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ORIGINAL PAPER

Assessment of rodenticide resistance, eradication units, and pathogen prevalence in black rat populations from a Mediterranean biodiversity hotspot (Pontine Archipelago)

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Abstract Biological invasions are a growing threat to biodiversity. The black rat, one of the worst pest in the world, is responsible for extensive population decline of many autochthonous and endemic species, particularly in island ecosystems. A number of rat eradication campaigns have been conducted, however, such endeavors do not always result in a complete removal of the pest. This may be due to the occurrence of individuals resistant to common rodenticides and/or a re-invasion of the same environment from interconnected areas when appropriate eradication units are not defined before starting an eradication campaign. Our study is a multidisciplinary approach whereby genetic and epidemiological methods were used to provide background information for successful eradication of black rats. We investigated the occurrence of mutations in the *VKORC1* gene known to confer resistance to rodenticides and evaluated the spread of zoonoses across three islands of the Pontine Archipelago, an Italian hotspot of endemic Mediterranean biodiversity and a possible mainland source of invasion. As part of an eradication campaign, we also assessed patterns of genetic diversity at 10 microsatellite loci in order to identify eradication units. We recorded a strong population structure and revealed at least two distinct eradication units. Some degree of admixture was recorded on Ponza, the largest island and likely the main source of rats invading the other two islands. We did not record the occurrence of rats resistant to anticoagulants, but we revealed transmission of vector-borne pathogens in commensal habitats of the Archipelago.

Keywords Eradication campaign · *Rattus rattus* · Biological invasions · Microsatellites · Zoonoses · *VKORC1*

Sara Fratini and Chiara Natali have contributed equally to this work.

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Introduction

Biological invasions are a pervasive global threat to biodiversity, human health and food security, have a

detrimental effect on the ecology of autochthonous species and a significant impact on ecosystem services (Vié et al. 2009; Vilà et al. 2010). The occurrence of alien species in particular, is one of the most severe menace to amphibians, reptiles, birds and mammals.

Rats are among the most invasive and dangerous pest species. They affect agriculture and natural vegetation and represent a threat to endangered and protected fauna, particularly in insular ecosystems (Doherty et al. 2016; Stenseth et al. 2003; Towns et al. 2006). Despite island invasions by rats occur mainly by passive transportation through sea routes, strict regulations involving ship inspections and biosecurity measures for docking ships are mostly lacking in many countries.

Besides being a threat to biodiversity, rats are also a danger to public and animal health (Capizzi et al. 2018; Davis and Calvet 2005; Webster et al. 1995). To date, more than 24 pathogens can be directly or indirectly transmitted to man by rodents (Meerburg et al. 2009). Vector-borne diseases (VBD), such as leishmaniasis, anaplasmosis, babesiosis and Lyme borreliosis, use rodents as intermediate or reservoir hosts and are the most noxious along with *Toxoplasma gondii*, which infects humans through the ingestion of contaminated food and water (Daszak et al. 2000; Dubey 1998; Rosso et al. 2017). The impact of VBD and *T. gondii* on public health is accentuated in insular habitats where epidemiologic conditions and population dynamics facilitate biological cycles of pathogens and their vectors (Zanet et al. 2014a).

Over the last decades, several rat eradication campaigns have been conducted to protect biodiversity and limit diffusion of zoonoses, particularly on islands (Graham and Veitch 2002; Howald et al. 2007; Keitt et al. 2011; Kerbiriou et al. 2004; Pascal et al. 2005; Towns et al. 2001). However, such endeavors do not always result in a complete eradication of the pest (Savidge et al. 2012). In fact, several factors may be responsible for the failure of an eradication program. One of this is the high capability of rats to re-invade the same environment by swimming across islands if the appropriate conditions are restored (Courchamp et al. 2003; Russell et al. 2008). Another possible cause of an ineffective eradication campaign is the failure to eradicate all individuals of a population because of, for example, the occurrence of resistance to rat poisons (Abdelkrim et al. 2007). Since the 1950s, rodent pest control has been conducted using first-generation anticoagulant rodenticides, such as warfarin, diphacinone and chlorophacinone. These compounds proved to be very effective in controlling rodent populations as they inhibit blood clotting by blocking Vitamin K reductase reaction and kill rodents relatively quickly. However, their massive use has been responsible for the establishment of resistance to anticoagulants, observed for the first time in 1958 in England (Boyle 1960). Anticoagulant resistance also leads to direct and indirect environmental damages. Rodents may continue to spread zoonotic diseases such as hantaviruses, echinococcosis or leptospirosis (e.g. Meerburg et al. 2009) and consume large amount of toxic baits, thus exposing their predators to secondary poisoning via bioaccumulation (Van den Brink et al. 2018).

The genetic basis of anticoagulant resistance in rats (and rodents in general) is the presence of point mutations in the three exons of the Vitamin K Epoxide Reductase Complex Subunit 1 (*VKORC1*) gene. The result is a few VKORC1 amino acid changes (Rost et al. 2004). Pelz et al. (2005) first described eight mutations in the *VKORC1* gene of brown rats, five of which were related to aminoacidic positions 128 and 139. Grandemange et al. (2009) then demonstrated that such mutations conferred resistance to first-generation anticoagulants. To date, approximately 30 mutations have been described in the *VKORC1* of several *Rattus* species worldwide (Goulois et al. 2016; Iacucci et al. 2018; Pelz et al. 2005; Rost et al. 2009; Tanaka et al. 2012).

The spread of invasive rodent species has strongly affected the entire Mediterranean area, especially insular ecosystems regarded as hotspots of endemic fauna and flora. In particular, the black rat *Rattus rattus*, classified as one of the world's worst invasive species, strongly impact on many natural habitats worldwide (Capizzi et al. 2014; Lowe et al. 2000). A number of eradication programs targeting invasive rodent species have been conducted to restore natural habitats in several western Mediterranean islands (e.g. Molara, Tavolara, Linosa, Pianosa, Montecristo, reviewed in Capizzi et al. 2016). The present study is itself part of a comprehensive habitat restoration and conservation of endemic plant and animal species program implemented in the Pontine Archipelago (http://www.ponderat.eu/en-home, Celesti-Grapow et al. 2017).

We investigated sequence polymorphisms at the three exons of the *VKORC1* gene in 119 black rats from Latium mainland and the Pontine Archipelago (Central Italy) to check for the occurrence of sequence variants conferring resistance to anticoagulants. While resistant phenotypes of brown rats and mice have been recently recorded in Italy (Iacucci et al. 2018; Iannucci et al. 2019), this is the first investigation of its kind on black rats in mainland Italy and nearby islands. We also assessed patterns of genetic diversity and population structure at 10 microsatellite loci in order to define the eradication units of black rats occurring in the Pontine Archipelago and describe the likely routes of colonization. The identification of such eradication units (defined as "the interconnected populations that must be eradicated at the same time to prevent rapid recolonization", sensu Robertson and Gemmell 2004) is, in fact, a quite important parameter to consider when planning an eradication plan. Finally, we evaluated the presence and prevalence of pathogens, with particular reference to those transmissible to humans, by analyzing a subsample of 38 black rats. As a whole, this study provides background information to help successful eradication of black rats in the Pontine Archipelago, limit the risks for human and animal health and devise biosecurity measures to prevent reinvasion.

