between pairs of loci was tested using the exact probability test implemented in GENEPOP v.4.2 (Raymond and Rousset 1995). Correction for multiple testing was performed using the false discovery rate (FDR) method (Benjamini and Hochberg 1995) implemented in R. GENETIX v.4.05 (Belkhir et al. 2004) was used to assess and test the significance of the unbiased inbreeding coefficient (F_{IS}) at each site, calculated according to Weir and Cockerham (1984). Significance of F_{IS} per site was determined using 10,000 F_{IS} bootstraps per population. Allelic richness (A_r) was estimated using the rarefaction procedure implemented in FSTAT v.2.9.3.2 (Goudet 2001) for a minimum sample size of six individuals (Goudet 1995).

MICRO-CHECKER v.2.2.3 (Van Oosterhout et al. 2004) was used to test for the possibility of scoring errors, allelic dropout, and null alleles.

BOTTLENECK v.1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999) was used to determine whether the populations had undergone a recent bottleneck. A two-tailed Wilcoxon sign rank test was performed under a two-phase model. We constrained the model by defining 70% of mutations as conforming to a stepwise mutation model and 30% as multi-step model. Variance was set at 10% and number of replications at 10,000.

Spatial genetic structure and migration

Genetic differentiation between all sites was quantified with F_{ST} statistics computed in GENEPOP v.4.2 (Raymond and Rousset 1995; Weir and Cockerham 1984) using the pairwise distance matrix, and significance was tested using Fisher’s exact probability test. Within each municipality, isolation by distance (IBD) was tested using a Mantel test implemented in R (*ade4* package) by regressing the pairwise estimates of $F_{ST}/(1 - F_{ST})$ against the logarithm of Euclidean geographical distances between sites (Rousset 1997).

First-generation migrants were detected in GENECLASS v.2.0 (Piry et al. 2004) using a likelihood computation (Paetkau et al. 2004) with 10,000 simulated genotypes and the L_h statistics (i.e. the likelihood of finding an individual in a given population in which it was sampled), as recommended when all source populations have not been sampled. Only individuals with a probability < 0.01 were considered as putative migrants (Paetkau et al. 2004).

Genetic structure within and genetic differentiation between sites and municipalities was investigated using discriminant analysis of principal components (DAPC) (Jombart et al. 2010) in the *adegenet* package (Jombart 2008) implemented in R. DAPC does not require the assumption of HWE, so all unlinked microsatellite loci were included in this analysis. For each analysis, the optimal number of clusters was determined using k-means clustering, run sequentially with increasing values of k, and the different clustering results were compared using Bayesian information criterion (BIC) (Jombart et al. 2010). For each migrant, the distance between sampling and assignment sites was calculated using the *fossil* R package.

To evaluate the power of the marker set for individual identification, the unbiased probability of identity ($PID_{unbiased}$) (i.e. PID corrected for small sample size) and PID for siblings (PID_{sib}) (i.e. PID among a population of siblings) were calculated using GIMLET v.1.3.3. (Nathaniel 2002). PID was calculated for each microsatellite locus and then multiplied across loci to give the overall PID (Waits et al. 2001). We sought $PID_{unbiased}$ and PID_{sib} values < 0.001 (Waits et al. 2001).

Results

Genetic diversity

A total of 355 brown rats were trapped (Table S2). The number of alleles at each microsatellite locus ranged from 5 to 17 (Table S3). Calculated at each site, A_r , computed for a minimum sample size of six individuals, ranged between 3.36 and 3.94 in GIV, 3.11–4.11 in ROM, and 3.12–3.82 in LYO (Table 1). There was no statistical difference in A_r between urban (Lyon city) and rural (GIV and ROM) sites (Wilcoxon $W=33.5$, $p=0.75$).

After FDR correction, 61/1326 (4.6%) pairs of neutral microsatellite combinations had significant LD ($p < 0.05$). These significant associations did not systematically affect the same pairs of loci in each site. Therefore, we concluded that the 13 microsatellite loci used were independent, which is consistent with their physical location on the rat chromosomal map. Results from LD tests are shown in Fig. S1.

MICRO-CHECKER did not detect evidence for scoring errors due to stuttering, neither for large allele dropout, nor for a high frequency of null alleles in any of the tested loci (van Oosterhout values are given in Table S3).

Three sites in GIV (GIV3, GIV4, GIV6), four in ROM (ROM1, ROM3, ROM5, ROM6), and two in LYO (LYO2, LYO4) showed significant deviation from HWE (Table 1). HWE deviation was associated with heterozygote deficiency at GIV6, ROM1, and LYO2 ($F_{IS}=0.024$, 0.118, and 0.014, respectively), whereas for the other sites the deviation was associated with heterozygote excess ($F_{IS}=-0.017$ to -0.180).

In rural habitat, H_o across the 13 loci ranged from 0.49 (ROM2) to 0.77 (GIV5) and H_e ranged from 0.55 (ROM4) to 0.68 (GIV2 and ROM1). In Lyon, H_o across the 13 loci ranged from 0.56 to 0.77 and H_e from 0.56 to 0.66 (Table 1). No statistical differences in H_o ($W=30.5$, $p=1$) and H_e ($W=38$, $p=0.43$) were observed between rural and urban populations.

Under the two-phase model, the results displayed a bottleneck signature for the populations in GIV1, GIV5, and LYO4 (Table 1).

Spatial genetic structure and migration

Pairwise $F_{STintra}$ estimates (between population pairs within each municipality) ranged from 0.095 to 0.217 in GIV, from 0.077 to 0.268 in ROM, and from 0.033 to 0.232 in LYO (Table 2). A significant IBD pattern was observed in GIV (Mantel $r=0.54$, $p=0.03$) and LYO (Mantel $r=0.87$, $p=0.04$), but not in ROM (Mantel

$r=0.37$, $p=0.20$) (Fig. S2). Pairwise $F_{STextra}$ estimates (between population pairs located in different municipalities) ranged from 0.127 to 0.355 (Table 2). Pairwise $F_{STintra}$ estimates were significantly smaller than pairwise $F_{STextra}$ estimates ($W=442$, $p < 0.001$) (Fig. S3).

