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Colony social structure and burrow architecture of the Lusitanian pine vole, *Microtus lusitanicus* (Gerbe, 1879)

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Mestrado em Biologia da Conservação

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2019

AGRADECIMENTOS (ACKNOWLEDGMENTS)

Antes de mais, queria agradecer às minhas orientadoras Ana Cerveira e Sofia Gabriel por todo o apoio que me deram desde do início desta louca e longa aventura. A vossa amabilidade, o vosso à vontade e o vosso entusiasmo contagiou-me. E soube logo que era a tese que queria. Porque iria trabalhar com mamíferos, porque englobava áreas científicas que me interessam desde que meti na cabeça que queria seguir Biologia e porque encontrei em vocês um ambiente extraordinário e estável para me fazer crescer. Ana, obrigado por tudo o que me ensinaste durante este projeto. Nunca me irei esquecer dos longos dias sobre sol escaldante e noites chuvosas e escuras como breu no centro da serra de Sintra, dos teus ensinamentos sobre como capturar *Microtus* e da nossa alegria cada vez que capturávamos um (principalmente a minha), dos cuidados a ter na manutenção de animais em cativeiro, das longas reuniões acerca dos sistemas de túneis e das confusões com as árvores genealógicas. Obrigado por seres como és, apaixonada pelo que fazes, polémica durante as reuniões, amiga e uma excelente orientadora. Sofia, obrigado igualmente por tudo o que me ensinaste neste projeto. Pelas looooongas horas no laboratório que às vezes iam para lá das 19h, pelas várias discussões sobre "quem é o pai da criança?", pelos teus ensinamentos no laboratório sobre tudo o que podia saber sobre análise de microssatélites (principalmente o tramado do PCR) e pelas várias gargalhadas e brincadeiras que ajudavam sempre aliviar o ambiente. Obrigado por tudo, por seres uma orientadora espetacular, uma amiga, polémica tal como a Ana e louca pelo que fazes e com muito gosto. Graças a vocês, tive tudo o que ambicionava no mestrado: duas opiniões que tanto podiam combinar como colidir mas que no final eram sempre produtivas, duas componentes que me permitissem alargar os meus horizontes e que fizeram perceber aquilo que gosto, um ambiente que me permitisse avançar com confiança, ser orador de um congresso pela primeira vez e duas orientadoras que, apesar dos bons e maus momentos que aconteceram, sempre acreditaram em mim (obrigado pelo raspanete!). Obrigado por tudo!

Queria agradecer ao grupo SMaLL por ter-me acolhido tão bem durante o meu trabalho todo. À professora Maria da Luz Mathias, ao Joaquim, à Sophie, à Rita Monarca (obrigado pelos ratinhos de Caldas!), à Rita Andrade, à Ana, ao Welvis, ao Diogo, ao Tomé, ao Flávio, ao Ricardo, ao Philippe, à Maílis e ao Guilherme, o meu muito obrigado pelo vosso acolhimento e camaradagem. Adorei todas as reuniões semanais em que participamos. Descobri novos mundos científicos graças a vocês que, muitas vezes, me deram ideias para o meu trabalho. Um agradecimento para os Parques de Sintra – Monta da Lua, ao Águia, à Inês e ao staff encarregue da gestão do património que é a serra de Sintra por terem permitido a execução do meu trabalho de campo numa das suas tapadas. O vosso trabalho é espetacular e graças a vocês, Sintra continua a ser linda e verde.

À minha turma de Mestrado! 2 anos sensacionais, pelas experiências que convivemos, mas também pelas muitas amizades que criamos. Mais do que uma turma, tornamo-nos numa família! Discussões pelo meio e gargalhadas, muitas gargalhadas, vocês foram A TURMA!

Ao Atlético Clube de Porto Salvo por ter sido sempre foi o meu templo, o lugar onde os meus problemas desaparecem e dão lugar à alegria de correr atrás de uma bola.

Aos meus melhores amigos Inês, Inês, Marco e Sousa. Vocês são as melhores amizades que formei na vida. As nossas brincadeiras são imensas e não cabem num parágrafo apenas. Atravessamos uma grande jornada em Biologia e, apesar de cada um ter seguido caminhos diferentes, a nossa amizade cresceu e agora temos sempre montes de histórias para contar uns aos outros. Os nossos bons momentos à tarde nas Arcadas, as nossas saídas à noite para aliviar a cabeça, as nossas jogatanas na casa Lolok. Obrigado a vocês por me aturarem e a mim por vos aturar. Eu sei que, independentemente do futuro, poderei contar convosco!

À minha família toda! Obrigado por serem um dos pilares da minha vida, por me suportarem e apoiarem em tudo o que fiz na minha vida. Não me arrependo de nada porque vocês sabem que o percurso que fiz tinha de ser feito com as minhas decisões, mesmo que fossem más. E olhem, acabou por dar certo! Adoro-vos!

Ao meu pai Paulo (Hernâni), à minha mãe Ermelinda (São) e à minha irmã Cláudia (Tita), obrigado. Sou o mais novo da casa. Tudo o que fiz na minha vida devo a vocês. Tudo o que alcancei devo a vocês. Tudo o que sofri e sofri mesmo muito, ultrapassei e devo a vocês. Deram-me pernas para andar, mãos para trabalhar, um coração valente e destemido e um cérebro curioso e pensador. E eu usei tudo bem até agora e prometo continuar a usufruir muito. Mãe, a mulher que tu és… Não tenho palavras para te descrever…és uma santa que caminha entre mortais. Como te disse uma vez, sei que vocês têm orgulho em mim e prometo que continuarei a mostrar-vos que merecem ter orgulho em mim. Xixo, obrigado por seres quem és. Não podia ter melhor figura paternal que tu. Sempre me protegeste, mesmo quando achava que não era necessário. És o maior! Mana…Big sis! Ai mana, maninha… tu és a causadora disto tudo. Desta tese. Deste licenciado quase mestre. Tu. Levaste-me a ter a tua ambição, a tua vontade de trabalhar, de nunca desistir, de ter sucesso. Sempre disse e continuarei a dizer: quero ter tanto sucesso como tu, big sis. Tenho muito orgulho em tudo o que fizeste e em tudo o que fizeres. Estarei cá sempre para ti, Coco.