Materials and methods

Study area, sample collection and DNA extraction

The Pontine Archipelago is a group of five volcanic islands, including Ponza, Palmarola, Ventotene, Santo Stefano and Zannone, located off the western coast of Central Italy (Fig. 1). The Archipelago is a popular

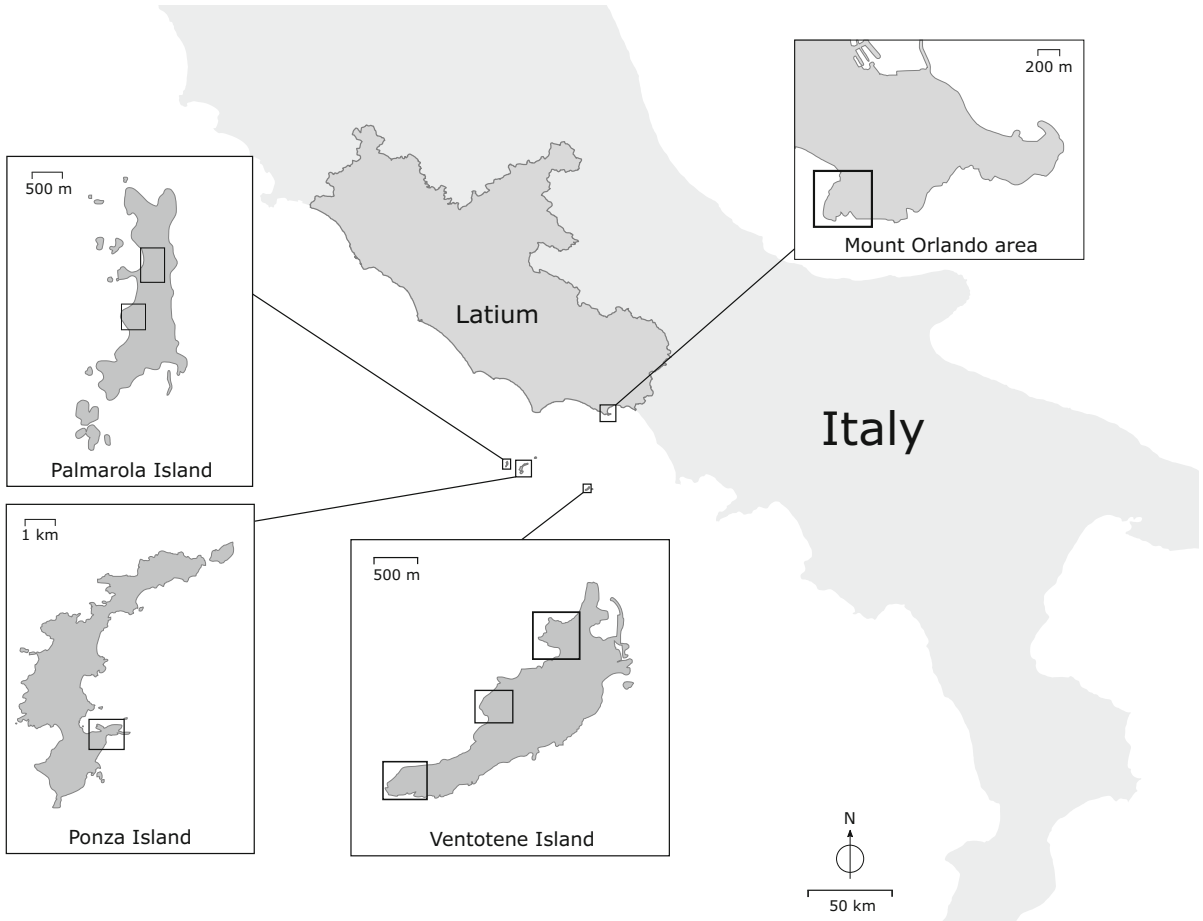

Fig. 1 Map of the study sites. Black rats were sampled on the islands of Palmarola (PLM), Ventotene (VNT), Mount Orlando (MNO), and Ponza (PNZ). Samples for epidemiological investigations were collected in PLM, VNT and PNZ

touristic destination in summer. In winter, on the other hand, some islands are uninhabited. Zannone is part of the Circeo National Park, while the other islands are included in the European Natura 2000 network.

We trapped a total of 119 *R. rattus* on the islands of Palmarola ($N = 36$), Ventotene ($N = 49$) and Ponza ($N = 10$), and in mainland Italy on the nearby Mount Orlando ($N = 24$). Mount Orlando rise close to the Gaeta and Formia harbours, from where ships depart to reach Ponza and Ventotene. Private and tourist boats depart daily from Ponza to the other Pontine islands. On Ponza Island, rats were trapped from the main harbour.

Approximately 10–50 mg of tail muscle tissue was collected from each rat and preserved in absolute ethanol. DNA was extracted by overnight digestion at 55 °C in a lysis buffer with proteinase K, followed by isopropanol-ethanol precipitation (Sambrook and Russell 2001). Samples were then resuspended in DNAase-free water and preserved at − 80 °C.

Genetic analyses

The *VKORC1* gene was amplified by polymerase chain reaction (PCR) using three sets of primer pairs designed to anneal to the adjacent intronic regions. The first two sets of primers were specifically designed for this study to amplify exon 1 and 2, respectively. Primer sequences were VKORC1_ex1_F: 5′-TCTTCCCTCCTGTSYCTGGG-3′ and VKORC1_ex1_R: 5′-AAATYATCTGGYAACCTGGC-3′ for exon 1, and VKORC1_ex2_F: 5′-GGTGGMGCTTCTTGCTAATC-3′ and VKORC1_ex2_R: 5′-GCTCAGTAATTAGCAGCTGGC-3′ for exon 2. Exon 3 was amplified using primers VKORC1_ex3_F: 5′–TTTCACCAGAAGCACCTGCTGYC-3′ and VKORC1_ex3_R: 5′-ACACTTGGGCAAGGSTCATGTG–3′ both modified from Grandemange et al. (2009).

