



A survey of *VKORC1* missense mutations in eleven Italian islands reveals widespread rodenticide resistance in house mice

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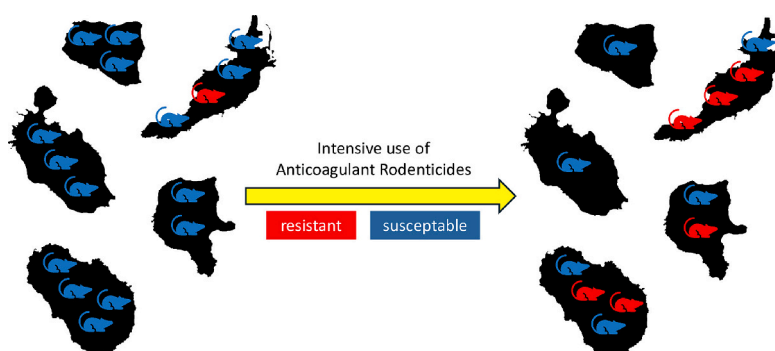
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HIGHLIGHTS

- Anticoagulant rodenticides (ARs) are commonly used in the Mediterranean islands.
- Almost no knowledge about ARs resistance is available for this area.
- The *VKORC1* gene was sequenced in 82 house mice from 11 islands in Italy.
- Resistance to the most used ARs is widespread in the study area.
- Different types of ARs should be adopted to reduce impacts on non-target species.

GRAPHICAL ABSTRACT



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ABSTRACT

To protect native wildlife, more than one hundred rodent eradications have been attempted in the Mediterranean islands by using anticoagulant rodenticides (ARs). Despite their high efficiency, resistance to ARs has been observed in many countries and it is mostly related to missense mutations (SNPs) in the *VKORC1* gene. The presence of resistant individuals reduces the efficiency of rodent management, leading to an excessive use of ARs. Thus, the risk of poisoning in non-target species increases. In this study, the first survey of ARs resistance in the house mouse *Mus domesticus* covering multiple islands in the Mediterranean was performed. Tissue samples of eighty-two mice from eleven islands in Italy were analysed and eight missense SNPs were found. In addition to some well-known missense mutations, such as Tyr139Cys, six new missense SNPs for the house mouse were discovered, four of which were new even for any rodent species. Furthermore, the frequency of Tyr139Cys significantly increased in Ventotene Island after a four-year long rat eradication. This could be due to the

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selective pressure of ARs that allowed the mice carrying the mutation to survive. This study demonstrates once again the importance of assessing resistance to ARs before undertaking rodent eradications. Indeed, this would allow an informed decision of the most effective AR to use, maximizing the success rate of the eradications and minimizing secondary poisoning and other deleterious effects for non-target species and the environment.

1. Introduction

Invasive alien species (IAS) represent the second most common cause of extinction worldwide (Bellard et al., 2016b; Maxwell et al., 2016). Control or eradication of IAS are widely recommended and undertaken by researchers and conservation organisations to mitigate their impact on native ecosystems (Veitch et al., 2011; Russell et al., 2017). This problem is further amplified on islands, especially considering that many of them host endemic species with very small distribution ranges and/or breeding sites (Bellard et al., 2017, 2021; Holmes et al., 2019; Russell and Kueffer, 2019). Human commensal rodents are widely recognised to be extremely harmful to small islands ecosystems and about 80 % of the archipelagos all over the world have already suffered rodents' invasions (Atkinson, 1985; Capizzi et al., 2014; Harper and Bunbury, 2015; Holmes et al., 2019; Moore et al., 2022; Nance et al., 2023). This has resulted sometimes in dangerous consequences for native species, even leading to some documented cases of extinctions (Hilton and Cuthbert, 2010; Bellard et al., 2016b; Bellard et al., 2016a; Nance et al., 2023).

The Western European house mouse, *Mus domesticus*, is among the most widespread synanthropic mammals worldwide and has been listed as one of the 100 most invasive species in the world by the IUCN (Lowe et al., 2000). Many studies have demonstrated its role in the transmission of zoonoses to humans and in foodstuff damages (Pocock et al., 2004; Witmer and Jójola, 2006; Capizzi et al., 2014; Bertolino et al., 2015; Marquez et al., 2019). Like for many other invasive rodents, the impacts of this species can get even greater on islands, where several cases of introduced mice causing severe damages to native ecosystems have already been reported (Angel et al., 2009; St Clair, 2011; Cuthbert et al., 2016; Russell et al., 2020). As a matter of fact, mice are known for preying on island-endemic plants, invertebrates, birds and reptiles (Wanless et al., 2007; Wedding, 2007; Frogley, 2013; Bolton et al., 2014; Norbury et al., 2014; Watts et al., 2017), thus possibly leading to altered ecosystem function (Eriksson and Eldridge, 2014). Mice are a constant presence in the Mediterranean basin, having arrived in this area from the Middle East between 10,000 and 3000 years ago (Cucchi et al., 2002; Cucchi et al., 2005; Gabriel et al., 2010; Solano et al., 2013; Sciandra et al., 2022). The Mediterranean basin is a worldwide important biodiversity hotspot, especially for the presence of >5000 islands and islets characterized by high levels of endemism (Vogiatzakis and Griffiths, 2008; Coll et al., 2010; Peyton et al., 2019). When compared to their Oceanic counterparts, Mediterranean islands are very anthropized, representing very popular tourist destinations and, in many cases, being inhabited throughout the year or for part of it. Many of them host settlements or villages where rodent control by local people is a common practice (Capizzi, 2020). In addition to this, as of 2020, about 139 mammal eradications had been attempted on 107 Mediterranean islands: in 115 cases, rodents were the target of the interventions, mostly the black rat (accounting for over 75 % of the eradications in the Mediterranean) and occasionally the house mouse (only 4.3 %) (Capizzi, 2020). With over 300 islands, Italy is among the island-richest countries in the area and about 40 eradications of alien rodents have been carried out on Italian islands to date, with very positive benefits for native ecosystems (Capizzi, 2020; Capizzi et al., 2023).