Por fim, à Beatriz Rosa. À pessoa que teve coragem de me aturar todos os dias, que olha por mim com tanto orgulho e amor hoje como olhou na primeira vez. És um dos pilares da minha vida. O futebol sempre foi o meu retiro até te conhecer. Agora, anseio todos os dias pelos teus braços pois lá encontro a minha paz. Obrigado por partilhares a tua vida comigo. Obrigado por acreditares em mim quando ninguém acreditava, por ajudares a levantar-me do chão várias vezes e a sorrir como sempre fiz. És tudo para mim, babe.

OBRIGADO!

“In a gentle way, you can shake the world.”

Mahatma Gandhi

COMMUNICATIONS

This work was presented in the following scientific meeting:

Resende JN*, Gabriel SI, Cerveira AM.

Colony social structure and burrow architecture of the Lusitanian pine vole, *Microtus lusitanicus*: preliminary results.

1st Workshop "Biology and management of Iberian voles: Progresses, challenges and prospects"

3-5 October 2018 - Vairão, Portugal.

(Oral communication)

*Presenting author

ABSTRACT

The Lusitanian pine vole (*Microtus lusitanicus*, Gerbe 1879) is a fossorial rodent known for its ability to build complex underground burrows system on which it lives. These burrows are usually several meters long, having multiple entrances, nests and food storages. Previous work indicates a single burrow system is shared by many individuals and may include more than one breeding pair. Recent work suggests the occurrence of alloparenting, as well as the formation of a strong pair bond between the breeding pair, thus strengthening the possibility of this species having a monogamous mating system and a complex social structure.

In the present study, an underground burrow system of *M. lusitanicus*, located in a semi-natural habitat (pasture field) in Sintra, was exposed and its structure mapped using a differential GPS system (DGPS). Additionally, the social structure of *M. lusitanicus* colonies from two distinct geographic locations (Sintra and Caldas da Rainha) was studied through microsatellite analysis.

The exposed burrow system was considerably large, reaching a total of 606.35 m in tunnel length, having a mean depth of 11.16±9.47 cm and reaching up to 52.8 cm in depth. A high number of openings (N=334) was found, however, only a single food chamber and no nests were detected. The low number of food chambers and nests can probably be explained by two main factors: (1) high food availability above-ground decreasing the need to storage food - the colony was located in a pasture field; (2) the burrow system was not completely exposed, and some of its deepest zones, where nests and food chambers are usually found, may have remained undug. This systems' fractal dimension and lacunarity (F_D and λ, 1.2678 and 0.7414, respectively) suggest a complex underground system with moderate heterogeneity, similar to that found in other fossorial and subterranean rodents.

The analysis of 10-12 microsatellite loci, in a total of 156 specimens of *M. lusitanicus*, allowed the identification of two family groups in Tapada do Mouco (distributed among 4 genetic clusters) and three family groups in Caldas da Rainha (5 genetic clusters). Mean family size is 15.2 (one of the families is smaller) which supports the observation of large family groups. With a single exception, all breeding pairs found exhibited a monogamous behavior, breeding with the same partner for successive generations and producing several litters. The only case of extra-pair mating occurred between a female and two males (polyandry), although this could have been due to external factors (severe disturbance of the colony's site). Despite close family members were found to share the same burrow system, no cases of inbreeding were detected. These findings, alongside with the sexual monomorphism observed, further support the hypothesis that the Lusitanian pine vole has a monogamous mating system and a complex social organization.

Keywords: DGPS, underground structure, fossorial, rodent, pedigree analysis, mating system, monogamy, microsatellites.

SUMÁRIO

O rato-cego (*Microtus lusitanicus*, Gerbe 1879) é um roedor fossador conhecido pela sua capacidade de construir um complexo sistema de túneis subterrâneos no qual habita. Estes sistemas podem ter vários metros de comprimento, com várias entradas, ninhos e despensas. Estudos anteriores indicam que um único sistema de túneis pode ser partilhado por vários indivíduos formando pequenos grupos familiares, podendo incluir mais de um par reprodutor. Trabalhos recentes sugerem a ocorrência de cuidados aloparentais, bem como a formação de um forte vínculo entre o par reprodutor, reforçando a possibilidade desta espécie ter um sistema de acasalamento monogâmico e uma estrutura social complexa.

No presente trabalho estudou-se a estrutura sistema de galerias subterrâneas de *M. lusitanicus*. O sistema subterrâneo, localizado num habitat semi-natural (campo de pastagem) em Sintra, foi exposto e a sua estrutura mapeada usando um sistema de GPS diferencial (DGPS). Adicionalmente, recorrendo à análise de 12 microssatélites, foi estudada a estrutura social e de parentesco da mesma. Para a recolha de amostras de tecido para análise de DNA, foram colocadas um total de 120 armadilhas de toupeira modificadas distribuídas pelo local de amostragem em Sintra (Tapada do Mouco), onde foram capturados 56 indivíduos. Com o mesmo objetivo, a 200 metros deste ponto, foram colocadas 40 armadilhas e capturados 12 indivíduos para comparação. Paralelamente, foram igualmente analisadas amostras de 88 espécimes provenientes de uma colónia de *M. lusitanicus*, resultado de um estudo anterior (2002-2003) num pomar de maçãs nas Caldas da Rainha.