PCR amplifications were performed in 10 µl total reaction using 1X reaction buffer, 300 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer and 0.5 U Taq DNA polymerase (Invitrogen). Thermal profiles consisted of an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, annealing for 45 s at 52–54 °C, extension for 90 s at 72 °C, and a final extension step for 10 min at 72 °C. Amplicons were visualized on a 1% agarose gel, purified using Sure Clean (Bioline) and cycle-sequenced using BigDye Terminator v3.1 chemistry (Life Technologies). Sequencing products were isopropanol-precipitated and resolved by capillary electrophoresis in an Applied Biosystems 3130xl Genetic Analyzer.

Allelic variation at 10 microsatellite loci was determined using primers described for *R. norvegicus* by Jacob et al. (1995) (D10Rat20, D5Rat83, D7Rat13, D19Mit2, D11Mgh5, D16Rat81 and D9Rat13) and for *R. fuscipes greyii* by Hinten et al. (2007) (RfgL3, RfgG3 and RfgD6) and successfully tested in *R. rattus* by Savidge et al. (2012), Ragionieri et al. (2013), Willows-Munro et al. (2016) and Iannucci et al. (2018). Each locus was PCR-amplified in 10 µl total reaction volume using 1X reaction buffer, 1.5 mM of $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP and 0.5 U of Taq DNA polymerase (Invitrogen). Thermal profiles consisted of an initial denaturation step of 5 min at 94 °C, 35 cycles of 60 s at 94 °C, annealing for 45 s at 54–57 °C and extension for 60 s at 72 °C, and a final extension for 7 min at 72 °C. PCR products were then pooled into three multilocus sets: R1 including D10Rat20, D5Rat83, D7Rat13 and D19Mit2, R2 including D11Mgh5, D16Rat81 and D9Rat13, and R3 including RfgL3, RfgG3 and RfgD6. Amplicons were resolved by capillary electrophoresis in an Applied Biosystems 3130xl Genetic Analyzer and allele sizes scored against a GeneScan500 LIZ size standard using GeneMapper 5.0 (Applied Biosystems).

Epidemiological investigations

Whole DNA was extracted from spleen (≈ 10 mg), skeletal muscle (≈ 25 mg of quadriceps femoris), kidney (≈ 25 mg) and the central nervous system (CNS) (≈ 25 mg of brain homogenate) of a subsample of 15 female and 23 male adult and young black rats (Palmarola, n = 13; Ventotene, n = 18; Ponza, n = 7) using the GenElute Mammalian Genomic MiniPrepKit (Sigma Aldrich, MO, USA) following the manufacturer's instructions.

A 145 bp fragment of *Leishmania infantum* kinetoplast DNA (kDNA) was amplified from spleen samples using the mRV1–mRV2 primer pair and the PCR amplification conditions reported in Zanet et al. (2014a). Spleen samples were also tested in order to detect tick-borne pathogens. Specifically, a 400 bp-long fragment of the V4 hypervariable region of the 18S rDNA of protozoa of the genera *Babesia* and

Theileria was amplified according to Zanet et al. (2014b). A 452 bp-long fragment of the 16 s rRNA gene of bacteria of the genera *Anaplasma* and *Ehrlichia* was amplified using primers PER1 and PER2 as reported by Beninati et al. (2006). *Borrelia burgdorferi* sensu lato was detected by PCR amplification of a 452 bp fragment of the 5S-23S intergenic spacer region using the 23SN1-23SC1 primer pair (Rijpkema et al. 1995). Skeletal muscle, kidney and CNS were tested in parallel to detect the presence of *Toxoplasma gondii* using a Loop-Mediated Isothermal Amplification (LAMP) as reported in Trisciuoglio et al. (2015). Positive and negative control samples were included in each PCR/LAMP assay and standard precautions were taken to avoid contamination. PCR positive samples were purified and cycle-sequenced as described above. Species identification was performed by comparison with sequences available in GenBank using MEGA X (Kumar et al. 2018).

Statistical analysis

VKORC1 gene sequence variation

The *VKORC1* sequences were edited using Geneious 8.0.5 (Kearse et al. 2012). Single nucleotide polymorphisms (SNPs) were identified by comparison with annotated reference sequences downloaded from GenBank.

Microsatellites variation

Microsatellite alleles were checked for scoring errors due to stuttering, allele dropout and evidence of null alleles using Microchecker 2.2.3 (Van Oosterhout et al. 2004). All populations showed heterozygote deficiencies at loci D10Rat20 and RfgG3. Since our analysis indicated the occurrence of null alleles, we used the software FreeNA (www.montpellier.inra.fr/URLB) to calculate allele frequencies corrected for null alleles following the INA method described in Chapuis and Estoup (2006). The new dataset was then used for subsequent analyses of population genetic variability.

The number of alleles and allelic richness for each locus and population were calculated using FSTAT 2.9.3.2 (Goudet 1995). Linkage equilibrium among loci and Hardy–Weinberg equilibrium (HWE) were assessed for each population using GENEPOP 4.2.1 (Rousset 2008). Significance levels were adjusted for multiple tests by using a sequential Bonferroni correction (Rice 1989).Statistical significance of deviation from HWE equilibrium was evaluated after 10,000 allele permutations performed in Genetix 4.05 (Belkhir et al. 1996–2004).

Population divergence, genetic structure and gene flow

We assessed whether individuals from a sampling site were more related than individuals from different sampling sites by comparing the mean pairwise relatedness calculated among individuals for each sampling site to average relatedness estimated across sampling sites using the Lynch and Ritland (1999) estimator implemented in GenAlex 6.5 (Peakall and Smouse 2006). Statistical significance was obtained after 10,000 permutations of genotypes.

Genetic divergence among sampling sites was estimated by the exact test for population differentiation implemented in GENEPOP 4.6 (Rousset 2008). Significance values were calculated using a Markov chain with 10,000 batches and 10,000 iterations per batch combined over loci using the Fisher method. Genetic differentiation was also assessed by the F_{ST} estimator θ using ARLEQUIN 3.5 (Excoffier and Lischer 2010). Statistical significance of θ values under the null hypothesis of no differentiation among sampling sites was assessed after 10,000 allele permutations.

Genetic structure was investigated using three clustering techniques: a multivariate discriminant analysis of principal components (DAPC, Jombart et al. 2010), a method based on sparse non-negative matrix factorization algorithms (snmf, Frichot and François 2015; Frichot et al. 2014) and a Bayesian clustering approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000).

The DAPC analysis was performed using ADEGENET version 1.2.8 (Jombart and Collins 2015) in the R statistical environment R 3.5.1. The optimal number of genetic clusters K describing the data was identified using Bayesian information criterion (BIC) scores and the "find.clusters" function. In this analysis, the optimal K is expected to be associated with a low BIC score positioned along the BIC curve where the following BIC scores either increase or are only slightly lower than the chosen BIC value (Jombart

et al. 2010). We determined the optimal number of principal components (PCs) for the DAPC by cross-validation using the "xvalDapc" function with 1,000 replicates. We selected the number of PCs associated to the lowest root mean squared error value. We ran DAPC using all the available discriminant functions and calculated the assignment probability of individuals to each cluster, which were graphically visualized using ADEGENET (Jombart and Collins 2015).