The most common method of invasive rodent management is the use of anticoagulant rodenticides (ARs). These compounds work by inhibiting the vitamin K 2,3-epoxide reductase complex (VKORC), a protein involved in the vitamin K synthesis cycle, an essential cofactor for blood clotting. There are two different categories of ARs: the first-generation

(FGARs) and the second-generation (SGARs). The FGARs were introduced in the 1940s and some of them, like warfarin, are still in use. They are less persistent with respect to the SGARs and require higher doses to be fatal. The SGARs are toxic at a much lower dose than FGARs and, for this reason, they rapidly became the most used substances for both rodent control and eradication. However, despite the high success rate of the ARs in the management of rodent populations, their prolonged use can result in the development of resistance in rodent populations (Buckle, 2013; Goulois et al., 2017a, 2017b). The phenomenon of resistance to ARs was first observed in UK at the end of 1950s (Boyle, 1960). Since then, many cases were detected in other European and non-European countries and in different rodent species (Iacucci et al., 2018; McGee et al., 2020; Díaz and Kohn, 2021; Rached et al., 2022; Chua et al., 2022; Yiğit et al., 2023; Aivelo et al., 2023; Krijger et al., 2023; Carromeu-Santos et al., 2023). Resistance is mainly due to the presence of Single Nucleotide Polymorphisms (SNPs) in one or more of the three exons of the VKORC1 gene. This gene encodes for the subunit 1 of the VKORC protein and missense SNPs cause aminoacidic substitutions leading to changes in the conformation of VKORC. This often results in a reduced basal activity of the protein, potentially linked to a lower VKORC-AR affinity and, thus, conferring resistance to all the FGARs and some of the SGARs (Pelz et al., 2005). Even though resistance alone is not considered among the main reasons for eradications to fail (Spatz et al., 2022), it may lower the effectiveness of control/management campaigns, leading to the release of excessive quantities of baits into the environment. This inevitably enhances the risk of non-target wildlife poisoning, either by direct consumption of bait or from predation on poisoned resistant rodents, with many cases of ARs contamination reported in invertebrates, reptiles, birds and mammals (Dowding et al., 2010; Alomar et al., 2018; Lohr, 2018; López-Perea and Mateo, 2018; Lettoof et al., 2020). Genotyping the VKORC1 gene allows the identification of known resistance-conferring mutations, thereby consenting an informed choice of what ARs to use and enhancing rodent management practices. On the other hand, even though every missense SNP causes aminoacidic substitutions, this does not necessarily mean they always confer resistance. As a matter of fact, the actual effects of newly described SNPs can be assessed only by running *in vivo* tests or biokinetic simulations. Without these analyses, their role in resistance can be only hypothesized based on previous studies. Nevertheless, the identification of any missense SNP still represents an important piece of knowledge to be considered in planning rodent management. A summary of the distribution of the known VKORC1 variations in mice, together with the type of substances for which a resistance effect is known, is presented in Tables 1, 2 and 3. Notably, the most prevalent non-synonymous mutations, Leu128Ser and Tyr139Cys, located in exon 3, have been demonstrated to significantly reduce VKORC1 activity in house mice, resulting in resistance to ARs (Pelz et al., 2012; Mooney et al., 2018; Krijger et al., 2023). Similarly, a combination of mutations found in exons 1 and 2, including Arg12Trp, Ala26Ser, Glu37Glu, Ala48Thr (exon 1), and Arg61Leu (exon 2), collectively known as the *spretus* genotype and attributed to adaptive introgression following hybridization between *Mus spretus* and *Mus domesticus*, have been observed to decrease VKORC activity in the presence of FGARs (Song et al., 2011; Banker et al., 2022). However, the type of resistance conferred by many other known SNPs is yet to be assessed.

Even though ARs-mediated rodent control is widely carried out in the Mediterranean islands and most of the mouse and rat eradications that occurred in this area involved the use of ARs (Capizzi, 2020; Gotti et al., 2022), resistance is a poorly studied phenomenon in the

Mediterranean islands. To date, only a single study has been performed in the island of Ventotene (Italy) (Iannucci et al., 2019, Table 3). Specifically, one mouse out of 19 analysed was found carrying a mutation (Tyr139Cys) in heterozygosity. However, right after this study, a black rat eradication took place on the island and went on for 4 years (Gotti et al., 2022). A prolonged use of ARs can positively select resistance-related mutations when they are already present in the population. Moreover, it is known that the presence of ARs resistant mice consuming baits originally placed for other pests' eradication may compromise the effectiveness of management operations against other sympatric invasive rodents, such as rats (Howald et al., 2007). This could be the case of Ventotene and other Mediterranean islands, where mice are constantly present and undergoing continuous control but only rats are targeted by most of the eradications (Capizzi et al., 2016; Capizzi et al., 2019).