O sistema de túneis exposto apresentou dimensões consideráveis, atingindo um total de 606.35 m de comprimento e uma profundidade média de 11.16±9.47 cm, atingindo um máximo de 52.8 cm de profundidade. Foi registado um número elevado de aberturas (N=334), no entanto, apenas foi encontrada uma única despensa mas nenhum ninho foi descoberto. O baixo número de despensas e a ausência de ninhos encontrados pode provavelmente ser explicado por dois fatores principais: (1) alta disponibilidade de alimentos à superfície, diminuindo a necessidade de armazenamento dos mesmos, pois a colónia estava localizada num campo de pastagem, com elevada disponibilidade alimentar; (2) o sistema de escavação não foi completamente exposto e algumas das suas zonas mais profundas, onde geralmente são encontrados ninhos e despensas, podem não ter sido encontradas. Os índices de linearidade, circularidade e convolução (1.5193, 0.4332 e 14.3714, respectivamente) indicam um sistema de túneis linear e moderamente convolscente. A dimensão fractal e a lacunaridade deste sistema (F_D e λ, 1,2678 e 0,7414, respectivamente), suportando os valores anteriores, sugerem um sistema subterrâneo complexo com heterogeneidade moderada, semelhante ao encontrado noutros outros roedores fossadores e subterrâneos.

A análise de 10 a 12 microssatélites, num total de 156 espécimes de *M. lusitanicus*, permitiu a identificação de dois grupos familiares na Tapada do Mouco (distribuídos em 4 *clusters* genéticos) e três grupos familiares em Caldas da Rainha (5 *clusters* genéticos). O tamanho médio da família foi de 15,2 indivíduos (sendo que uma das famílias era consideravelmente menor), estando de acordo com o anteriormente observado (organização dos indivíduos em grandes grupos familiares). Com uma única exceção, todos os pares reprodutores encontrados exibiram um comportamento monogâmico, reproduzindo-se com o mesmo parceiro, inclusivamente, num dos casos, por gerações sucessivas e produzindo várias ninhadas. O único caso de acasalamento extra-par ocorreu entre uma fêmea e dois machos (poliandria), embora isso possa também ter sido devido a fatores externos (alta perturbação no local da colónia). Apesar de familiares próximos partilharem o mesmo sistema de túneis, não foi detectado qualquer caso de consanguinidade, com reprodução entre irmãos ou parentes próximos. Este dado é reforçado por baixos valores de consanguinidade a nível de ambas as populações analisadas.

Estes resultados, juntamente com o monomorfismo sexual observado, sustentam e reforçam a hipótese de que o rato-cego tem um sistema de acasalamento monogâmico e uma organização social complexa.

Palavras-chave: DGPS, sistema subterrâneo, fossador, roedor, análise de parentesco, sistema de acasalamento, monogamia, microssatélites.

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1. Introduction

1.1. Burrow function and architecture

Burrow systems are more than just "holes in the ground" (Kinlaw 1999), food storage sites or refuges. Quite the contrary, for many species, burrow systems represent a vital micro-ecosystem, essential to their survival. In vertebrates, the burrowing trait is thought to have evolved as early as the Carboniferous (358.9-298.9 million years ago) (Olson and Bolles 1975), and it may have been responsible for the success of many early animal life-forms. Thanks to this, the burrowing trait can be found across taxonomic and ecological boundaries worldwide, from insects to mammals but also reptiles, amphibians and even birds (Rau 1929; Coulombe 1971; Bailey and Dale Roberts 1981; Kinlaw 1999; Eubanks et al. 2003).

Burrows are usually classified according to the function they provide its user. According to Kinlaw (1999) nine main functions can be identified: (1) protection from environmental extremes; (2) reduction in predation risk; (3) food storage; (4) place to heal from injury/disease; (5) safe place for reproduction; (6) "comfort" - provided by the downward temperature gradient in deeper burrows; (7) place to save both energy/time - most common in species that use other organisms' burrows; (8) socialization and (9) communication. However, despite the numerous roles attributed to burrows, three stand out as the most important: protection against predators, providing a stable microclimate, and potentiating socialization (Kinlaw 1999; Burda et al. 2007).

In terms of protection against predators, besides providing a place to hide, burrows can also act as anti-predator traps; potential predators can be discouraged by the diameter, depth, and length of the burrow and first timer hunters can be confused by the complexity of the underground system and thus fail to capture the resident(s). For example, some species of gerbils, such as *Desmodillus auricularis* (Nel 1967), escape through burrows that they use as exits when being pursued. Others, like the female Columbian ground squirrel *Spermophilus columbianus,* dig small, inconspicuous chambers on the periphery of the major burrow system which they use as escape pods from aggressive males (McLean 1978).

In terms of microclimate, burrows offer their inhabitants a relatively constant environment, both in terms of temperature and humidity, often offering more moderate temperatures than those felt in the outside environment. Such is the case of the Cape ground squirrel *Xerus inauris* (Van Heerden and Dauth 1987) and the banner-tailed kangaroo rat, *Dipodomys spectabilis* (Kay and Whitford 1978). In deeper burrows it is even possible to observe a downward gradient in temperature (i.e., a drop in temperature with increasing burrow depth); when in hyperthermia *Meriones hurrianae,* a subterranean gerbil from the Thar Desert (India), move to deeper and cooler areas of the burrow to cool down and avoid excessive heat (Prakash 1997).

With respect to socialization, the burrow represents a key environmental resource when considering philopatry (i.e., remaining at the natal burrow). Despite the high energetic cost associated with the construction and maintenance of a complex and extensive underground system with nests and food chambers (Ebensperger and Bozinovic 2000), in the long term, the investment not only seems to increase the species' breeding success but also ensures a stable structure that the offspring will inherit (Reichman and Smith 1990).

Besides the functions burrows provide their inhabitants, burrows also have a great impact on the surrounding environment, namely in terms of the site's geomorphology (by mixing surface and lower profile soils), hydrology, vegetation patterns (by redistributing seeds) and animal community diversity,

promoting soil dynamics (by loosening and aerating the soil), and adding organic matter (through their excrements and food residues) (Smith et al. 1991; Laundré 1993; Kinlaw 1999; Davidson and Lightfoot 2008).