$PID_{unbiased}$ and PID_{sib} were $9.64e^{-15}$ and $5.46e^{-06}$, respectively (Table S3).

The DAPC run on all sampled individuals (355) assigned most individuals to their municipality of capture (Fig. 1b and 1c). However, two clusters from LYO largely overlapped with ROM clusters, indicating potential gene flow between LYO and ROM (Fig. 1b). ROM and GIV clusters were highly differentiated by DAPC although two individuals from ROM were assigned to a GIV cluster (green bars in ROM, Fig. 1c) and one individual from GIV was assigned to a ROM cluster (orange bar in GIV, Fig. 1c).

The most likely number of genetic clusters, as determined by DAPC performed on each municipality, was five in GIV (Fig. 2e), seven in ROM (Fig. 3e), and four in LYO (Fig. 4e). Cluster 3 in ROM included only two individuals while cluster 5 consisted of one animal (Fig. 3e). These three animals could not be assigned to any of the sampled populations in ROM but two were assigned to GIV and one to LYO in the global DAPC (Fig. 1c). Similarly, cluster 1 in LYO was composed of two individuals (Fig. 4e) which were assigned to ROM in the global DAPC (orange bars in LYO, Fig. 1c). Interestingly, these two individuals, detected as first-generation migrants (LYO829 and LYO830, Table 3), were also the only two individuals from LYO with the Y139F mutation. Rat populations were highly structured in GIV (Fig. 2d and 2e). In contrast, DAPC analysis highlighted potential gene flow between ROM1, ROM2, ROM3, and ROM6 (Fig. 3d, e) and substantial gene flow was also detected between LYO1, LYO2, and LYO3 (Fig. 4d, e).

GENECLASS and DAPC conducted on each site identified 25 (7%) and 36 (10%) migrants, respectively (20 migrants were identified by both methods) (Table 3). GENECLASS evidenced 12/25 (48%) migrants as males (9/12 were adults) and 12 as females (7 adults, 1 NA data on sex) while 10/25 (40%) presented genotype *Y139F/Y139F* at locus *Vkorc1* (homozygote resistant to AVKs) and 7 (28%) presented the wild genotype (homozygote susceptible *-/-*). DAPC evidenced 19/36 (53%) migrants as males (13/19 were adults) and 12 (33%) as females (8 adults, 5 NA data on sex) while 13/36 (52%) presented genotype *Y139F/Y139F* at locus *Vkorc1* and 9 (36%) presented the wild genotype (Table 3, Figs. 2b, c, 3b, c, 4b, c).

Based on DAPC results, median distance between sampling and assignment sites did not statistically differ between rats sampled in Lyon city (3.6 km) and individuals captured in rural habitats (2.0 km) ($W=84$, $p=0.08$). Five individuals were unlikely to originate from any of the sampled sites.

Table 1 Summary statistics for each site

Site	N	Vkor1			Vka			Vkc			13 neutral microsatellites					Bottleneck probability			
		f(Y139F)	He	Ho	p(HWE)	A _r	He	Ho	p(HWE)	A _r	He	Ho	p(HWE)	A _r	He		Ho	F _{IS}	p(HWE)
GIV1	21	0.90	0.18	0.20	1.000	1.93	0.19	0.20	1.000	2.92	0.57	0.65	0.637	3.49	0.62	0.69	-0.111*	0.560	<0.001*
GIV2	28	0.68	0.44	0.36	0.389	2.63	0.50	0.43	0.830	2.72	0.50	0.39	0.341	3.94	0.68	0.73	-0.072*	0.202	0.057
GIV3	64	0.92	0.14	0.15	1.000	1.55	0.14	0.15	1.000	1.83	0.17	0.18	1.000	3.69	0.65	0.68	-0.047*	0.002*	0.414
GIV4	29	0.26	0.39	0.31	0.336	1.98	0.39	0.31	0.334	1.98	0.37	0.28	0.297	3.36	0.57	0.64	-0.132*	<0.001*	0.497
GIV5	6	0.50	0.56	0.60	1.000	2.00	0.56	0.60	1.000	2.00	0.56	0.60	1.000	3.62	0.64	0.77	-0.217*	0.981	0.048*
GIV6	22	0.62	0.48	0.45	1.000	2.47	0.50	0.55	0.815	2.00	0.47	0.50	1.000	3.66	0.62	0.60	0.024	<0.001*	0.946
ROM1	18	0.66	0.47	0.19	0.025*	3.28	0.43	0.25	0.043*	2.45	0.37	0.25	0.136	4.11	0.68	0.60	0.118	0.010*	0.191
ROM2	6	0.83	0.30	0.00	0.091	2.00	0.30	0.00	0.089	2.00	0.30	0.00	0.091	3.15	0.57	0.49	0.150	0.422	0.946
ROM3	8	0.78	0.36	0.43	1.000	4.00	0.40	0.43	1.000	2.70	0.39	0.43	1.000	3.79	0.64	0.74	-0.161*	0.003*	0.735
ROM4	28	0.35	0.47	0.48	1.000	3.69	0.66	0.56	0.437	2.43	0.51	0.41	0.291	3.11	0.55	0.58	-0.043	0.560	0.735
ROM5	40	0.96	0.08	0.08	1.000	1.15	0.03	0.03	-	1.43	0.77	0.08	1.000	3.17	0.57	0.58	-0.017	<0.001*	0.273
ROM6	18	0.58	0.50	0.61	0.622	3.57	0.59	0.61	0.214	3.57	0.59	0.61	0.204	3.30	0.60	0.62	-0.050	0.030*	0.127
LYO1	18	0.11	0	0.2	0.003*	3.24	0.60	0.33	0.008*	0.00	0.39	0.28	0.257	3.47	0.58	0.60	-0.029	0.134	0.146
LYO2	12	0.00	0	0	-	4.02	0.70	0.83	0.138	2.00	0.51	0.67	0.549	3.82	0.62	0.62	0.014	0.047*	0.244
LYO3	9	0.00	0	0	-	2.67	0.54	0.44	0.639	2.00	0.47	0.44	1.000	3.12	0.56	0.56	0.008	0.796	0.635
LYO4	22	0.00	0	0	-	4.13	0.68	0.59	0.098	2.23	0.37	0.36	1.000	3.70	0.66	0.77	-0.180*	<0.001*	0.027*
LYO5	6	0.00	0	0	-	4.00	0.77	1.00	1.000	3.00	0.83	0.83	1.000	3.15	0.56	0.68	-0.241*	0.800	0.233