Given the importance of understanding the spread of the phenomenon of resistance in insular contexts, in this study, we carried out the first survey covering several islands in the Mediterranean basin in the presence of effective or potentially resistance-related mutations in the VKORC1 gene in the Western European house mouse. Specifically, this

study aims to: a) investigate the frequency and distribution of resistance-related mutations in the VKORC1 gene in the Western European house mouse in the study area; b) identify possible novel SNPs and evaluate their potential role in resistance based on the available knowledge from literature; c) compare SNPs diversity and frequency with those observed in different geographic regions in previous studies; d) compare pre- and post-eradication frequency of SNPs in mice in islands where rat eradications occurred to test the role of the intense and prolonged use of ARs in selecting mutant genotypes.

2. Materials and methods

2.1. Samples collection and DNA amplification

The study area consists of eleven islands in Italy: Ventotene, Pantelleria, San Domino, San Nicola, Lipari, Vulcano, Panarea, Salina, Stromboli, Alicudi and Filicudi. All these islands are inhabited and have ARs use history (see Table 4 for details). Particularly, two large black rat eradications were carried out in Ventotene Island (2018–2022) and in

Table 1
Distribution of known missense VKORC1 mutations in *Mus domesticus* in exon 1.

EXON 1	Arg12Trp	Ala21Thr	Ala26Ser	Ala26Thr	Glu37Gly	Ala48Thr	Arg58Gly	References
France	X		X	X	X	X	X	Goulois et al., 2017a
Germany	X				X		X	Rost et al., 2009 Song et al., 2011 Pelz et al., 2012 Endepols et al., 2013 Carromeu-Santos et al., 2023
Portugal	X		X			X		Šćepović et al., 2016 Song et al., 2011 Ruiz-López et al., 2022 Carromeu-Santos et al., 2023
Serbia		X						
Spain	X		X			X		Díaz and Kohn, 2021 Pelz et al., 2012 Marquez et al., 2019 Carromeu-Santos et al., 2023
USA		X						
Switzerland	X		X			X		
Martinique				X		X		
Azores	X		X			X		
Type of resistance	Coumatetralyl ^a Chlorophacinone ^a Bromadiolone ^a Difenacoum ^a	Bromadiolone	Coumatetralyl ^a Chlorophacinone ^a Bromadiolone ^a Difenacoum ^a Difethialone ^b	Coumatetralyl ^b Chlorophacinone ^b Brodifacoum ^b Bromadiolone Difenacoum ^b Difethialone ^b	No resistance	Coumatetralyl ^a Chlorophacinone ^a Bromadiolone ^a Difenacoum ^a	Warfarin	Rost et al., 2009 Song et al., 2011 Šćepović et al., 2016 Goulois et al., 2017a, 2017b

^a Only when in the *spretus* genotype.

^b Only when Ala26Ser or Ala26Thr occur together with Leu128Ser.

Table 2
Distribution of known missense VKORC1 mutations in *Mus domesticus* in exon 2.

EXON 2	Trp59Gly	Trp59Leu	Trp59Ser	Arg61Leu	Arg61Gln	References
France	X			X		Lasseur et al., 2006 Goulois et al., 2017a
Germany	X	X	X	X		Song et al., 2011 Pelz et al., 2012 Carromeu-Santos et al., 2023
Portugal				X		
Italy					X	This study
Spain				X		Song et al., 2011 Ruiz-López et al., 2022 Carromeu-Santos et al., 2023
USA		X				Díaz and Kohn, 2021
Switzerland				X		
Azores				X		Pelz et al., 2012 Carromeu-Santos et al., 2023
Martinique				X		Marquez et al., 2019
Type of resistance	Warfarin	Unknown	Unknown	Coumatetralyl ^a Chlorophacinone ^a Bromadiolone ^a Difenacoum ^a	Unknown	Lasseur et al., 2006 Song et al., 2011 Goulois et al., 2017a, 2017b

^a Only when in the *spretus* genotype.

Table 3
Distribution of known missense VKORC1 mutations in mice in exon 3.

EXON 3	Ile104Val	Val114Phe	Val118Leu	Leu124Gln	Leu124Met	Leu128Ser	Tyr139Cys	Ser149Asn	Ser149Ile	Gln151His	Glu155Lys	Lys157Asn	References
France					X	X	X						Goulois et al., 2017a
Germany				X	X	X	X						Rost et al., 2009 Pelz et al., 2012
United Kingdom						X	X						Endepols et al., 2013 Pelz et al., 2005 Song et al., 2011
Ireland						X	X						Prescott et al., 2018
Italy	X						X	X	X	X	X	X	Mooney et al., 2018 Iannucci et al., 2019 This study
Lebanon						X	X		X				Rached et al., 2022
Netherlands							X						Krijger et al., 2023
Portugal						X	X						Carromeu-Santos et al., 2023
Serbia						X	X						Šćepović et al., 2016
Spain						X	X						Blazić et al., 2019 Song et al., 2011 Ruiz-López et al., 2022
USA	X		X			X	X						Díaz and Kohn, 2021
Switzerland						X	X						Pelz et al., 2012
Finland						X	X						Aivelo et al., 2023
Martinique							X	X					Marquez et al., 2019
Azores						X	X			X			Rost et al., 2009 Pelz et al., 2012 Carromeu-Santos et al., 2023
Madeira Archipelago							X						Carromeu-Santos et al., 2023
Type of resistance	Unknown	Unknown	Unknown	Unknown	Unknown	Warfarin Coumatetralyl Chlorophacinone Bromadiolone Difethialone Brodifacoum ^b Difethialone ^b	Warfarin Coumatetralyl Chlorophacinone Bromadiolone	Low resistance	Brodifacoum	Unknown	Bromadiolone	Unknown	Pelz et al., 2005 Rost et al., 2009 Šćepović et al., 2016 Goulois et al., 2017a, 2017b Mooney et al., 2018 Blazić et al., 2019 Marquez et al., 2019 Díaz and Kohn, 2021 Bermejo-Nogales et al., 2022

^a Only when in the *spretus* genotype.