The architecture of burrow systems can vary greatly between species. Among mammals, rodents are responsible for building some of the most complex systems known to date (Hansell 2007). Hickman (1990) breaks down the complexity of the underground system into its various structural components: i) tunnels - opening tunnels, surface tunnels and connection tunnels; ii) functional chambers - food storages, waste disposal and nest chambers; and iii) mounds. The complexity of the burrow system is associated not only to tunnel usage but also with its physical characteristics. For example, a single Brants' whistling rat (*Parotomys brantsii*), can build up to 500 entrances within an underground system (Hansell 2007), while a naked mole-rat (*Heterocephalus glaber*) burrow can exceed 3 km in length, including many different sections, such as foraging galleries, nests, food and sanitary chambers (Le Comber et al. 2002). Most species, however, produce much simpler structures, such as those built by the deer mouse (*Peromyscus maniculatus*), its burrow simply consisting on a single tunnel with an entrance and a nest chamber, or those of the wood mouse (*Apodemus sylvaticus*) - a loop like tunnel often supported by the roots of a tree, with five or six entrances and no nest area (Tew and Macdonald 1993; Hansell 2007).

1.2. Subterranean rodents

The burrowing trait is a multi-functional tool that allows animals to change their habitat into a more favorable state. As mentioned above, building a simple underground tunnel allows a more efficient alternative to escape from predation, creates a microhabitat with a more suitable environment, a food storage system, and a safer den for females to give birth and rear their pups. However, of all possible functions attributed to burrows, the sociality component remains the least understood. Even though there is already information regarding the social behavior of some subterranean rodents, with highlight on bathyergids (Šklíba et al. 2012; Šumbera et al. 2012), the mating system and sociality levels of most subterranean rodents remains unknown, thus making it difficult to assess the generality of ecological and evolutionary hypotheses developed for subterranean rodents. In recent years, studies on other social subterranean taxa were published (e.g., Rovatsos et al. 2011; Rekouti 2018) but much remains to be learned regarding the nature and extent of behavioral diversity in these animals.

African mole-rats (Bathyergidae, Rodentia) are among the most specialized mammals to have adapted to a subterranean lifestyle. Because of their high degree of specialization to this microecosystem, a vast number of scientific studies have been conducted regarding their morphological, physiological, and sensory adaptations to this particular microhabitat. However, as mentioned by Šumbera and colleagues (2012) most scientific interest is drawn to the social divergence found within this family, where both highly social mole-rat species and strictly solitary ones are found. To explain this variability in social structure, Jarvis and colleagues (1994) formulated the aridity food distribution hypothesis (also known as AFDH), where sociality evolves as an adaptive response to increased aridity. In the case of African mole-rats, living in arid environments with low and unpredictable rainfall, harder soils to work for long periods of the year and where the plants' underground storage organs (mole-rats' main food resources) are large and more widely spaced, sociality should be favored, as cooperative foraging spreads the energetic costs of burrowing and increases the chances of finding food (Šumbera et al. 2012). On the contrary, when living in mesic habitats, with softer soils, and smaller but denser and more regularly distributed food sources, the costs of living alone are not so high and may even be favored (Jarvis et al. 1994). The AFD hypothesis is able to generate predictions regarding the mole-rats' social structure that are generally accepted. However, there are some constrains, as Burda and colleagues (2000) point out in their work with naked mole-rats, where the AFDH is able to explain group size dynamics as a

consequence of the distribution and availability of resources but not the eusocial structure of naked mole-rat populations.

1.3. Mating systems

The term "mating system" is defined as the way males and females of a species or population pair to mate. There is a great diversity of mating systems in nature, namely: a) monogamy (each sex, male and female, mates with a single individual), b) polygyny (a male mates with multiple females), c) polyandry (a female mates with several males), d) harem (also known as polygyny with defense of resources), e) polygynandry (both males and females mate with several partners within the social group), and f) promiscuity (both males and females mate with several partners outside the social group) (Clutton-Brock 1989). Among different animal groups, a predominance of certain mating systems can be observed. For example, in birds, more than 90% of the species are monogamous, whereas in mammals monogamy is only found in c. 3-9% of the species, with c. 90% of mammals being polygynous (Clutton-Brock 1989; Lukas and Clutton-Brock 2013).

Which mating system evolves in each species or population is the result of a conflict between the sexes in order to maximize their own reproductive success. Since each sex has its specific constraints on reproduction, it follows that males and females usually adopt different mating strategies. For example, male strategies are usually a balance between the advantage of copulating with as many females as possible with the advantage of providing parental care. Depending on female availability, both in space and time, the competitive ability with other males but also the female's own reproductive strategies, males may opt for different mating behaviors (e.g., guarding females or competitive mate searching), resulting in different mating systems (Clutton-Brock 1989; Mulder 2009).

Monogamy is a relatively rare mating system in mammals but is among the most evolved forms of social organization, since it often involves a considerable degree of tolerance towards an individual over a long period of time and generally outside a mating context (Kleiman 1977). Monogamous species are characterized by reduced physical or behavioral sexual dimorphism, low reproductive potential, delayed sexual maturation of the young when in the presence of their parents, juvenile assistance to the younger siblings (i.e., alloparenting), and a strong pair bond between the breeding pair (Kleiman 1977). When group coexistence is favored in relation to individual isolation, monogamy becomes more advantageous because it requires a low energetic cost in social and sexual interactions, and allows the allocation of energy towards the defense of scarce resources such as nesting and food areas (Lukas and Clutton-Brock 2013).