Snmf was run using the "snmf" function of the R package LEA (Frichot and François 2015). The entropy criterion values were used to choose the number of ancestral populations *K* that best explained the genotypic data (i.e. the *K* value with minimal cross-entropy or for which the cross-entropy curve reached a plateau). We tested $K = 1$–6 using 100 replicates for each *K*. Robustness of snmf to the regularization parameter (alpha) was assessed by running preliminary analyses with alpha = 1, 10, 100, and 1,000. The best entropy scores were obtained with alpha = 100. The best *K* was determined by the run with the lowest entropy value (Frichot et al. 2014). The Q-matrix computed from the snmf run was graphically visualized in a bar chart representation using the "barchart" function.

The Bayesian clustering method implemented in STRUCTURE 2.3.4 was used to infer the most likely number of genetically distinct clusters (populations) given the observed genotypes and to evaluate the proportion of each individual's genotype belonging to each inferred population. We used the admixture model as the most appropriate for populations that may have recent ancestors from more than one population. We run 1,000,000 Markov Chain Monte Carlo (MCMC) iterations without prior population information for a number of populations *K* ranging from 1 to 6 using a burn-in period of 20,000 iterations. We calculated the mean likelihood over 20 runs for each *K* with correlated allele frequencies and estimated the most likely number of clusters as described in Evanno et al. (2005). The *K* value with the highest ΔK was then used as prior information to estimate the proportion of membership of each genotype in each of the *K* populations. Results were graphically visualized using STRUCTURE PLOT (Ramasamy et al. 2014).

We also estimated recent migration rates using BayesAss 1.3 (Wilson and Rannala 2003). This Bayesian approach relies on MCMC to estimate the proportion of migrants in a population over the last few generations. The run consisted of 3×10^6 iterations with a sampling frequency of 2,000 and the first 1×10^6 steps discarded. We used default setting delta values.

Demographic inference

Evidence of bottleneck in the four *R. rattus* sampling sites was assessed using BOTTLENECK 1.2.02 (Cornuet and Luikart 1996). Populations that have experienced a recent reduction in their effective population size are expected to exhibit a corresponding reduction in the number of alleles and gene diversity (Piry et al. 1999). The average gene diversity expected at equilibrium was calculated from a distribution of 10,000 simulated values under the two-phase mutation model (TPM) and the stepwise mutation model (SMM) of microsatellite evolution. For the TPM, we set 70 multi-step mutations with a 12% variance among multi-steps (Piry et al. 1999), and obtained statistical significance based on 1,000 replications. Gene diversity excess was assessed using a Wilcoxon sign-rank test (Luikart et al. 1998). We also assessed whether the observed allele frequencies at each locus deviated from an L-shaped distribution expected under mutation-drift equilibrium (Luikart et al. 1998). A similar test was performed by calculating the Garza and Williamson (2001) index (M) to test for a reduction in rat population size using ARLEQUIN 3.5. Garza and Williamson (2001) reported that M values greater than 0.82 should be representative of stable populations that have not suffered a known reduction in size, whereas values of the M index lower than 0.68 indicate a bottleneck or a founder event.

Epidemiological investigations

Variables associated with infection of each pathogen were identified using generalized linear models with PCR results as dichotomous response variables in R 3.5.1.

Results

VKORC1 gene sequence variation

We amplified and sequenced three fragments of the *VKORC1* gene 253 bp, 272 bp and 308 bp long,

Table 1 *VKORC1* mutations found in *Rattus rattus* from the Pontine Archipelago and Latium mainland

Mutations	Exon	WT codon	Mut codon	WT AA	Mut AA
A41A	1	GCG	GCA	Ala	Ala
L97L	2	TTA	CTA	Leu	Leu
S110S	3	TCC	TCT	Ser	Ser
A143A	3	GCG	GCA	Ala	Ala

WT codon, wild-type codon; Mut codon, mutated codon; WT AA, wild-type amino acid; Mut AA, mutated amino acid

Table 2 Genetic diversity measures in *R. rattus* from four sampling sites in the Pontine Archipelago and Latium mainland

Sampling site	*N*	*Na*	A_R	*Ap*	*Ho*	*He*
Mount Orlando	24	7.0 ± 0.61	5.5 ± 0.34	19	0.67 ± 0.05	0.63 ± 0.04
Palmarola	36	7.3 ± 0.94	5.04 ± 0.55	15	0.53 ± 0.07	0.52 ± 0.07
Ventotene	49	6.7 ± 0.73	5.10 ± 0.46	13	0.65 ± 0.05	0.64 ± 0.05
Ponza	10	6.5 ± 0.75	6.29 ± 0.69	7	0.66 ± 0.09	0.66 ± 0.08

N, number of analysed individuals; N_A, number of alleles; A_R, allelic richness; A_P, number of private alleles; H_O, observed heterozygosity; H_E, expected unbiased heterozygosity. Mean ± SE values

respectively. These allowed analysis of the entire 160 amino acids encoding region of the gene. We found a total of 4 SNPs, of which one in exon 1, one in exon 2 and two in exon 3 (Table 1). All were silent mutations.

Population genetic diversity

No significant linkage disequilibrium was recorded across either populations or loci. All loci were polymorphic, with a number of alleles ranging from 4 (locus RfgD6) to 20 (locus D7Rat13) (Table A1, Supporting Information). Relatively high levels of genetic variation were recorded at all sampling sites (Table 2). Allelic diversity and average allelic richness were similar across sampling sites, ranging from 6.50 ± 0.75SE to 7.30 ± 0.94SE and from 5.04 ± 0.55SE to 6.29 ± 0.69SE, respectively (Table 2, Table A1, Supporting Information). The total number of private alleles ranged from 7 to 19 with the highest number assessed in the Mount Orlando sampling site. The lowest values of expected and observed heterozygosity were recorded in Palmarola (Table 2, Table A1, Supporting Information). No deviation from HWE was recorded after Bonferroni correction, except for Palmarola at locus RfgD6 (Table A1, Supporting Information).

Average pairwise relatedness was $r = 0.074$ for Mount Orlando, $r = 0.103$ in Palmarola, $r = 0.076$ in Ventotene and $r = 0.076$ in Ponza. All values were significantly higher than average relatedness calculated among all individuals across locations ($r = -0.004 \pm 0.001$SE, $P < 0.001$).