N number of individuals per site, f(Y139F) Y139F allelic frequency, He expected heterozygosity, Ho observed heterozygosity, p(HWE) p value resulting from the test for deviation from Hardy-Weinberg equilibrium, A_r corrected allelic richness, F_{IS} inbreeding coefficient, bottleneck probability 2-tailed p value of the Wilcoxon test for bottleneck probability

Significant p values, i.e. p < 0.05, are indicated with an asterisk

Table 2 Pairwise F_{ST} values (lower half of the matrix) and Euclidean distances in km (upper half of the matrix) between the sampling sites

	GIV1	GIV2	GIV3	GIV4	GIV5	GIV6	ROM1	ROM2	ROM3	ROM4	ROM5	ROM6	LYO1	LYO2	LYO3	LYO4	LYO5
GIV1 (21)		4.572	4.776	2.843	2.762	4.532	37.762	37.069	35.995	38.273	31.717	37.114	23.605	23.78	23.676	21.461	27.142
GIV2 (28)	0.173		0.298	1.945	1.879	1.816	38.984	38.324	37	39.294	32.505	38.519	20.562	20.735	20.667	18.954	24.716
GIV3 (64)	0.171	0.098		2.215	2.128	2.082	39.259	38.6	37.269	39.563	32.769	38.798	20.621	20.794	20.73	19.061	24.822
GIV4 (29)	0.184	0.166	0.156		0.306	1.776	37.769	37.096	35.858	38.15	31.431	37.24	21.185	21.359	21.271	19.276	25.016
GIV5 (6)	0.126	0.095	0.095	0.118		1.992	38.056	37.382	36.15	38.441	31.727	37.522	21.427	21.601	21.515	19.544	25.287
GIV6 (22)	0.216	0.103	0.115	0.218	0.142		37.179	36.52	35.187	37.481	30.689	36.723	19.441	19.616	19.531	17.61	23.363
ROM1 (18)	0.199	0.180	0.175	0.191	0.160	0.191		0.736	2.627	1.803	7.147	1.68	32.314	32.335	32.09	29.326	28.33
ROM2 (6)	0.255	0.254	0.247	0.340	0.228	0.271	0.128		2.417	2.301	6.683	1.293	31.927	31.951	31.705	28.898	28.028
ROM3 (8)	0.210	0.188	0.217	0.179	0.207	0.236	0.077	0.201		2.294	4.699	3.617	29.714	29.733	29.489	26.76	25.703
ROM4 (28)	0.281	0.265	0.274	0.253	0.268	0.262	0.082	0.237	0.134		6.933	3.473	31.647	31.658	31.417	28.78	27.455
ROM5 (40)	0.250	0.185	0.221	0.234	0.221	0.237	0.150	0.268	0.133	0.213		7.493	25.284	25.312	25.064	22.219	21.575
ROM6 (18)	0.261	0.209	0.203	0.231	0.217	0.232	0.081	0.190	0.111	0.178	0.167		32.776	32.805	32.557	29.682	28.999
LYO1 (18)	0.293	0.190	0.191	0.274	0.250	0.205	0.190	0.309	0.252	0.295	0.267	0.244		0.175	0.244	3.647	5.582
LYO2 (12)	0.256	0.149	0.185	0.217	0.217	0.198	0.161	0.299	0.201	0.247	0.229	0.187	0.068		0.257	3.761	5.485
LYO3 (9)	0.293	0.196	0.220	0.282	0.273	0.227	0.200	0.355	0.268	0.314	0.312	0.249	0.102	0.033		3.517	5.35
LYO4 (22)	0.241	0.177	0.199	0.209	0.231	0.220	0.127	0.267	0.151	0.221	0.208	0.166	0.174	0.129	0.175		5.762
LYO5 (6)	0.247	0.182	0.214	0.265	0.219	0.206	0.174	0.276	0.197	0.258	0.217	0.215	0.166	0.172	0.232	0.179	

Pairwise $F_{STintra}$ values (populations pairs located within the same municipality) are shown in dark grey
 Sample size for each population is indicated in brackets. The F_{ST} value in bold type is not significant (Fisher’s method)

Genetic resistance to rodenticides

The Y139F mutation was present in all rural sites, with allelic frequency varying between 0.26 and 0.96 (Table 1). In Lyon, Y139F was found in one single site (LYO1) with frequency 0.11 (two homozygous individuals *Y139F/Y139F*). Y139F frequencies were significantly different in rural versus urban habitat ($p < 0.01$). The proportion of individuals carrying the homozygous *Y139F/Y139F* genotype varied, depending on the site, between 0 (LYO2, LYO3, LYO4, LYO5) and 0.92 (ROM5); the proportion of heterozygous *Y139F/-* individuals varied from 0 (ROM2, LYO1, LYO2, LYO3, LYO4, LYO5) to 0.61 (ROM6), while the proportion of individuals with the non-mutated (*-/-*, wild type) genotype ranged between 0 (GIV1, GIV3, ROM3) and 1 (LYO2, LYO3, LYO4, LYO5) (Table 1). No other mutations were detected in *Vkorc1* following sequencing. HWE deviations

at locus *Vkorc1* occurred in ROM1 ($p = 0.025$) and LYO1 ($p = 0.003$) (Table 1).