Among mammals, monogamy is most commonly found in canids, primates and rodents (Solomon et al. 2004). One of the best studied monogamous rodents is the prairie vole, *Microtus ochrogaster*. The males provide parental care, help in nest construction and the protection of the vital area of their burrow, both in the wild and in captivity (Williams et al. 1992; Carter and Getz 1993). Additionally, during studies in captivity, *M. ochrogaster* individuals showed a preference for their partner in relation to other unknown conspecifics not belonging to their family group, revealing the ability to recognize family members, as well as a strong pair bond between the breeding pair (Williams et al. 1992). A similar behavior was also observed in *Microtus pinetorum*, both sexes showing a preference for their partner and providing parental care to their offspring (Oliveras and Novak 1986). Although several examples of monogamous mating systems can be found in the genus *Microtus* (Marfori et al. 1997; Wu et al. 2012)(Marfori et al. 1997), some species exhibit a promiscuous mating system (*M. oeconomus*, Borkowska et al. 2009; *Microtus pennsylvanicus*, Berteaux et al. 1999; *Microtus arvalis*, Fink et al. 2006), while others, such as the ground squirrels (*Spermophilus* spp.) and prairie dogs (*Cynomys* spp.) are polygynous (Wolff 2007).

1.4. *Microtus lusitanicus*, a social fossorial rodent

Voles from the genus *Microtus* can be divided in two distinct groups according to the ecotope they occupy: surface-dwellers i.e., voles mostly living aboveground, such as *M. cabrerae* (Pita et al. 2006) and *M. agrestis* (Ranchelli et al. 2016); and fossorial i.e., voles mostly living underground, but coming to the surface to forage, such as *M. lusitanicus, M. duodecimcostatus* (Borghi et al. 1994), *M. arvalis* (Brügger et al. 2010), *M. savii, M. multiplexus, M. subterraneus* (Salvioni 1988) *and M. pinetorum* (FitzGerald and Madison 1983).

The Lusitanian pine vole, *Microtus lusitanicus* (Gerbe, 1897) is a small rodent of the subgenus *Terricola* (Mira and Mathias 2007). Like most of the subterranean rodents, *M. lusitanicus* excavates its burrows with the help of its front legs and teeth (Giannoni et al. 1993). Previous work in orchards indicates that the gallery system built by this species consists of two types of tunnels: i) shallow tunnels (up to 15 cm of depth), built to come to the surface and search for food or for quick escapes, and ii) a set of deeper tunnels (up to 40 cm) to store food and nest (Mira and Mathias 2007). As the majority of burrow inhabitants, *M. lusitanicus* is morphologically adapted to underground conditions, presenting a compact body, short and powerful limbs, and a reinforced and robust skull, reflecting the conditions found in the subterranean ecotope (Mathias 1990; Mira and Mathias 2007).

When present in high populational densities, *M. lusitanicus* can constitute an agricultural pest, the most affected crops being fruit trees and horticultural produces, mainly due to voles gnawing the base of young fruit trees (Vinhas 1993; Mira and Mathias 2007). Sprinkler irrigation, a very common practice in agricultural systems, favors the growth of vegetation near the tree trunks, increasing the humidity and disintegration of the soil. These conditions seem to be optimal for voles, guaranteeing not only an abundant food source but also more amenable soil conditions to build underground tunnels and allowing potential local populational peaks (Mira and Mathias 2007).

The social organization of *M. lusitanicus* remains unclear. Research suggests that its populations are likely to be organized into small family groups, composed by a reproductive pair and other individuals of different ages (Godinho 1982; Mira and Mathias 2007). Lack of sexual dimorphism, small testis and a 1:1 sexual ratio suggest this species has a monogamous mating system (Ventura et al. 2010). However, Ventura's study is based on results obtained through the morphological analysis of sampled individuals and comparative analysis with other *Microtus* species, not from actual behavioral tests. A more recent study (Duarte et al. 2015) indicates that a strong bond between breeding pair members exists and should be indicative of a monogamous mating system. Additional laboratory work indicates parental care is performed by both parents and older siblings (alloparenting), further suggesting this species has a complex social structure (Cerveira, in prep.). Social structure studies usually require extensive observation to evaluate the behavioral interactions between two or more individuals. However, in many cases such as subterranean species, it is very difficult to access such data in the wild or even in captivity. Estimating the relatedness through genetic analysis can be a good alternative (Queller and Goodnight 1989). This methodology requires highly polymorphic genetic markers which are easily surveyed and interpreted. Microsatellites, although, posing some issues when used for the determination of relationships between individuals separated by many generations, are the ideal markers for assessing familial relationships and characterizing fine-scale population structure (Garza et al. 1997).

1.5. Main goals

The main goals of this project were to study the architecture of *M. lusitanicus* subterranean gallery systems and its colonies social structure. To accomplish this, the following objectives were defined:

• Determine the architecture and complexity of a Lusitanian pine vole subterranean gallery system in a semi-natural setting (length, width, depth, number of nests, food chambers, entrances and exits) using a differential GPS system;

• Determine the social structure of two Lusitanian pine vole populations, specifically, to determine the relatedness between the individuals constituting the colonies, by sampling individuals and performing subsequent parentage and kinship analysis using microsatellite loci.

2. Methods

2.1. *Microtus lusitanicus*

The study species was the Lusitanian pine vole, *Microtus lusitanicus*, a fossorial rodent (Order Rodentia) from the family Cricetidae. This species has a small geographical distribution restricted to the North and centre of the Iberian Peninsula and South of France (Figure 2.1.) (Mira and Mathias 2007). Morphologically, *M. lusitanicus* shows some adaptations to the fossorial life style, namely: incisor prognathism (Mathias 1990), a small and elongated body, rounded head, short tail, small eyes and ears, the latter hidden in the fur (Figure 2.2.).

Figure 2.1. Geographic distribution of the Lusitanian pine vole, *Microtus lusitanicus*. Adapted from Cerveira et al. 2019.

Figure 2.2. Specimen of a Lusitanian pine vole, *M. lusitanicus*, captured during this study.