Table 3 Pairwise comparison matrix of the F_{ST} estimator θ between black rat sampling sites (below diagonal) and corresponding *P* values (above diagonal)

	Mount Orlando	Palmarola	Ventotene	Ponza
Mount Orlando	–	< 0.001	< 0.001	< 0.001
Palmarola	0.34	–	< 0.001	< 0.001
Ventotene	0.19	0.31	–	< 0.001
Ponza	0.22	0.22	0.17	–

Significant *P* values < 0.008 after Bonferroni correction

Population divergence and genetic structure

The Fisher exact test rejected the hypothesis of genetic homogeneity of allele frequency distributions ($X^2 = \infty$, $df = 20$, $P < 0.001$). Significant genetic differentiation was also recorded by *F*-statistics among populations ($\theta = 0.27$, $P < 0.001$) and for all pairwise population comparisons (Table 3). Migration rates between localities were very low and varied between 0.004 and 0.012. On the other hand, proportion of self-recruitment in each site was always higher than 0.95 (Table 4).

Table 4 Mean (± SE) and 95% confidence intervals (in parentheses) of Bayesian posterior distribution of recent migration rates of *R. rattus* between sampling sites

	From			
	Mount Orlando	Palmarola	Ventotene	Ponza
To				
Mount Orlando	**0.987 ± 0.013** (**0.954–1.00**)	0.003 ± 0.004 (0.000–0.017)	0.002 ± 0.003 (0.000–0.011)	0.012 ± 0.016 (0.000–0.057)
Palmarola	0.004 ± 0.007 (0.000–0.023)	**0.991 ± 0.008** (**0.968–1.00**)	0.002 ± 0.004 (0.000–0.012)	0.011 ± 0.015 (0.000–0.056)
Ventotene	0.004 ± 0.006 (0.000–0.023)	0.003 ± 0.004 (0.000–0.015)	**0.993 ± 0.007** (**0.972–1.00**)	0.025 ± 0.025 (0.000–0.091)
Ponza	0.004 ± 0.006 (0.000–0.021)	0.003 ± 0.004 (0.000–0.016)	0.002 ± 0.004 (0.000–0.014)	**0.950 ± 0.035** (**0.864–0.996**)

Bold values along the diagonal axis represent the proportion of resident individuals in each site

The DAPC analysis suggested the presence of three distinct genetic clusters (Fig. 2), as indicated by a rapid decrease of the BIC values from K = 1 to K = 3 and a further decrease for K > 3 (Fig. A1, Supporting Information). Individuals from Palmarola, Ventotene and Mount Orlando were all assigned to three different clusters (1, 2 and 3 respectively), while individuals from Ponza were partitioned among cluster 1 (80%)

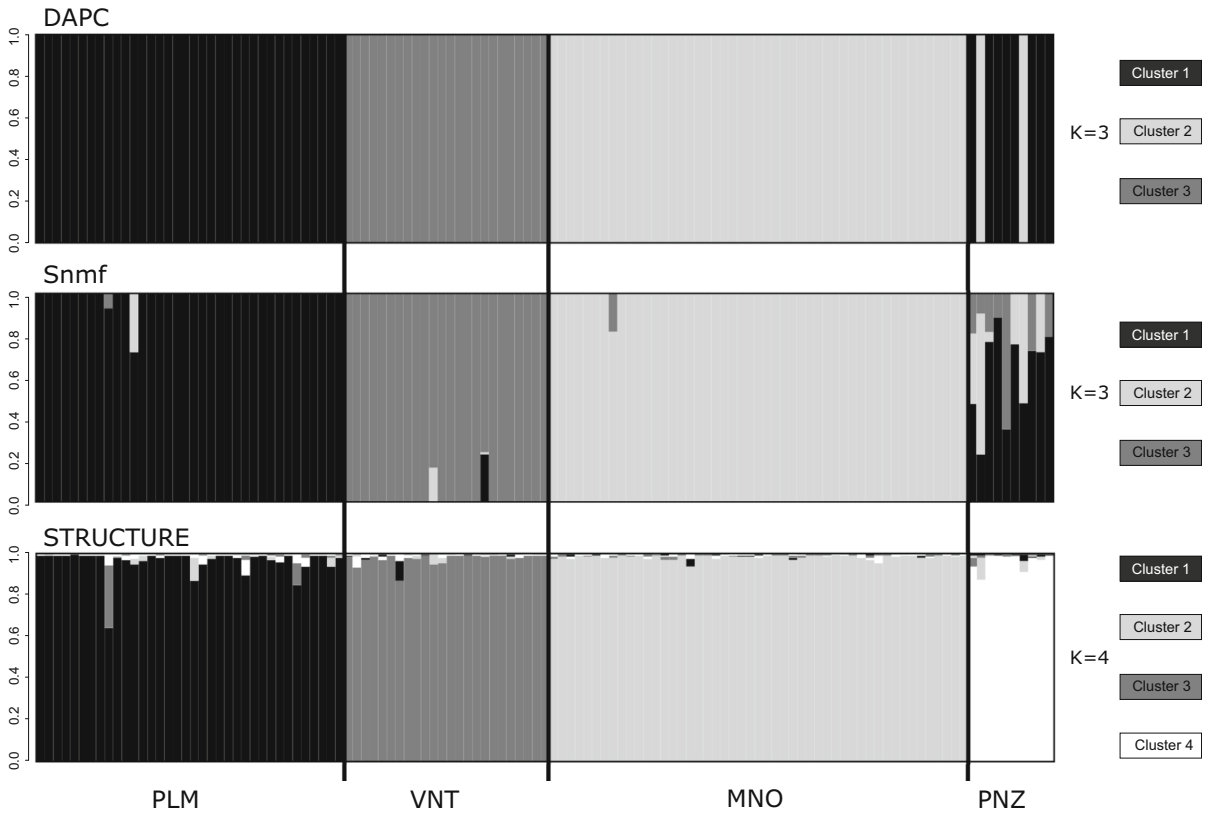

Fig. 2 Clustering analyses for 119 black rats performed using a multivariate discriminant analysis of principal components (DAPC), a method based on sparse nonnegative matrix factorization algorithms (snmf) and a Bayesian clustering approach (STRUCTURE). Each individual is represented by a vertical line partitioned into *K* segments with lengths corresponding to the proportion of its genome originating from each of the *K* inferred clusters. Black vertical bars define distinct island sampling sites. Location acronyms as in Fig. 1

and 2 (20%, Fig. 2; Fig. 3a, b). Absence of overlap among genetic clusters in the ordination plot indicated a high degree of differentiation (Fig. 3c).

Snmf results were concordant with the DAPC analysis. The most likely number of clusters based on the value of K with the minimal cross-entropy value was $K = 3$ (Fig. A1, Supporting Information). Individuals from Palmarola, Mount Orlando and Ventotene were assigned to cluster 3 (99%), cluster 2 (96%) and cluster 1 (98%) respectively. The Ponza sampling site was admixed, with 11% of individuals assigned to cluster 1, 21% to cluster 2 and 68% to cluster 3 (Fig. 2).