^b Only when Ala26Ser or Ala26Thr occur together with Leu128Ser.

Table 4
Number of samples, year of collection and ARs history of the islands included in this study.

Island	n samples	Year of collection	ARs history
Alicudi	5	2005	No eradications, rodent control by local people
Filicudi	2	2005	No eradications, rodent control by local people
Lipari	5	2005	No eradications, rodent control by local people
Panarea	5	2005	No eradications, rodent control by local people
Pantelleria	17	2021–2023	No eradications, rodent control by local people
Salina	10	2005	No eradications, rodent control by local people
San Domino	10	2022–2023	Rat eradication (2022–2023), rodent control by local people
San Nicola	3	2022	Rat eradication (2022–2023), rodent control by local people
Stromboli	2	2005	No eradications, rodent control by local people
Ventotene	14	2022–2023	Rat eradication (2018–2022), rodent control by local people
Vulcano	9	2005	No eradications, rodent control by local people

Table 5
Primers used in this study for VKORC1 amplification.

VKORC1	Forward	Reverse	Annealing Temperature	Reference
Exon 1	TCTTCCCTCTGTSYCTGGG	AAATYATCTGGYAACCTGGC	56 °C	Iannucci et al., 2019
Exon 2	CTGTGCTGAGGGGACAAAGT	TTGCCATAAAACTGAGATTGTGA	49 °C	Iannucci et al., 2019
Exon 3	TTCACCAGAAGCACCTGCTGTC	ACACTTGGGCAAGSTCATGTG	61 °C	Grandemange et al., 2010

the Tremiti Archipelago (2022–2023), where San Domino and San Nicola Islands are located (Gotti et al., 2022; Capizzi et al., 2023). Rodent eradications usually take place over weeks or months so, Ventotene offers a unique opportunity of evaluating the effects of an exceptionally long (4 years) and intense ARs exposure on mice, that were never the target of the eradication but still being affected by ARs. Pantelleria is the largest among the studied islands, it hosts Italy's youngest national park and over 7000 people who often use ARs to control rodents in their properties (Gallozzi and Castiglia's observation). Lipari, Vulcano, Panarea, Salina, Stromboli, Alicudi and Filicudi all belong to the Aeolian Archipelago which represents an important biodiversity hotspot as well as one of the main touristic hubs in Southern Italy with intense human activity and ship traffic especially in Summer.

Eighty-two mice were caught between 2005 and 2023 using Sherman live traps baited with peanut butter. Tissue samples were stored in 96 % pure ethanol until used for DNA extraction. Genomic DNA was extracted by means of the universal extraction protocol (Salah and Martinez, 1997). Three fragments (253 bp, 801 bp and 308 bp long) of the VKORC1 gene were amplified through PCR using the primers shown in Table 5 and then sequenced. The fragments included the three exons of VKORC1 and this allowed the analysis of the entire coding region of the gene (486 bp long). Exons 1, 2 and 3 are 174 bp, 110 bp and 202 bp long respectively and they encode in total 161 amino acids.

2.2. Genetic analysis

Sequence chromatograms were visually checked and analysed in FinchTV (version 1.4.0; Geospiza Inc., 2006) to confirm mutations in homozygosity and heterozygosity. The FASTA files for each sequence were exported and exons 1, 2 and 3 were merged together to build a single sequence covering the whole VKORC1 encoding region. A complete VKORC1 sequence including all the three exons was downloaded from GenBank (NM178600) and used as wild type (WT) reference. An alignment was then built in MEGA (version 11.0.13; Tamura et al., 2021) comparing all the sequences against the WT. Finally, we translated the DNA sequences into amino acids sequences to identify missense SNPs.

2.3. Statistical analysis

After detecting the presence of SNPs, the frequency of mice carrying at least one missense mutation in the study area was assessed and

compared to the frequencies observed in previous studies in other mainland areas in the Mediterranean basin and archipelagos by running a z test in R (R package *BSDA*). To do so, we collected data from the paper with the largest sample size available for each location and selected only those with a sample size ≥ 20 . In addition, the diversity of the VKORC1 variants observed was also evaluated for the study area and for all the comparison mainland areas and archipelagos by calculating the Simpson diversity index (Simpson, 1949) in R (R package *abdiv*). To avoid multiple counting of the same individual in assessing the diversity index, when more than one SNPs co-occurred in a single individual, they were considered together as a single variant (e.g., *spretus* genotype is considered as one variant and not as five distinct SNPs).

Ventotene is the only island with previous data, with Tyr139Cys already being detected in samples collected before the beginning of the 2018–2022 rat eradication (see above in Section 2.1; Iannucci et al., 2019). So, to evaluate the presence of ARs-mediated positive selection of this resistance-giving SNP in mice during the rat eradication, the allelic frequency of Tyr139Cys was calculated for the samples from this study and compared to the frequency reported in Iannucci et al. (2019) by running a z test.