M. lusitanicus' body mass ranges between 14-19 g, having a total body length of 77.5-105.0 mm (head-tail) (Mira and Mathias 2007). This species does not show sexual dimorphism (Ventura et al. 2010), with females reaching sexual maturity before males (5 and 7 weeks, respectively). Litter size varies between one and five offspring, with a mean number of two pups per litter. It is an herbivorous species, having a diet mostly based on geophytes but also consuming small bulbs, seeds and rhizomes (Mira and Mathias 2007). The Lusitanian pine vole has a conservation status of 'Least Concern' (according to the IUCN and the Portuguese Red Book of Vertebrates), being an abundant species throughout its distribution, with no specific conservation requirements. It is a common prey of the barn owl (*Tyto alba*) and the tawny owl (*Strix aluco*) (Mira and Mathias 2007), as well as a diverse array of small and medium size carnivores such as the fox (*Vulpes vulpes*), wildcat (*Felis silvestris*), genet (*Genetta genetta*) and stone marten (*Martes foina*) (Carvalho and Gomes 2004).

2.2. Study site

This study was developed in the Sintra mountain range, in the Lisbon district. The region has a Mediterranean climate with Atlantic influence. Its proximity to the Atlantic Ocean, orography and altitude (max of 528 m) result in a relatively small variation in annual temperature (average of 19°C in the warmest month and 10°C in the coldest month) and high degree of humidity (Soares et al. 2016). The condensation of ocean air, favored by the mountain's tree cover, promotes the occurrence of precipitation (annual mean of 860 mm) comparatively higher to the surrounding areas, as well as the frequent occurrence of fog and a characteristic cloud cap formation over the mountain range (Soares et al. 2016).

Located in the central area of the Natural Park of Sintra-Cascais, Tapada do Mouco is one of the most intervened forest units in the Park. With a forest area of approximately 45 hectares, it is probably the most recent forested unit in the park, since it had large extensions of deforested areas until about 1895, and later suffered great damages caused by the 1966 fire. The site chosen for this study was a semi-natural area located within the boundaries of Tapada do Mouco (38° 47'2.29 "N; 9° 24'0.88" W, altitude 405 m) (Figure 2.3.).

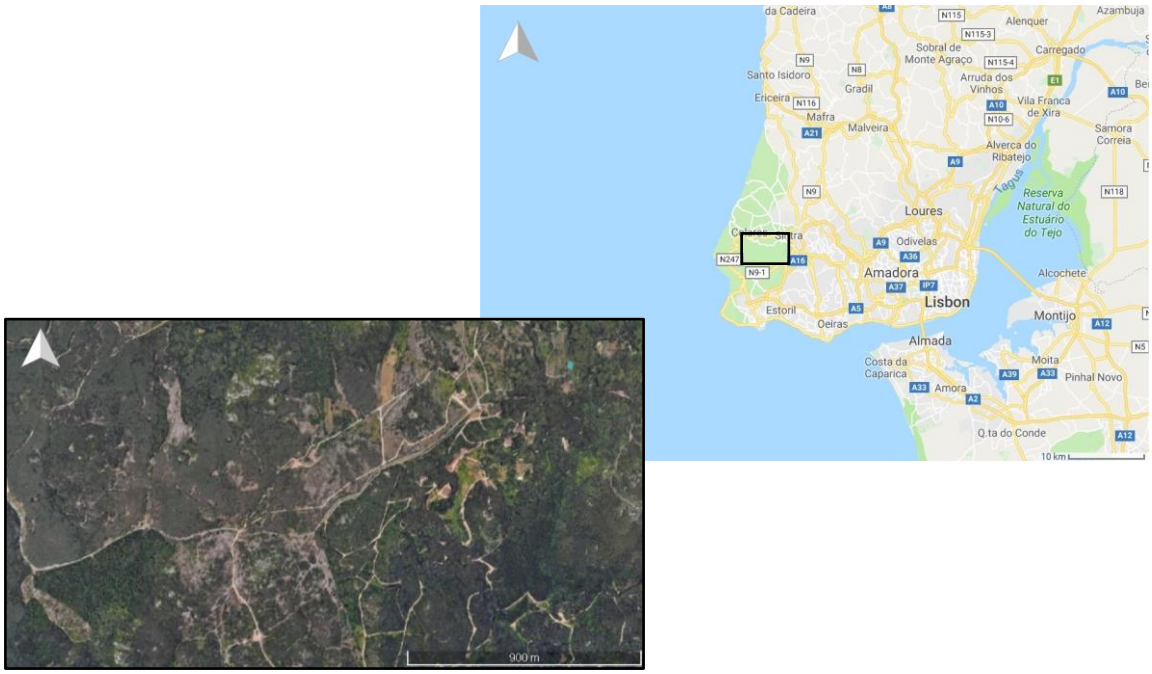

Figure 2.3. Study site at Tapada do Mouco, Natural Park of Sintra-Cascais, Portugal. Satellite view extracted from Google Earth and map extracted from Google Maps.

The main study site is a pasture field (J1 - c. 150 m in length, 50 m in width), intervened every spring by cutting and thinning the vegetation to produce bales of hay by Parques de Sintra - Monte Lua, S.A. – PSML, the entity responsible for managing the area (Figure 2.4.).The vegetation in the pasture field is mainly composed of herbaceous species (c. 50-100 cm high), such as the Spanish iris, *Iris xiphium*, the European yellow lupin, *Lupinus luteus*, oat, *Avenula sulcata* and crinkled hairgrass, *Deschampsia stricta*. The surrounding landscape that characterizes both Tapada do Mouco and the Sintra mountain range presents very different ecological characteristics, being mostly composed by maritime pine trees (*Pinus pinaster*), different species of oaks (*Quercus* sp., *Ilex aquifolium*), acacia (*Acacia longifolia*), strawberry trees (*Arbutus unedo*), and a variety of common plants (*Rubus* sp., *Hedera* sp., *Vinca* sp., etc).