The number of alleles at loci *Vka* and *Vkc* was 10 and 8, respectively (Table S1), while per site it ranged from 2 to 6 and from 2 to 4, respectively. Corrected allelic richness (A_r), computed for a minimum sample size of six individuals, ranged between 1.15 and 4.13 for *Vka* and 0.00–3.57 for *Vkc* (Table 1). Locus *Vka* demonstrated HWE deviations in ROM1 ($p = 0.019$) and LYO1 ($p = 0.01$). All populations were in HWE for locus *Vkc*. Mutation Y139F was almost exclusively associated with allele 328 at locus *Vka* (99.5% of the haplotypes) and with allele 327 at locus *Vkc* (98.3%) (Fig. S4). Linkage disequilibrium was examined at the 13 sites where Y139F was present. Significant LD ($p < 0.05$) both between Y139F and *Vka*, and between Y139F and *Vkc*, was found at nine sites. Significant LD reflected the physical proximity between loci *Vkorc1* and *Vka/Vkc* on chromosome 1. However, in GIV5 and ROM2

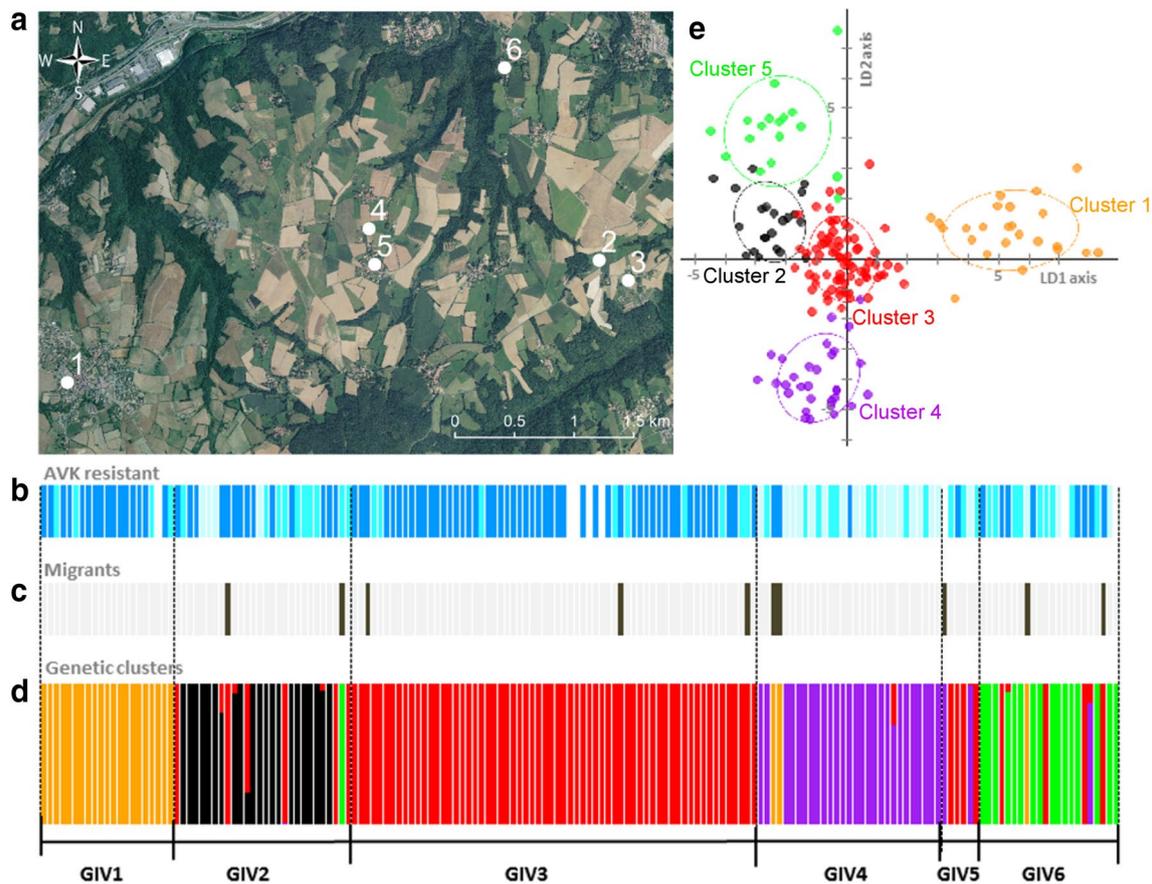


Fig. 2 Results of the genetic analyses of 170 brown rat genotypes collected in the rural municipality of Givors (GIV), eastern France. **a** Sampling sites in Givors. Map data: BD ORTHO® 5 m, National Institute of Geographic and Forestry Information (IGN). **b** Individual *Vkorc1* genotypes determining resistance to AVK rodenticides. Dark blue: resistant homozygote *Y139F/Y139F* (mutated on both alleles), cyan: resistant heterozygote *Y139F/-*, light blue: susceptible *-/-* (wild type, non-mutated), white: data not available. **c** First-generation migrants detected with GENECLASS (dark grey bars). **d** Individual

membership assignment of rats to the genetic clusters identified by DAPC conducted on animals sampled in GIV using data from 13 microsatellite loci. Individuals are represented by vertical bars, colours correspond to different genetic clusters, and each individual's colour proportion indicates its membership to the corresponding cluster. **e** Scatterplot of the genetic structure in GIV using 13 microsatellite loci, showing the individuals (points) and clusters (ellipses) in the first two axes of the DAPC space (horizontal: axis 1, vertical: axis 2). For **b–d** sampling site for each individual is indicated at the bottom

no LD was observed between *Y139F* and *Vka* or *Vkc*, perhaps due to weak statistical power caused by low sample sizes ($N=6$ at both sites). No LD was detected at ROM5 despite a large sample size ($N=40$).