3. Results

A total of 239 PCR products were successfully amplified and sequenced from 82 individuals. Specifically, 79 sequences were obtained for exon 1, 78 sequences for exon 2, and 82 sequences for exon 3. Several SNPs in the VKORC1 gene were found in mice from seven of the eleven sampled islands. Particularly, eleven different mutations were identified: one synonym SNP in exon 1 (Ala18Ala), one missense SNP in exon 2 (Arg61Gln), two synonym (Gln151Gln, Lys157Lys) and seven missense SNPs (Val114Phe, Tyr139Cys, Ser149Ile, Ser149Asn, Gln151His, Gln155Lys and Lys157Asn) in exon 3 (Fig. 1 and Table 6).

3.1. Exon 1 and 2 mutations

No mice carrying the *spretus* variant of VKORC1 were found in this study. Collectively, only six of the eighty-two genotyped mice showed mutations in exon 1 and 2.

The synonym SNP Ala18Ala is the only mutation that was found in exon 1. It was detected in one heterozygous individual from Pantelleria and in two heterozygous individuals from San Nicola.

The missense mutation Arg61Gln was the only SNP found in exon 2

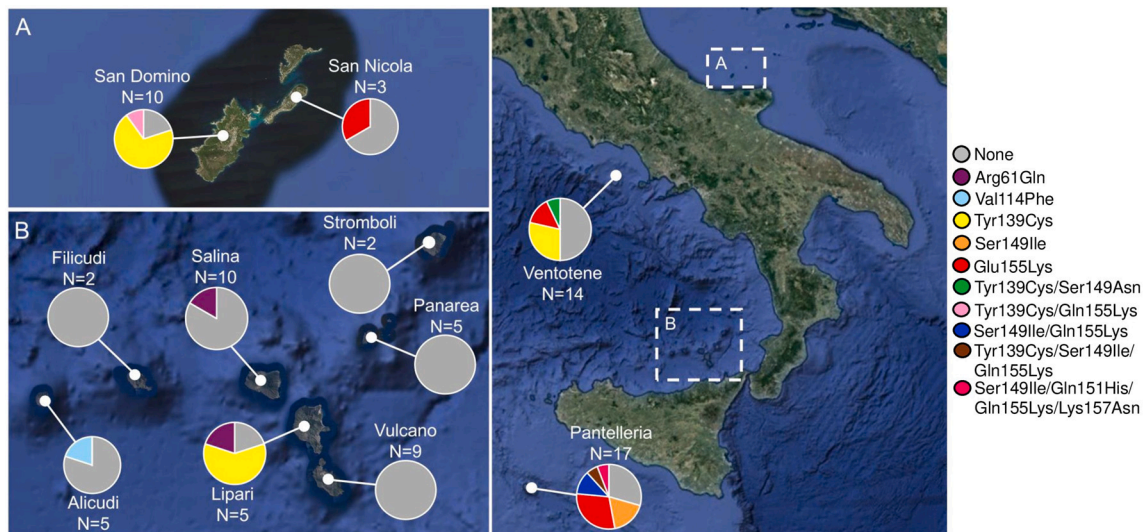


Fig. 1. Frequency and distribution of mice carrying missense VKORC1 mutations in the study area.

and it appears to be restricted to the Aeolian Archipelago. Specifically, it was detected in the islands of Salina (two heterozygous individuals) and Lipari (one heterozygous individuals).

3.2. Exon 3 mutations

A total of nine SNPs were found in exon 3. Two of them, Gln151Gln and Lys157Lys, are synonym mutations and they were found exclusively in Pantelleria Island always together and in heterozygosity in seven individuals.

The Val114Phe mutation was found in a single heterozygous individual from the island of Alicudi in the Aeolian Archipelago. The well-known SNP Tyr139Cys is the most widespread mutation among the ones described in this paper. In fact, it was detected in four different islands: Ventotene (five heterozygous), Pantelleria (one heterozygous), San Domino (seven heterozygous, one homozygous) and Lipari (one heterozygous, two homozygous). Two different SNPs were found in the codon 149: Ser149Ile in five heterozygous and one homozygous individual from Pantelleria and Ser149Asn in one heterozygous individual from Ventotene. The Gln155Lys mutation was always found in heterozygosity in five samples from Pantelleria, two from Ventotene, one from San Domino and one from San Nicola. The two SNPs Gln151His and Lys157Asn were detected together and exclusively in one heterozygous individual from Pantelleria.

Six cases of SNPs co-occurrence were found in the islands of Pantelleria, San Domino and Ventotene. Specifically, in four mice from Pantelleria, two up to four missense SNPs co-occurred in the same individual (two mice: Ser149Ile/Gln155Lys; one mouse: Tyr139Cys/Ser149Ile/Gln155Lys, Ser149Ile/Gln151His/Gln155Lys/Lys157Asn). The other two co-occurrence cases were Tyr139Cys/Ser149Asn in one individual from Ventotene and Tyr139Cys/Gln155Lys in one mouse from San Domino.