The Bayesian population structure analysis revealed that the most probable number of clusters for interpreting the observed genotypes was $K = 4$ based on the highest modal value of $\Delta K = 521.79$ estimated using the Evanno et al. method (Fig. A1, Supporting Information). Four main partitions were used as prior population information for calculating the posterior probability of individual assignment (Fig. 2). Individuals from Mount Orlando were almost entirely assigned to cluster 4 (97.5%), those from Palmarola to cluster 2 (98.4%), Ventotene to cluster 1 (96.2%) and Ponza to cluster 3 (96%).

Demographic inference

The heterozygosity excess approach performed under the stepwise and two-phase models of microsatellite mutation reported no evidence of reduction in effective population size (Table 5). The Palmarola black rat population showed evidence of heterozygosity deficiency under the stepwise model of microsatellite. Moreover, all four locations had a clear L-shaped allele frequency distribution. On the other hand, the M ratio index varied from 0.22 to 0.26 suggesting a bottleneck event in all island populations. The highest M values were recorded for black rats from Palmarola (Table 5).

Epidemiological investigations

The 38 rats tested for zoonotic pathogens had an average weight of 162.98 gr (± 7.60 gr). All animals appeared to be in satisfactory body conditions and no gross lesions were reported at necropsy.

Leishmaina infantum DNA was detected in the spleen of two females from Ventotene Island with a prevalence of 5.26% (CI 95% 1.46–17.29%). *Babesia* piroplasms were detected in 14 rats, corresponding to 36.84% (CI 95% 23.38–52.72%) of the tested animals. No significant difference was recorded in infection prevalence among sexes, age classes or islands of origin (Table 6). All isolates were sequenced and identified to the species level as *B. microti*. *Borrelia afzelii* DNA was amplified from an adult female rat from Ventotene. This rat was also infected by *B. microti*. None of the rats analysed resulted positive to *Anaplasma/Ehrlichia* spp. *Theileria gondii* DNA was detected with a prevalence of 42.11% (IC 95% 27.85–57.81%). Fifteen rats tested positive on muscle-extracted DNA and one on kidney-extracted DNA. A relatively high infection prevalence was recorded in rats from Palmarola ($P = 46.15\%$, CI 95% 23.21–70.86%, $P > 0.05$). However, no significant difference was recorded in infection prevalence among sexes, age classes or island of origin (Table 6).

Discussion

VKORC1 sequence variation

In this study, we recorded no black rats from the Pontine islands carrying mutations in subunit 1 of the *VKORC1* gene known to confer resistance to common anticoagulants. To date, about 30 such mutations have been detected in *R. norvegicus* (Pelz et al. 2005; Rost et al. 2009), while only six were reported for *R. rattus* (Goulois et al. 2016; Tanaka et al. 2012). In Europe, resistance to rodenticides is frequently observed in *R. norvegicus*, but very rarely in *R. rattus*, probably because of the lower number of studies performed on black rats. Only recently, Goulois et al. (2016) detected the first mutation (Y25F) in the *VKORC1* gene of *R. rattus* in Spain and suggested an association to resistance to bromadiolone. However, this newly discovered mutation was not found in our sample set.

We detected only five synonymous mutations in the *VKORC1* coding sequence, suggesting that resistance to anticoagulants in *R. rattus* had not yet affected our study area. However, the extensive use of anticoagulants (second-generation compounds such as bromadiolone and difenacoum, in particular) as pest control in the Pontine Archipelago has been shown to exert selective pressure on *VKORC1*, resulting in resistance in house mice as shown by Iannucci et al. (2019). For