Discussion

Rat population dynamics and dispersal

Because urban habitats present several physical barriers to rat movements, urban rat populations are expected to be more fragmented than in rural landscapes (Combs et al. 2018b; Gardner-Santana et al. 2009; Kajdacs et al. 2013). On the contrary, we showed that, as in urban habitats, rural rat colonies present low gene flow between populations from

nearby farms. In particular, the demographically meaningful genetic parameters we tested were not significantly different between rural and urban populations. Genetic diversity, estimated by H_e , averaged 0.61 in farms and 0.60 in urban sites, which is close to values reported from urban rat populations investigated near Paris, France ($H_e=0.63$) (Desvars-Larrive et al. 2017), in Salvador, Brazil (mean $H_e=0.66$) (Kajdacs et al. 2013), and in Baltimore, USA (mean $H_e=0.73$) (Gardner-Santana et al. 2009). The mean genetic differentiation between rat populations within the three investigated municipalities, measured by mean $F_{STintra}$, was 0.15, a value close to those reported in Salvador (mean $F_{ST}=0.17$) (Kajdacs et al. 2013) and Baltimore (mean $F_{ST}=0.10$) (Gardner-Santana et al. 2009). In comparison, the mean genetic differentiation between rat populations located in different municipalities ($F_{STextra}$) was 0.23. These results are indicative of a

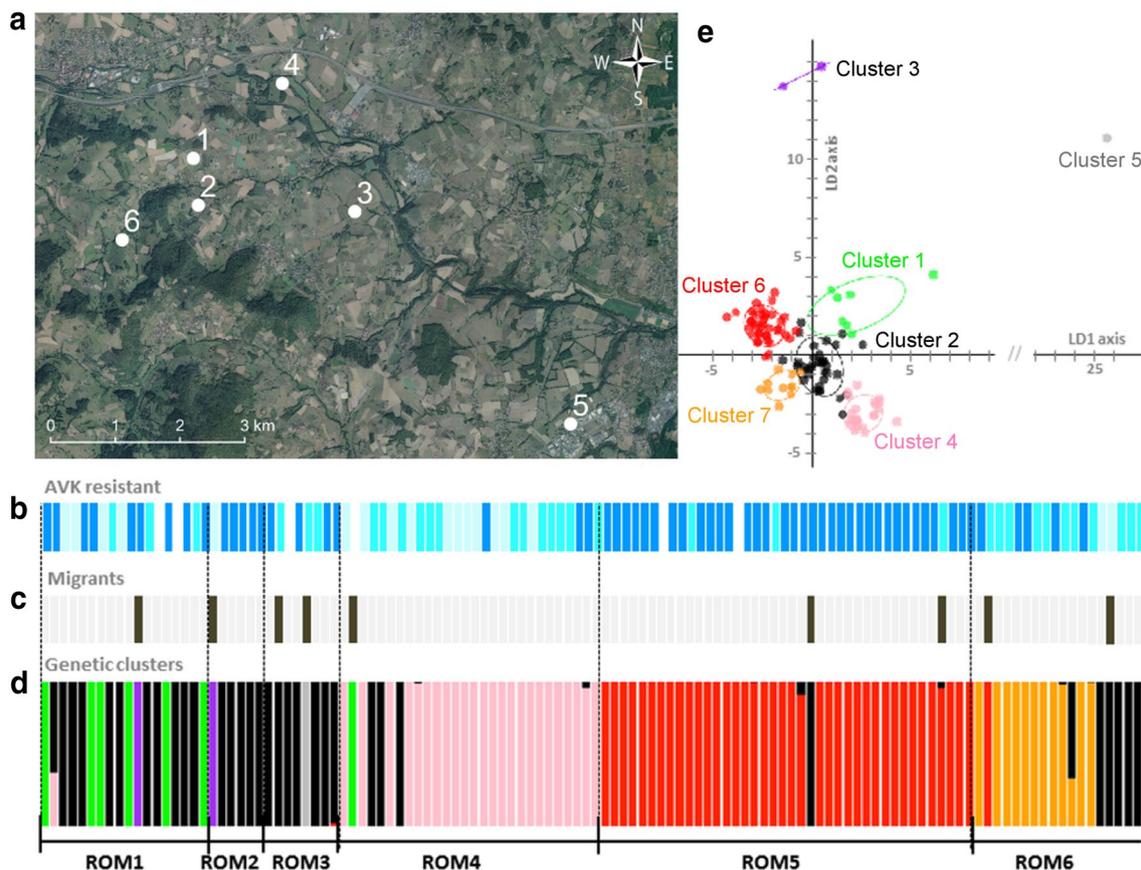


Fig. 3 Results of the genetic analyses of 118 brown rat genotypes collected in the rural municipality of Saint-Romain-de-Popey (ROM), eastern France. **a** Sampling sites in Saint-Romain-de-Popey. Map data: BD ORTHO® 5 m, National Institute of Geographic and Forestry Information (IGN). **b** Individual *Vkor1* genotypes determining resistance to AVK rodenticides. Dark blue: resistant homozygote *Y139F/Y139F* (mutated on both alleles), cyan: resistant heterozygote *Y139F/-*, light blue: susceptible *-/-* (wild type, non-mutated), white: data not available. **c** First-generation migrants detected with GENECLASS (dark grey bars). **d** Individual membership assignment of

rats to the genetic clusters identified by DAPC conducted on animals sampled in ROM using data from 13 microsatellite loci. Individuals are represented by vertical bars, colours correspond to different genetic clusters, and each individual's colour proportion indicates its membership to the corresponding cluster. **e** Scatterplot of the genetic structure in ROM using 13 microsatellite loci, showing the individuals (points) and clusters (ellipses) in the first two axes of the DAPC space (horizontal: axis 1, vertical: axis 2). For **b–d** sampling site for each individual is indicated at the bottom

low gene flow between sites within each municipality and quasi null exchanges among municipalities, and therefore suggest a low level of effective dispersal (dispersal followed by reproduction in the new location) and a strong isolation of the populations. The presence of physical barriers between the investigated municipalities (e.g. major waterways and roads, valleys) can explain the intra-municipal isolation of the rat populations in GIV, ROM, and LYO (Combs et al. 2018a; Richardson et al. 2017).

Effective dispersal between adjacent populations is expected to induce a stepping-stone pattern of IBD (Gardner-Santana et al. 2009; Kimura and Weiss 1964). Our results are unclear on this; the pattern of IBD was verified in GIV and LYO, although only on the outer margin of significance, but not in ROM. Absence of correlation between genetic diversity and geographical distances is expected

when effective dispersal is a rare event and/or when passive (human-assisted) dispersal occurs, for example, when the intensity of connections between different locations is not correlated to inter-location distance but to anthropogenic parameters, such as frequency of social or commercial exchanges (Fountain et al. 2014; Holland and Cowie 2007). We surmise that intrinsic differences in habitat traits (e.g. in resource abundance and quality, number of harbourages, and rat control actions) can impact connectivity and gene flow between neighbouring populations, leading to deviations from IBD and genetic structuring at small spatial scales. In line with this hypothesis, we detected large variations in the genetic diversity of the investigated rat populations ($H_e = 0.49–0.77$), a finding consistent with similar studies in Baltimore (0.57–0.84) (Gardner-Santana et al. 2009) and Salvador, Brazil (0.57–0.72) (Kajdacsi et al. 2013).