Since one of the main goals of this work is to determine the social structure of Lusitanian pine vole populations, an additional site (J2) located in the vicinity of the pasture field (c. 200 m) was chosen as a secondary trapping site to obtain samples for parentage analysis (see below for details; Figure 2.5.). This site was composed mainly by herbaceous and berry fruit plants (*Rubus sp.*), oak and strawberry trees.

Figure 2.4. Pasture field - *Microtus lusitanicus* main trapping site, J1, at Tapada do Mouco, Sintra.

2.3. Trapping and sampling

Lusitanian pine voles (*M. lusitanicus*) were captured during February, April, May and July of 2018. The choice of the study area was based on *M. lusitanicus* presence signs, namely mounds and holes found at the surface. Given the use of underground traps and the associated risk of captured individuals dying between trap checks (due to potential flooding of the tunnels and traps), trapping sessions were only carried during periods when there was little or no rainfall. Animals were captured using 120 modified mole traps (Figure 2.6.) at J1 and 40 at J2.

Figure 2.5. Tapada do Mouco with the localization of the two trapping sites: J1, the main study site, marked in red and J2 in yellow. Satellite view extracted from Google Earth.

Figure 2.6. Modified mole trap used for live trapping of Lusitanian pine voles (view from above).

Unlike the conventional mole traps (a metal tube equipped with two one-way doors, one at each end), this trap is equipped with an additional zinc chamber attached to the top of the tube. The tube and the box are connected through a hole inside the tube. This chamber allows the accommodation of the animal after capture, providing a nesting area between trap checks.

Traps were supplied with vegetation from the surrounding habitat, hydrophobic cotton as nesting material, and apple pieces as bait - known to increase the capture success of this species (Sezinando 1982). The traps were installed at the entrances and openings of the tunnel system by positioning one side of the tube (or both) against the inside of gallery tunnels. All traps were numbered to know in which part of the gallery system animals were captured (Figure 2.7.).

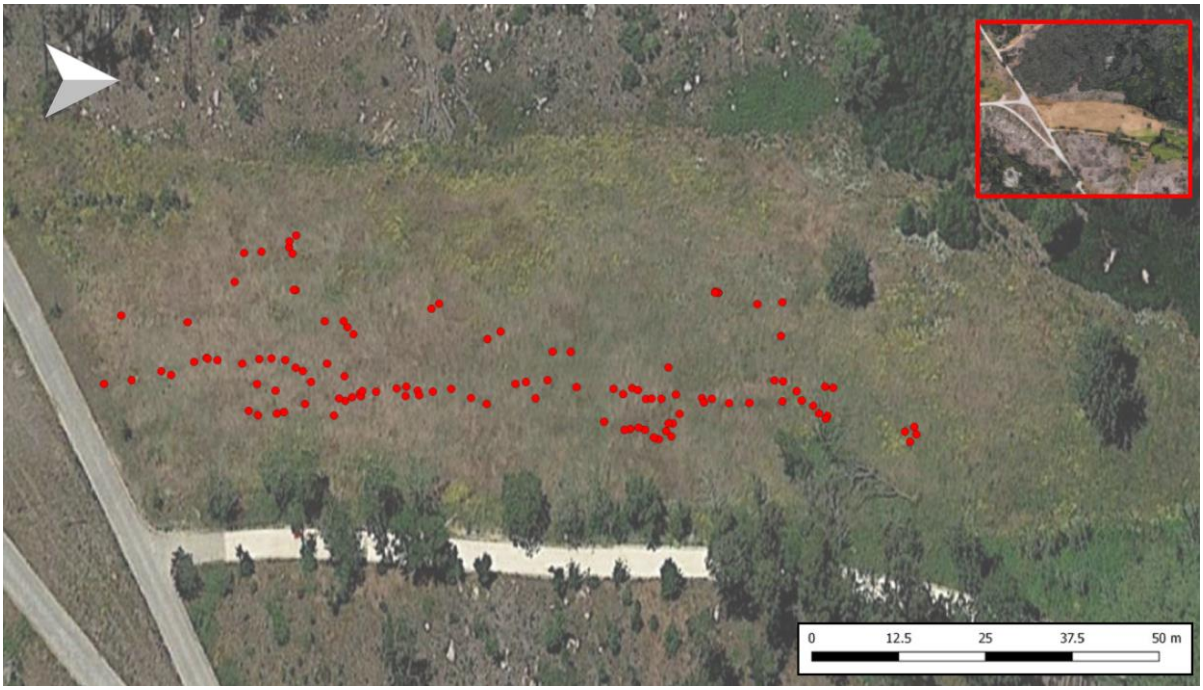

Figure 2.7. Trap map with the location of the 120 traps within the main study area, J1. Satellite view extracted from Google Earth.

Given the low temperatures during the night, trap-associated stress, and the fact that *M. lusitanicus* is active both during day and night, traps were checked twice a day, at early morning and before dusk to minimize the mortality of captured individuals. At each trap check, trap identification number, total number of traps with captured animals and number of captured animals. To avoid the recapture of individuals and to maximize the number of captures, all trapped individuals were transported to the Animal facilities at the Faculty of Sciences of the University of Lisbon. Individuals were sexed, weighed and housed individually in rodent-specific cages (Makrolon Type III) and kept under a controlled temperature (± 20 ºC) and photoperiod (12L:12D, lights on at 07:00). Given the species fossorial habits, soil collected from the study site was used as cage bedding and hay was provided as nesting material. Cages were environmentally enriched with small twigs and cardboard rolls. Food (apple, carrot and *Trifolium* sp.) was provided *ad libitum* three times a week. Individuals were returned to their natural habitat as soon as mapping of the burrow system (see below) was completed.

To determine if there were significant differences between the weight of males and females, a t-test of independent samples was performed. Normality and variance homogeneity were tested for body weight Animals from the adjacent site (J2) were not included in this analysis.