3.3. SNPs frequency and diversity

In total, 42.68 % ($n = 35$) of the genotyped mice carried at least one missense mutation (Tables 6 and S1). Tyr139Cys is the SNP with the highest allelic frequency (12.2 %). Specifically, in Ventotene Island, Tyr139Cys was found in 5 mice in heterozygosity, meaning that the allelic frequency of this SNP on the island has grown from 2.6 % in 2018 (Iannucci et al., 2019) to 17.9 % in 2022/2023, when the present study was conducted. The z test confirmed the statistical significance of this variation (p -value < 0.05). Together with Tyr139Cys, Gln155Lys is the

most widespread SNP found in this study but with a lower allelic frequency (6.7 %). All the remaining mutations show allelic frequencies lower than 5 %: 1.8 % for Arg61Gln, 0.6 % for Val114Phe and Ser149Asn, 4.2 % for Ser149Ile, 4.9 % for Gln151His and 3.1 % for Lys157Asn.

Among the archipelagos and the Mediterranean countries with available rodenticide resistance data, six locations had a sample size >20 and were considered suitable for our analysis: France, Lebanon, Portugal, Spain and the archipelagos of the Azores and Madeira (Song et al., 2011; Goulois et al., 2017a; Rached et al., 2022; Carromeu-Santos et al., 2023). The number of mutant mice found in our study appears significantly lower than expected when compared through a z test to other European Mediterranean countries (France, Portugal and Spain; p -value < 0.001) and slightly lower than the Azores Archipelago (p -value < 0.05). On the other hand, it follows the same pattern observed in Lebanon and the Madeira Archipelago (p -value > 0.05). The Simpson diversity index (D) for the SNPs found in the islands included in this study ($D = 0.63$) is comparable to most of the countries and both of the archipelagos included in the analysis. Only France and Portugal show higher values with $D = 0.82$ and $D = 0.70$, respectively (see Tables 7 and S2 for details).

4. Discussion

An increasing number of investigations regarding VKORC1 mutations distribution and their relationship with ARs resistance in rodents have contributed to a deeper knowledge of the phenomenon in many parts of the world in the last years. Nonetheless, the Mediterranean basin was barely covered. Despite the importance of this area as a biodiversity hotspot and the high number of ARs mediated rodent eradications (Coll et al., 2010; Capizzi, 2020; Capizzi et al., 2023), the only available paper to date investigating VKORC1 polymorphism in mice in a Mediterranean island was a short note by Iannucci et al. (2019) focused on Ventotene Island. In our study, the first survey of ARs resistance in mice in multiple island systems in the Mediterranean basin was conducted.

Some of the SNPs we found have already been detected in rodents (Tyr139Cys, Ser149Ile, Ser149Asn, Glu155Lys) and, among these, two (Ser149Ile and Glu155Lys) are new for the house mouse. In addition, seven other mutations are newly described and never found in any rodent species (Ala18Ala, Arg61Gln, Val114Phe, Gln151His, Gln151Gln, Lys157Asn, Lys157Lys).

Table 6
List of the SNPs found in this study. Missense SNPs are reported in bold. Several SNPs co-occurred in the same individual (see Fig. 1 and Table S1). n: number of samples, WT: number of wild type individuals (individuals showing only synonym SNPs are considered as wild type), HET: number of heterozygous individuals, HOM: number of homozygous individuals.

	Ala18Ala		Arg61Gln		Val114Phe		Tyr139Cys		Ser149Ile		Ser149Asn		Gln151Gln		Gln151His		Glu155Lys		Lys157Lys		Lys157Asn	
	n	WT	HET	HOM	HET	HOM	HET	HOM	HET	HOM	HET	HOM	HET	HOM	HET	HOM	HET	HOM	HET	HOM	HET	HOM
Pantelleria	17	5	1	-	-	-	1	-	5	1	-	-	7	-	1	-	6	1	-	-	1	-
Ventotene	14	7	-	-	-	-	5	-	-	-	1	-	-	-	-	-	2	-	-	-	-	-
S. Domino	10	2	1	-	-	7	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
S. Nicola	3	2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
Alicudi	5	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Filicudi	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Panarea	5	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salina	10	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lipari	5	1	-	-	-	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Stromboli	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vulcano	9	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	82	47	3	-	1	-	14	3	5	1	-	-	7	-	1	-	9	1	-	7	1	-
Type of resistance				Unknown	Unknown	Unknown	Warfarin	Coumatetralyl Chlorophacinone Bromadiolone	Clorophacinone Brodifacoum	Low resistance				Unknown	Unknown	Bromadiolone					Unknown	
References			This study	This study	This study	This study	Pelz et al., 2005; Rost et al., 2009; Šćepović et al., 2016; Goulois et al., 2017a, 2017b; Mooney et al., 2018; Blazić et al., 2019	Bermejo-Nogales et al., 2022	Marquez et al., 2019	This study	This study	Bermejo-Nogales et al., 2022	This study	This study	This study	This study	This study	This study	This study	This study	This study	This study

Table 7

Number of samples (n), frequency of mice carrying at least one missense SNP (% mutant), number of different variants found in each locality and Simpson diversity index for the observed missense SNPs in each country/archipelago (SNPs diversity).

	n	% mutant	VKORC1 variants	Diversity index (D)	References
France	266	72.56 %	12	0.82	Goulois et al., 2017a
Portugal (mainland)	34	88.23 %	5	0.70	Carroumeu-Santos et al., 2023
Spain	29	93.10 %	1	0.13	Song et al., 2011
Lebanon	24	58.33 %	2	0.60	Rached et al., 2022
Azores	151	55.63 %	5	0.66	Carroumeu-Santos et al., 2023
Madeira Archipelago	49	46.94 %	1	0.50	Carroumeu-Santos et al., 2023
Italian Islands	82	42.68 %	10	0.63	This study