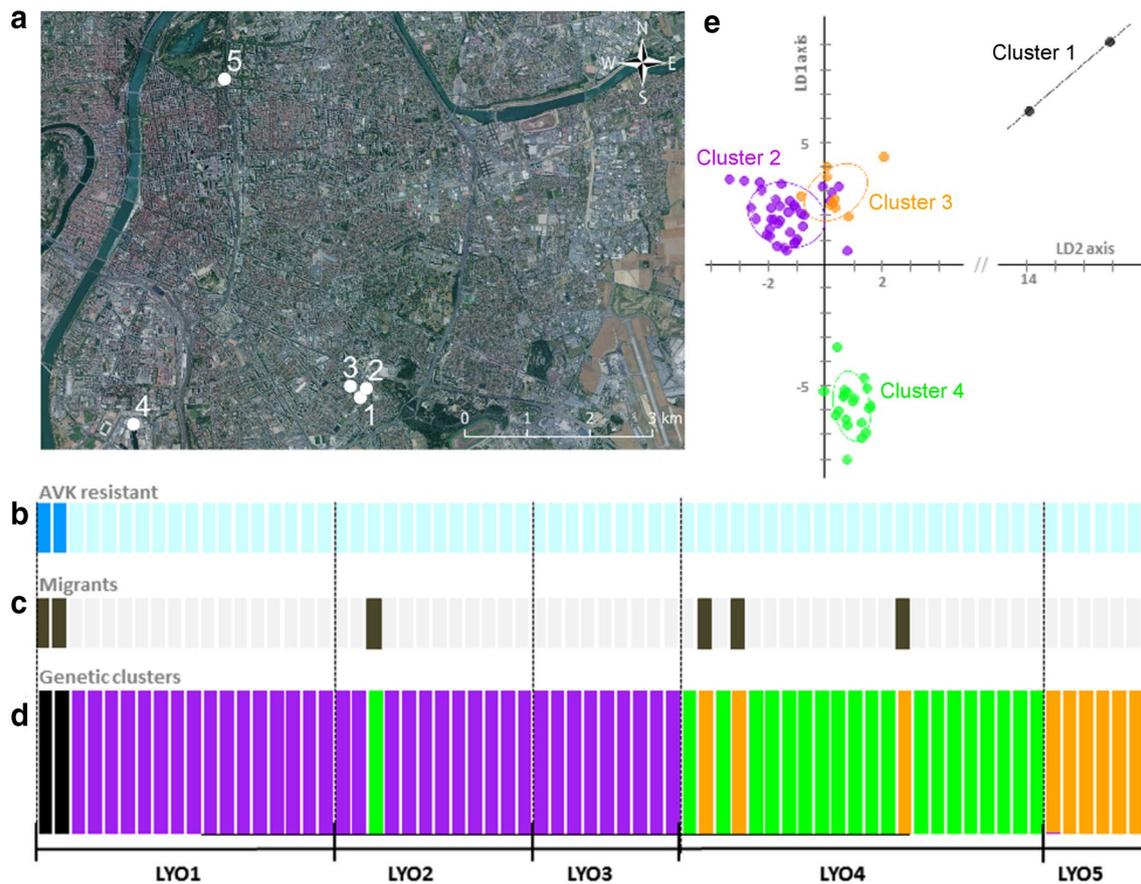


Fig. 4 Results of the genetic analyses of 67 brown rat genotypes collected in Lyon city (LYO), eastern France. **a** Sampling sites in Lyon. Map data: BD ORTHO[®] 5 m, National Institute of Geographic and Forestry Information (IGN). **b** Individual *Vkorc1* genotypes determining resistance to AVK rodenticides. Dark blue: resistant homozygote *Y139F/Y139F* (mutated on both alleles), cyan: resistant heterozygote *Y139F/-*, light blue: susceptible *-/-* (wild type, non-mutated), white: data not available. **c** First-generation migrants detected with GENECLASS (dark grey bars). **d** Individual membership assignment of

rats to the genetic clusters identified by DAPC conducted on animals sampled in LYO using data from 13 microsatellite loci. Individuals are represented by vertical bars, colours correspond to different genetic clusters, and each individual's colour proportion indicates its membership to the corresponding cluster. **e** Scatterplot of the genetic structure in LYO using 13 microsatellite loci, showing the individuals (points) and clusters (ellipses) in the first two axes of the DAPC space (horizontal: axis 1, vertical: axis 2). For **b–d** sampling site for each individual is indicated at the bottom

As we could not investigate all rat populations within the three surveyed municipalities, it is difficult to give a precise estimate of dispersal distances. However, several movements between farms or between urban sites located a few kilometres apart were detected (one-fourth of migrants were assigned to locations > 2.5 km). Such instances of long-distance dispersal, albeit infrequent, have been described in other genetic studies on urban brown rats (Gardner-Santana et al. 2009; Glass et al. 2016; Heiberg et al. 2012; Richardson et al. 2017). In Combs et al. (2018b), several rats were assigned to areas between 2 and 11.5 km apart. Long-distance movements were also suspected for five rats in our study, although none of them could be assigned to any identified genetic clusters.