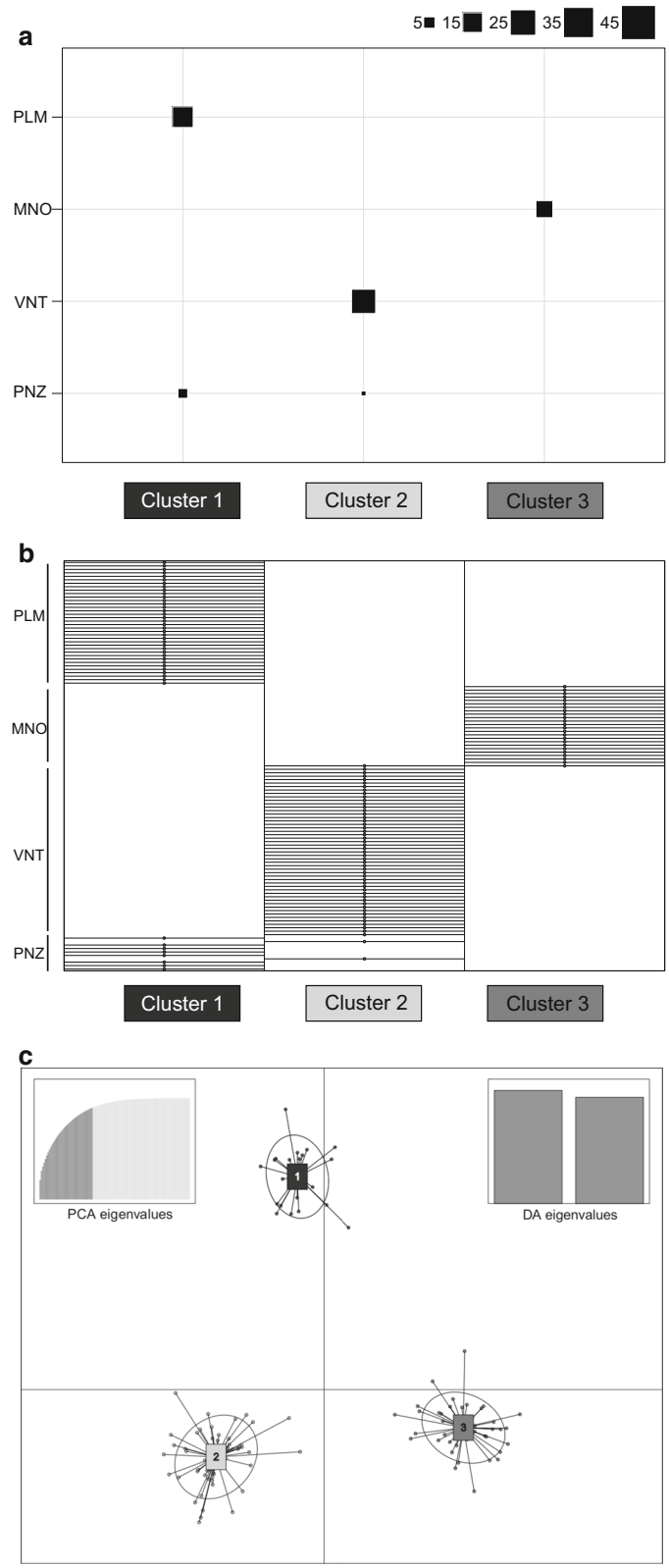

Fig. 3 Assignment of *Rattus rattus* to population of origin based on DAPC analysis. **a** The number of individuals from each population (vertical axis) assigned to each of the three inferred genetic clusters (horizontal axis). The size of black squares is proportional to the number of individuals assigned to each cluster (upper legend). **b** Assignment based on discriminat functions of individuals (vertical axis) to each genetic cluster determined by K-means analysis. Thick horizontal lines show the proportion of each multilocus genotype belonging to one or more clusters. Bullets indicate the cluster where individuals were originally assigned by K-means analysis. **c** Ordination plot for the first two discriminant axes. Dots represent individual rats connected to the centre of an inertia ellipsis, which indicates assignment to one of the three genetic clusters inferred by DAPC. The upper-left inset shows the variance explained by the principal component axes used for DAPC (in dark grey). The upper-right inset shows in relative magnitude the variance explained by the two discriminant axes. Location acronyms as in Fig. 1

Table 5 Results of the heterozygosity excess and M-ratio tests performed to assess evidence of population bottleneck in black rats from Mount Orlando and the Pontine Islands

Location	Heterozygosity excess test						M-ratio test	
	TPM			SMM				
	N_{exc}	Ratio	P_{TPM}	N_{exc}	Ratio	P_{SSM}	Mode	M-ratio
Mount Orlando	5.96	5:5	0.54	5.92	9:1	0.99	L-shaped	0.22
Palmarola	5.90	5:5	0.65	5.91	8:2	0.99	L-shaped	0.26
Ventotene	5.95	2:8	0.08	5.94	8:2	0.98	L-shaped	0.24
Ponza	5.95	4:6	0.38	6.20	5:5	0.68	L-shaped	0.24

TPM, two-phase model of microsatellite mutation; SMM, stepwise model of microsatellite mutation; N_{exc}, expected number of loci with heterozygosity excess under mutation-drift equilibrium; Ratio, number of microsatellite loci exhibiting heterozygosity deficiency versus excess; P_{TPM} and P_{SSM} are probability values of the Wilcoxon test for heterozygote excess under the TPM and SMM models, respectively. Mode indicates a L-shaped normal distribution or a shifted distribution

Table 6 Number of black rats resulted positive to infections for each tested pathogen

	Number of rats tested positive/total number of rats (%)			
	Toxoplasma gondii	*Babesia microti*	*Borrelia afzelii*	*Leishmania infantum*
Island of origin				
Ponza	3/7 (42.86%)	2/7 (28.57%)	0/7 (0.00%)	0/7 (0.00%)
Ventotene	7/18 (38.89%)	7/18 (38.89%)	1/18 (5.56%)	2/18 (11.11%)
Palmarola	6/13 (46.15%)	5/13 (38.46%)	0/13 (0.00%)	0/13 (0.00%)
Age				
Juvenile	4/14 (28.57%)	3/14 (21.43%)	0/14 (0.00%)	0/14 (0.00%)
Adult	12/24 (50.00%)	11/24 (45.83)	1/24 (4.17%)	2/24 (8.33%)
Sex				
Male	12/23 (52.17%)	7/23 (30.43%)	0/23 (0.00%)	0/23 (0.00%)
Female	4/15 (26.67%)	7/15 (46.67%)	1/15 (6.67%)	2/15 (13.33%)

this study, we sampled black rats from both sparsely populated islands (Palmarola) with no or very limited control activities, and populated areas (e.g. Ponza, Ventotene, and Mount Orlando) where rat control has been very intense over the last decades. It was therefore conceivable to record resistant phenotypes of black rats at least from areas where anticoagulants have been extensively used.

Anticoagulant resistance in Europe is much more common in the house mouse than in the genus *Rattus* (Goulois et al. 2017). This is because of a stronger selective pressure exerted on the house mouse by both first-generation rodenticides, commonly used in the last decades, and current second-generation anticoagulants (Goulois et al. 2017; Pelz and Prescott 2015). House mice control is mainly carried out by non-professionals, who tend to exceed in the use of rodenticides unaware of the consequences that a massive use of these molecules can have on the spread of resistance. On the contrary, rat control is usually performed by informed professionals, well aware of the risks associated with the use of such chemicals (Goulois et al. 2017).

Definition of eradication units

Allelic variation and heterozygosity values observed in black rats from the Pontine Archipelago were similar to those reported for other island systems (e.g. Abdelkrim et al. 2009; Iannucci et al. 2018; Ragionieri et al. 2013; Savidge et al. 2012). In all sites, relatedness coefficients were higher than average relatedness among individuals across sites. This may be due to the presence of family groups and inbreeding, as well as the occurrence of a founding event followed by limited gene flow (Frankham et al. 2010).

We recorded a strong population structure and very limited gene flow among islands and mainland Mount Orlando. Differences in allele frequencies distribution, *F*-statistics, DAPC, snmf and Bayesian clustering analysis, all suggested that black rats from Palmarola and Ventotene are two distinct populations and should be therefore considered separate eradication units.

While STRUCTURE recorded the occurrence of four distinct clusters each corresponding to one of the sampling sites, the DAPC and snmf analyses showed a strong degree of admixture of rats from Ponza Island with individuals from Palmarola and Ventotene. These contrasting results can be due to different methodological approaches. STRUCTURE relies on the assumptions of absence of genetic drift as well as Hardy–Weinberg and linkage equilibrium in the ancestral populations. Evidence of disequilibrium is therefore attributed to population structure. On the other hand, snmf and DAPC are more relaxed in their assumptions and therefore more appropriate for analyses of inbred lineages and perhaps more complex patterns of population genetic structure (see Frichot et al. 2014; Jombart et al. 2010).

Under the hypothesis of the occurrence of three clusters, the rat population of the island of Ponza cannot be considered a distinct eradication unit. Ponza is the main island of the Archipelago from where ferries and private boats leave on daily basis to the other Pontine islands and the ports located on the Latium coast (i.e. Gaeta, Formia, Terracina and Anzio). This may suggest that one of the possible sources of black rats for Palmarola and Ventotene may be the harbour of Ponza, even if the relatively lower number of rats sampled on this island with respect to the other sampling sites might affect clustering computations. Therefore, we cannot rule out the presence of other source populations not included in this study and that movement of black rats may follow an inverse flow, from Ventotene and Palmarola to Ponza. However, considering the very low degree of migration across the Archipelago and the high self-recruitment rates recorded within islands, it seems plausible to assume that in recent times exchange of individuals among Pontine islands has been negligible.