4.1. Known missense SNPs – Tyr139Cys and Ser149Asn

The mutation Tyr139Cys is the most common resistance-related SNP both in mice and brown rats in Europe and in other non-European country and it is known to confer resistance also in heterozygous condition to all FGARs and some of the SGARs (see Table 1; Grandemange et al., 2010; Hodroge et al., 2011; Pelz et al., 2005, 2007; Markussen et al., 2008; Meerburg et al., 2014; Rost et al., 2009; Iacucci et al., 2018; McGee et al., 2020; Díaz and Kohn, 2021; Rached et al., 2022; Chua et al., 2022; Yiğit et al., 2023; Avelo et al., 2023; Krijger et al., 2023). This mutation is also the one detected in heterozygosity in Ventotene Island by Iannucci et al. (2019) in only one sample out of the 19 mice they collected. The sampling by Iannucci et al. (2019) took place right before the beginning of the rat eradication, that was carried out on the island between 2018 and 2022. The 14 mice from Ventotene Island used for our study were collected in 2022 and 2023, after 4 years of intense ARs exposure. Tyr139Cys is found at a significantly higher frequency in our samples when compared to the results shown in Iannucci et al. (2019) and this is probably the consequence of the selective pressure of the rat eradication that allowed the mice carrying the mutation to survive in an ARs-dense environment. Unfortunately, the sample sizes used in this study and in Iannucci et al. (2019) do not allow us to be conclusive on this point. However, an ARs-mediated selection still seems to be a likely scenario for the increase in Tyr139Cys frequency we observed in Ventotene.

The mutation Ser149Asn, also found in Ventotene, has already been observed in mice in Martinique and it seems to give very low or no resistance to both FGARs and SGARs (Marquez et al., 2019).

4.2. Missense SNPs new to mice – Ser149Ile and Glu155Lys

This paper represents the first documented case of Glu155Lys in mice. Previous studies found this mutation in brown rats in France (Grandemange et al., 2010) and in black rats in Spain (Bermejo-Nogales et al., 2022) showing an increase in the VKORC affinity for vitamin K and a decrease in affinity for bromadiolone (SGARs). Therefore, ARs resistance is likely for this SNP.

Ser149Ile was found for the first time in mice in this study in the island of Pantelleria. This mutation was already known in the European water vole (Abi Khalil et al., 2021) and in both brown and black rats in Lebanon and Spain (Rached et al., 2022; Bermejo-Nogales et al., 2022). Decreases in affinity for clorophacinone (FGAR) and brodifacoum (SGAR) have been observed in rats carrying this mutation (Bermejo-

Nogales et al., 2022), suggesting that Ser139Ile may play a role in giving resistance to ARs.

4.3. Missense SNPs new to any rodent species

The missense SNPs Arg61Gln, Val114Phe, Gln151His and Lys157Asn have never been detected in any rodent species and this study represents their very first record. Therefore, it is difficult to assess their effects on ARs resistance. Nevertheless, Gln151His and Lys157Asn are adjacent to some known SNPs which may cause similar conformation changes in the VKORC protein and, indeed, have similar roles in resistance: Lys152Thr, found in Spain in brown rats and apparently not involved in ARs resistance (Damin-Pernik et al., 2022), and Glu155Lys, previously described in brown rats (and for the first time in mice in this study) and potentially involved in resistance as discussed above. Similarly, Arg61Gln is located in the same codon of the known mutation Arg61Leu, found in different countries and associated, together with four more SNPs in exon 1, with the *spretus* variant of VKORC1 (Lasseur et al., 2006; Song et al., 2011; Pelz et al., 2012; Goulois et al., 2017a; Marquez et al., 2019; Díaz and Kohn, 2021; Ruiz-López et al., 2022; Carromeu-Santos et al., 2023). Mice with a *spretus* genotype have been observed to be resistant to different kinds of ARs (Goulois et al., 2017b). Nonetheless, the effect of Arg61Leu alone has never been tested to date and therefore the role of Arg61Gln in resistance is not possible to assess.

4.4. Mutations co-occurrence

Six cases of missense SNPs co-occurrence were found: one in Ventotene, one in San Domino and four in Pantelleria. Mice with multiple mutations are not uncommon and were detected in several previous studies (Pelz et al., 2012; Šćepović et al., 2016; Goulois et al., 2017a; Díaz and Kohn, 2021) but all the combinations of SNPs we found are newly described ones. Except for the mutations involved in *spretus*-like genotypes, the co-occurrence of which results in giving resistance to some ARs (Song et al., 2011), the effects of other SNPs co-occurrence are poorly known. So, even though all our cases involved at least one known mutation, it is difficult to assess if more SNPs in the same individual can enhance or lower the effects of known resistance-giving mutations. In addition, the sample size of some of the islands considered in this study ($n < 5$) is not large enough for any statistical inference concerning potential association between mutations occurring together and this could be the subject for further investigations.