We observed a small but consistent percentage of first-generation migrants across rural and urban sites. Seven and

ten per cent of migrants were detected using GENECLASS and DAPC, respectively, percentages relatively close to those reported in Baltimore (6.5%) (Gardner-Santana et al. 2009) and Salvador (6.8%) (Kajdacs et al. 2013). Moreover, our results strongly suggest that not only males migrate (Calhoun 1963; Kajdacs et al. 2013) but also females (33–48% of migrants were females). These results are consistent with the study of Gardner-Santana et al. (2009) who did not see sex-biased dispersal in urban brown rats. Although their study identified only mature adults as first-generation migrants, our results showed that a third of the identified migrants was composed of young animals and subadults. It is unclear whether rats are capable of mating after dispersal (attacks from socially dominant males towards new males have been described) (Blanchard and Blanchard 1977; Calhoun 1963; Davis and Christian 1956), but our data presume

Table 3 Migrants detected using GENECLASS (exclusion probability = 0.01) and by discriminant analysis of principal components (DAPC)

ID	Site of sampling	Sex	Weight	<i>Vkorc1</i> genotype	GENECLASS probability of exclusion	Genetic clustering (DAPC)		
						Main cluster at sampling site	Membership assignment ^a	Assigned cluster
GIV757	GIV2	M	470	<i>Y139F/Y139F</i>	0.036	Clust2	<0.001*	Clust3
GIV758	GIV2	F	213	<i>Y139F/Y139F</i>	<0.001*	Clust2	<0.001*	Clust3
GIV762	GIV2	M	168	<i>Y139F/-</i>	0.164	Clust2	0.002*	Clust3
GIV773	GIV2	M	299	<i>Y139F/-</i>	0.113	Clust2	<0.001*	Clust3
GIV783	GIV2	M	264	<i>Y139F/Y139F</i>	0.176	Clust2	<0.001*	Clust3
GIV774	GIV2	F	148	<i>Y139F/-</i>	0.001*	Clust2	<0.001*	Clust5
GIV647	GIV3	F	262	<i>Y139F/Y139F</i>	0.002*	Clust3	0.999	Clust3
GIV737	GIV3	M	231	<i>Y139F/-</i>	0.002*	Clust3	0.998	Clust3
GIV792	GIV3	F	180	<i>Y139F/Y139F</i>	0.008*	Clust3	0.999	Clust3
GIV407	GIV4	F	248	<i>Y139F/Y139F</i>	<0.001*	Clust4	<0.001*	Clust1
GIV408	GIV4	F	305	<i>Y139F/Y139F</i>	0.002*	Clust4	<0.001*	Clust1
GIV238	GIV5	NA	NA	-/-	0.086	Clust3	0.009*	Clust4
GIV540	GIV5	M	378	NA	0.009*	Clust3	0.007*	Clust4
GIV215	GIV6	M	55	<i>Y139F/Y139F</i>	0.114	Clust5	0.002*	Clust3
GIV738	GIV6	M	335	<i>Y139F/-</i>	0.193	Clust5	<0.001*	Clust3
GIV775	GIV6	M	49	-/-	0.002*	Clust5	<0.001*	Clust1
GIV795	GIV6	M	25	<i>Y139F/Y139F</i>	0.119	Clust5	<0.001*	Clust3
GIV804	GIV6	NA	NA	<i>Y139F/Y139F</i>	0.081	Clust5	<0.001*	Clust4
GIV808	GIV6	M	360	<i>Y139F/Y139F</i>	<0.001*	Clust5	<0.001*	Clust3
LYO829	LYO1	F	260	<i>Y139F/Y139F</i>	0.002*	Clust2	<0.001*	Clust1
LYO830	LYO1	F	285	<i>Y139F/Y139F</i>	0.003*	Clust2	<0.001*	Clust1
LYO881	LYO2	M	205	-/-	<0.001*	Clust2	<0.001*	Clust3
LYO855	LYO4	F	200	-/-	0.006*	Clust4	<0.001*	Clust3
LYO857	LYO4	F	100	-/-	0.001*	Clust4	<0.001*	Clust3
LYO901	LYO4	M	374	-/-	0.001*	Clust4	<0.001*	Clust3
ROM779	ROM1	M	351	<i>Y139F/Y139F</i>	<0.001*	Clust1/2	<0.001*	Clust3
ROM223	ROM2	F	44	-/-	<0.001*	Clust2	<0.001*	Clust3
ROM761	ROM3	M	265	<i>Y139F/-</i>	0.006*	Clust2	0.998	Clust2
ROM770	ROM3	M	100	<i>Y139F/-</i>	0.003*	Clust2	<0.001*	Clust5
ROM776	ROM4	M	358	NA	<0.001*	Clust4	<0.001*	Clust1
ROM573	ROM4	F	289	<i>Y139F/-</i>	0.044	Clust4	<0.001*	Clust2
ROM575	ROM4	M	326	<i>Y139F/-</i>	0.080	Clust4	<0.001*	Clust2
ROM593	ROM4	M	388	<i>Y139F/-</i>	0.066	Clust4	<0.001*	Clust2
ROM479	ROM5	M	212	<i>Y139F/Y139F</i>	<0.001*	Clust6	<0.001*	Clust2
ROM382	ROM5	NA	NA	NA	0.002*	Clust6	0.953	Clust6
ROM185	ROM6	NA	NA	<i>Y139F/-</i>	0.059	Clust7	<0.001*	Clust2
ROM221	ROM6	F	259	-/-	0.028	Clust7	<0.001*	Clust2
ROM222	ROM6	F	107	-/-	0.006*	Clust7	<0.001*	Clust2
ROM232	ROM6	NA	NA	<i>Y139F/-</i>	0.168	Clust7	<0.001*	Clust2
ROM233	ROM6	NA	NA	<i>Y139F/-</i>	0.493	Clust7	<0.001*	Clust2
ROM576	ROM6	M	198	<i>Y139F/-</i>	0.009*	Clust7	<0.001*	Clust6

Cluster numbering refers to those in Fig. 2 (GIV), Fig. 3 (ROM), and Fig. 4 (LYO). Migrants highlighted in bold are long-distance migrants, i.e. rats which were not assigned to any identified clusters in their municipality of origin

M male, F female, NA data not available

Significant *p* values, i.e. <0.01, are indicated with an asterisk

^aMembership assignments to the main cluster in the site of sampling

that female and young rats may contribute considerably to gene flow.

Significant deviation from HWE was observed in more than half of our investigated sites. HWE deviation is indicative of a limited population size where a reduced number of males produce descendants and/or of a recent admixture of two or more populations or families (Berdoy et al. 1995). Indeed, in brown rat colonies, dominant males tend to monopolise females and have privileged access to reproduction (Calhoun 1963). Also, population recovery after a control event might result from both the recovery of local survivors and the colonisation by migrants from the nearby areas.