Mount Orlando is close to the Gaeta and Formia harbours from where boats regularly sail to Ponza and Ventotene islands. Black rats from this mainland sampling site are genetically distinct from those sampled in Palmarola and Ventotene, but they appear to be slightly admixed with Ponza, indicating that Mount Orlando and mainland locations nearby may be one of the main sources of rodents of the Pontine Archipelago. Again, this conclusion must be taken with caution given the low sampling size from Ponza.

Population demographic history

We described the demographic history of the Pontine black rat populations by using three different methods based on evidence of heterozygosity excess, shifts in allele frequencies and low ratios of allelic number to allelic size range, respectively. We obtained apparently incongruent results as previously reported by Iannucci et al. (2018) for the Tuscan Archipelago. Clear evidence of bottleneck events for all populations was in fact recovered by the M-ratio test only. Methods based on differences in the rate of reduction in the number of alleles versus gene diversity and those assessing shift in allele frequencies from an L-shaped distribution generally record recent evidence of bottlenecks, while the M-ratio test recovers historical reduction in population size (Garza and Williamson 2001; Marshall et al. 2009). The low M-ratio values recorded in this study therefore suggested that all black rat populations of the Pontine Archipelago went through a drastic reduction in population size, perhaps due to an old founder event (Abdelkrim et al. 2005; Iannucci et al. 2018).

Our results confirm previous hypothesis on the pattern of colonization of the Pontine Archipelago. Black rats were found in the western Mediterranean basin since Roman times (Ruffino et al. 2009). In Central Italy, in particular, records of *R. rattus* date back to the sixth century (Colangelo et al. 2015; De Grossi Mazorin 1987). Although several colonization and/or introduction events could have occurred over centuries, the Western Mediterranean black rat population appears to have originated following a single colonization event (Colangelo et al. 2015). A relatively high haplotype and nucleotide diversities and the presence of private haplotypes were, in fact, recorded especially for the island of Ponza, suggesting a recent in situ genetic diversification after colonization (Colangelo et al. 2015). Our results advocate this hypothesis, for we found a basically null gene flow between Ponza and Mount Orlando, and a low admixture between the two sampling sites, probably

the result of a past colonization of Ponza from the mainland.

Epidemiological investigations

Relatively high relatedness values and evidence of past bottleneck may be explained by a number of factors. Among these, intra-specific competitions against new invaders may lead to the establishment of familiar groups of conspecifics (Granjon and Cheylan 1989; Fraser et al. 2015), while a massive use of rodenticides may result in human-driven bottlenecks of rat populations. Additionally, the spread of zoonotic diseases, carried by parasites as flies and ticks, can be another cause of a drastic reduction in population size, and it was probably one of the main factors affecting demography of the Pontine rat populations. In fact, our epidemiological assessment revealed the presence of *T. gondii* and some vector-borne pathogens, such as *L. infantum*, *B. microti* and *B. afzelii*, in the study area.

Leishmania infantum was detected with a lower prevalence with respect to southern Italy, where the parasite is hyperendemic, and Montecristo Island in the Tuscan Archipelago (Cringoli et al. 2002; Rossi et al. 2008; Zanet et al. 2014a). On the other hand, *T. gondii* infection was recorded with a high prevalence. The castor bean tick (*Ixodes ricinus*) is one of the most common tick in Italy and the main vector of *Babesia* sp. In this study, we recorded the co-infection of rats with two *Babesia* species, as already reported by Mehr-Scherrer (1999). The difference in prevalence found among the three Pontine islands (although not statistically significant) is evidence of a higher risk of infection by rats from Palmarola and Ventotene rather than those from Ponza. Based on the biology of *I. ricinus*, the less urbanized environment of Ventotene and Palmarola probably facilitates the persistence of the vector (Medlock et al. 2013).

Conclusions

In the last decades, several studies have described the detrimental impact of invasive black rats on Mediterranean endemisms and island ecosystems (e.g. Baccetti et al. 2009; Capizzi et al. 2010). For instance, in the Tuscan Archipelago, predation upon bird nestlings and eggs resulted in a decrease of several protected species. After pest control measures were adopted in this area, an increase in population size of autochthonous bird species was recorded (Baccetti et al. 2009; Sposimo et al. 2019). Similar results were recently documented for other insular ecosystems of the Mediterranean basin (e.g. Bourgeois et al. 2013; Canale et al. 2019). However, there are examples where rat eradication can fail, mainly as a consequence of a rapid re-colonization of the area from nearby sites and/or survival of rodenticide-resistant individuals (Howald et al. 2007).

In this study, which is the first of its kind on black rats in Italy, we did not record rats carrying mutations in *VKORC1* coding gene that would confer resistance to common anticoagulants. This result is particularly important as it suggests that less powerful second-generation anticoagulants (i.e. bromadiolone and difenacoum) can be used in the Pontine Archipelago as they appear to be efficient against rat populationsto be eradicated. This action should nevertheless be carefully evaluated since such compounds are highly bio-accumulative and thus potentially harmful to species of conservation concern, rodent predators and pets (Eason et al. 2002; Laakso et al. 2010; Vein et al. 2013). However, the importance of investigating the occurrence of resistance to anticoagulants is not limited to island ecosystems. Such an investigation should be performed beforehand in every eradication programs, both in urban and rural areas, in order to avoid the use of first-generation anticoagulants where resistant phenotypes are not recorded.

We also showed that in the Pontine Archipelago there are at least two distinct black rat eradication units located on the islands of Palmarola and Ventotene. Although it is likely that colonization of these islands occurred in historical times, our study suggests that the port of Ponza might have been a main source of the Ventotene and Palmarola rat populations. Eradication campaigns conducted on the islands of Palmarola and Ventotene should therefore be extended to Ponza and adopt long-term biosecurity measures in the harbours as well as regular monitoring of vessels connecting these islands.

Finally, the presence of *T. gondii*, *B. microti*, *L. infantum* and *B. afzelii* in black rats from the Pontine islands revealed the presence of vector-borne pathogens in commensal habitats of the Archipelago. Precautions and safety measures should therefore be adopted when coming in contact with rodents,

especially during rat control campaigns. These results are therefore important not only for the preservation of local biodiversity but also for the protection of public health.

This study used for the first time a multidisciplinary approach where genetic and epidemiological methods are integrated to provide background information for devising and implementing eradication programs of black rats. We characterized the presence of resistant phenotypes, defined the boundaries of eradication units and evaluated the spread of zoonoses (with particular attention to those transmissible to humans). Such information is crucial to ensure successful eradication of rats, set up biosecurity measures to prevent future re-invasions and limit the risks to human and animal health in the study area. In a broader context, we advocate the importance of multidisciplinary studies in the implementation of eradication programs of rodents, and particularly for such initiatives conducted in island ecosystems.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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