4.5. Mutations frequency and diversity

It is worth stressing that six new mutations for the house mouse were discovered in the study area, four of which were new even for any rodent species. This diversity appears similar to that observed in other island systems in the world with comparable frequencies of mutant mice (Table 7). On the other hand, the diversity (Simpson index) and frequency of SNPs observed in mainland areas, as in France and Portugal is higher than the one observed in this study and in other archipelagos. Possibly, this difference is due to the frequent unregulated use of ARs in big cities and urbanized areas. In fact, the number of mutant mice may rapidly increase even though their frequency is low if ARs are used without following management best practices. When intensive rodent control is carried out, this is also particularly true on islands, where small population sizes and high inbreeding rates can even accelerate this process and lead to the fixation of mutations in a few generations. In this regard, Ventotene Island is probably an example on how the prolonged and intensive use of ARs can positively select resistance-related SNPs. Nonetheless, a larger sample size is probably needed to describe these processes with certainty and higher statistical accuracy but our results surely provide valuable knowledge about this topic. It is not clear to what extent VKORC1 variants arise independently in different geographical regions or they are the result of the colonization of the

house mouse, both globally and locally (Díaz and Kohn, 2021). Azores and mainland Portugal shares similar SNPs identity and frequency (Carromeu-Santos et al., 2023) and this suggests that the mutations found on islands may have been inherited from mainland mice. Even though there is no record of SNPs from mainland Italy, only a small number of mice has been genotyped so far (Song et al., 2011). Thus, it is not possible to make this kind of inference for the Italian islands. Anyway, since the Mediterranean basin has played an important role as a possible source area for the spread of the house mouse worldwide (Jones et al., 2013), our study provides a greater understanding of the diversity and distribution of mutations in the area and can contribute to better understand the phenomenon.

4.6. Management implications

The results of this study provide evidence towards seeking a safer and less impactful management of invasive rodents in an island conservation perspective. It is of crucial importance that insular populations of mice are screened for VKORC1-mediated ARs resistance before any practice involving the use of ARs is undertaken. Even though resistance alone is not among the main causes for eradication failures (Spatz et al., 2022), having knowledge about the presence of resistant rodents on islands would be a powerful tool for eradication planning, allowing an informed decision of the most effective AR to use. Bromadiolone and brodifacoum are the most used ARs for both rodent eradication and control by local people in Italy (Gotti et al., 2022). However, according to our results, resistance to bromadiolone is already present in five islands (Ventotene, Pantelleria, San Domino, San Nicola and Lipari) and resistance to brodifacoum is found in Pantelleria. In addition to this, mutations with unknown effects on resistance are found in two more islands (Alicudi and Salina). Therefore, laboratory trials and biokinetic tests should be carried out as the next step to carefully assess if ARs are still effective and at what level. The use of different and more effective ARs may also be considered for ongoing and future operation on these islands. Previous studies suggested brodifacoum as the most effective choice when bromadiolone resistance is present (Blazić et al., 2019). However, mice from Pantelleria show resistance to both bromadiolone and brodifacoum, thus requiring alternative solutions. Flocoumafen and difethialone may be suitable substances for such cases, since, to the current state of knowledge, none of the SNPs we found can give resistance to these ARs. This kind of practices may reduce secondary poisoning and other deleterious effects for non-target species and the environment. However, these ARs are rarely used, they have never been proven successful for rodent eradication and their efficiency needs to be validated (Capizzi, 2020; Gotti et al., 2022). It is important to keep in mind that eradication success should always be the priority and, even when resistant rodents are present, most of the time they are not immune to ARs but just require higher doses to die. Therefore, using a given AR may still represent the best eradication tool. In such scenarios, having knowledge about VKORC1 mutations allows an informed decision of the correct ARs dosage to use, avoiding prolonged ARs exposures.

5. Conclusions

Resistance to ARs is a crucial matter that must be carefully addressed in planning rodent management operations, the effectiveness of which may be jeopardized by the presence of resistant populations. As a matter of fact, eradication attempts targeting insular mouse populations may be extremely challenging if resistance-related SNPs in the VKORC1 gene are present (Pelz and Prescott, 2015). This is even more critical when ARs usage is present before eradication campaigns, as it is often the case in human-inhabited islands, increasing the selective advantage of mutant mice. According to our results, this might be the case of Ventotene Island, where the frequencies of missense SNPs significantly increased in mice after a rat eradication. Since we found at least one missense SNPs in seven of the eleven islands we studied, we suggest that

resistance to ARs is a widespread phenomenon in Italian islands. Even though there are very few studies investigating ARs resistance on islands, similar scenarios have been already observed in other archipelagos around the world (Rost et al., 2009; Pelz et al., 2012; Marquez et al., 2019; Carromeu-Santos et al., 2023) and our study indicate that it might be the same in the Mediterranean basin. Therefore, ARs resistance screening should be a part of standard operating procedures for eradication planning, especially on inhabited islands where rodent control takes place continuously. This paper aims to be a starting point for future rodent management plans in the Mediterranean islands under a safer and more conscious use of ARs.

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CRediT authorship contribution statement

Francesco Gallozzi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Lorenzo Attili:** Writing – review & editing, Investigation, Data curation. **Paolo Colangelo:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Davide Giuliani:** Formal analysis, Data curation. **Dario Capizzi:** Writing – review & editing, Writing – original draft, Conceptualization. **Paolo Sposimo:** Writing – review & editing, Data curation. **Filippo Dell'Agnello:** Writing – review & editing, Data curation. **Rita Lorenzini:** Writing – review & editing, Supervision, Data curation. **Emanuela Solano:** Writing – review & editing, Data curation. **Riccardo Castiglia:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesco Gallozzi reports financial support, administrative support, and travel were provided by Pantelleria National Park. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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