Another future study could use slower-evolving markers (e.g. cytochrome oxidase gene I) to enlighten global brown rat phylogeographic patterns in eastern France, but also over the whole country. Elucidating global routes of brown rat expansion, genomic contribution of the first migrants within invaded areas, urban/rural population differentiation, and global population structure will allow for a better design of rat control efforts.

Resistance to rodenticides and rat control strategy

Although more than one mutated allele of the *Vkorc1* gene can coexist within a region (Grandemange et al. 2010) and also within the same population (Pelz et al. 2005), our results clearly established the presence of one single mutation (Y139F) in the investigated region. The Y139F mutation was almost exclusively associated with allele 328 at locus *Vka* and allele 327 at locus *Vkc* which demonstrates that Y139F was introduced, together with the haplotype allele 328 at locus *Vka* and allele 327 at locus *Vkc*, to the region of Lyon via a single introduction event or via recurrent introductions from the same source. The low genetic diversity at nearby loci suggests that the introduction of the Y139F mutation to this part of France was recent (Barton 2000) and was most likely followed by a wide geographical dispersal of Y139F across the region.

Mutation Y139F was highly prevalent in the two rural municipalities, while it was almost absent in Lyon city. Disparities in the prevalence of the mutation Y139F could derive from differences in rat control practices. Pest control in Lyon city involves the Department of Urban Ecology, which mainly uses bromadiolone and difenacoum in the public urban green spaces and buildings, while pest management professionals are in charge of the sewer system (where difenacoum is mostly used) and private buildings. Rat control measures are administered at the macro-city scale and follow a strict protocol, with concerted rotation of the molecules and fixed bait stations. Therefore, urban rats undergo a continuous and strong selection pressure that keeps resistance alleles at a low

prevalence in the rat populations. In contrast, pest control in rural areas is handled at the farm level, by the farmers themselves. It involves mostly the use of bromadiolone outdoor and FGARs indoor, typically without rotation of the compounds, and at a frequency mostly depending on rat (or rat signs) sightings and economic considerations. This type of management creates a fluctuating selection pressure that helps to promote the selection of alleles which confer resistance to some rodenticides (Bishop et al. 1977; Grandemange et al. 2009; Pelz et al. 2005). In our study, 78% of the migrants carried one or two Y139F mutated alleles. In spite of intense aggression reported towards migrants (Blanchard and Blanchard 1977; Calhoun 1963) resistance to rodenticides is probably advantageous for migrants to establish locally during a rat control event. A fine-scale longitudinal monitoring programme of rat population recovery after control events is needed to understand the underlying ecological processes.

Implications for rat control programmes

The presence of rat populations resistant to FGARs often encourages the use of more potent, and more ecotoxic, SGARs, presenting higher risks of secondary poisoning and environmental contamination. There is little published evidence, however, about the practical effectiveness of anticoagulants against Y139F-mutated rats. Grandemange et al. (2009) recommended not using FGARs and bromadiolone when this mutation is present. They suggested that difenacoum might be efficient, although its use could increase the frequency of the resistance mutation. Highly potent compounds, such as difethialone (and by extrapolation presumably brodifacoum and flocoumafen), may also be effective (Grandemange et al. 2009).

Information campaigns for an educated and safe use of rodenticides, combined with technical and possibly financial support, will be essential to change rodent control practices in rural habitats. Resistance is likely to spread locally, either by natural dispersal or through human transport. Within the investigated region in eastern France, attempts to eradicate rat populations at the local scale of an urban patch or a farm are most likely doomed to fail because interconnectivity with neighbouring populations is quite common. In the rural municipalities, a coordinated pest control strategy employed by several neighbouring farms would likely yield the most immediate positive impact and thus be the best strategy to consider. Non-chemical control methods must also be developed through integrated pest management programmes, involving both environmental (habitat modification, sanitation, harbourage reduction, rat-proofing) and mechanical (trapping)

measures (Meerburg et al. 2004; Mughini Gras et al. 2012).

Limitations of the study

Studies that compare population characteristics (e.g. pathogen prevalence, population genetics, genotyping diversity) in urban versus other habitat types, without considering any detailed environmental parameters or meaningful features, do not allow understanding the specific habitat characteristics that may effectively contribute to the observed differences (Rothenburger et al. 2017). Nevertheless, they can highlight a trend. Further investigations, taking into account micro-environmental and societal parameters (e.g. farmers' practices), are needed to highlight fine-scale environmental specificities that could explain the observed differences. For example, a landscape genetics study along presumed pathways across the rural–urban gradient may help to identify genetic units, mode of migration, and corridors, providing critical information for rat population management.

Sociological data are missing to support our different hypotheses. No data are available on truck movements within and between rural municipalities, neither on the intensity of connections between Lyon city and the surrounding municipalities. Detailed data about the use of anticoagulants in the field were not available and constitute the major limitations of this study. Most of the interviewed farmers did not wish to answer our questions regarding their practices, whereas in the city, the number of rodenticide users is high and pest management professionals were difficult to contact, which rendered the collection of precise data infeasible.

Conclusion

This research provides the first comparative genetic study on rural and urban rat populations and produces substantial advances on the understanding of rat population dynamics and dispersal in these two contrasting landscapes. Our results highlight the interest of population genetics and genotyping as tools for determining the most appropriate spatial scale for rat control measures. Overall, our study calls for greater coordination of rat management between neighbouring farms in order to limit the frequency and spread of anticoagulant resistance. We feel that we must now bridge the gap between rodenticide users and researchers. In a mutually beneficial collaboration, rodenticide users would supply ground data (about rodenticide use and rodent observations) while researchers would share results which could provide support to implement best-practice guidelines for a responsible use of rodenticides.

Author contributions

JFC, EB, PB designed the research, obtained funding, and conducted the field work. AHa, AHo, PB, EB, VL contributed reagents and analytical tools. ADL and JFC performed the data curation, analysis, and visualisation. ADL and JFC wrote the manuscript. All authors read and approved the manuscript.

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