Tissue samples were collected from all captured individuals by sectioning a small (c. 2-3 mm) portion of the tail tip. Tail sectioning has been previously used in this species and is not known to cause significant damage or affect the fitness of the individuals (Cerveira pers. comm.). Tissue samples were stored in absolute ethanol at -20 ºC for later genetic analysis (see below).

An additional set of 88 *M. lusitanicus* tissue samples was used, collected in an orchard in Caldas da Rainha, Portugal. The individuals from this site were captured over three different seasons (autumn, winter and spring) in 2003. Moreover, the methodology adopted was to place the traps along a 4-line grid of fruit trees, moving along the orchard's tree lines each season. The captured animals were not released into their habitat.

2.4. Burrow architecture

Mapping the underground architecture of *M. lusitanicus*' burrow system was carried out with a Differential Global Positioning System (DGPS) during late May 2018. While the common GPS has a precision of ± 15 m, the DGPS has an associated error of ± 10 cm. This gain in precision is due to a set of fixed ground-based reference stations that transmit the difference between the positions indicated by the GPS satellite system and known fixed positions. The differential correction of the position from which the point is taken using different reference stations allows a massive decrease in error compared to the common GPS (Rempel and Rodgers 1997; Tomkiewicz et al. 2010). Another advantage of the DGPS is that this system provides three-dimensional rather than two-dimensional information, i.e., it allows taking depth measurements. It is a well-known method in geological studies (Aguirre et al. 2006) but also used in ecology, being mainly used in geomorphological studies of the habitat of several species (Fukushima 2001; Hewitt et al. 2004), and more recently in studies of subterranean and fossorial rodents (Rekouti 2018).

For the mapping to be carried out it was necessary to expose the underground burrow system. This was done by first cutting the vegetation and then carefully opening the tunnels with small picks, making sure the structure of the burrow system, regarding shape and depth, was kept as intact as possible (Figure 2.8.)

Figure 2.8. Section of an exposed burrow system, illustrating the complexity of the underground tunnels produced by a population of *Microtus lusitanicus* in Tapada do Mouco, Sintra

Due to time and weather constraints, it was not possible to expose and dig the entire burrow system. After exposing most of the main galleries, the DGPS was used to create a map of the system. Each GPS point provided the following information: x coordinate, y coordinate, altimetric quota, point quality (the device does not register values with less than 5 cm of error) and a code (ID for each point). The used DGPS system consists of three devices: a *Leica Viva* CS10 Field Controller, a SmartAntenna CS10 antenna and a GLS30 Telescopic carbon fiber GNSS pole, on top of which the antenna is placed (Figure 2.9.). The altimetric quota is the distance, in altitude, between the GPS point and the reference point at sea level. To obtain the depth values at each point, it is necessary to create a topographic profile of the study area; the difference between the surface topographic GPS point quota and the value of the same attribute of the burrow system points give the depth value of that specific point.

Soil hardness was also recorded in randomized points of the burrow system, using a manual penetrometer.

Figure 2.9. DGPS fieldwork at Tapada do Mouco, Sintra.

The DGPS data collected was processed in QGIS 2.18 (QGIS Development Team 2018) to map and characterize the architecture of the burrow system, namely: 1) number of burrow entrances/exits, 2) number of tunnel ramifications, 3) number of nests, 4) number of food chambers, 5) number of backfilled tunnels, 6) number of main tunnels, 7) minimum and 8) maximum burrow depth, and 9) total burrow excavated length. Mean depth of the burrow system was also calculated, however, only depth values >5 cm were considered as lower values would refer to burrow entrances/exits which, if used, would bias the system's mean depth. The percentage of tunnels with depths of more and less than 5 cm was also calculated.

A two-dimensional map of the excavated burrow system was created with the GPS points collected in the study site. The fractal dimension (F_D) of the burrow system was calculated using the FracLac plug-in (Karperien 2013) developed for ImageJ (Abràmoff et al. 2004). This value provides information on the level of complexity of the system, varying from 1 in a simple system, to 2 in a highly complex and reticulated system (Block et al. 1990). Its calculation is done using the box-counting method, which analyses complex patterns by gradually breaking an image into smaller and smaller parts (typically "box-shaped") and analysing each part at smaller scale. The methodology is easily compared to zooming in or out using optical or computer methods to examine how detail changes with changes in scale. However, rather than changing the resolution of a common lens, the size of the element used to inspect the object or pattern is changed instead (Karperien 2013). Lacunarity (λ) was also calculated in order to identify areas where information may be lacking, in this case, the absence of lines (i.e., unaccounted tunnels). Although most studies do not calculate this variable, here it was considered because it provides an indication of the map's heterogeneity and allows the estimation of how much of the burrow system was unaccounted for. This was particularly relevant because it was not possible to completely excavate the burrow system. Lacunarity was calculated using ImageJ, by performing multiple rotations of the map and evaluating whether changes in image orientation increase the level of information. Lacunarity values range between 0, for no lacunarity and 2, for high lacunarity. To complement the results obtained by the fractal dimension and lacunarity and allow an easier interpretation of the burrow architecture, three indexes were also calculated: *index of linearity* (I_{lin}), which allows the user to understand how linear is the burrow (a circular burrow system has values near 1.0 and a linear one has values higher than 1.0) (Reichman et al. 1982); *index of circularity* (I_{circ}), ranging from 0 (straight line) to 1 (circle) (Romañach and Le Comber 2004), and *index of convolution* (I_{conv}), where low values indicate less convoluted areas (Cameron et al. 1988). In this work, the index of convolution was corrected since according to the literature, there is a negative correlation between this index and the total length of the burrow, which if not corrected, may lead to inaccurate results (Šumbera et al. 2008; Šklíba et al. 2012).

2.5. Microsatellite analysis

Alongside with the tissue samples obtained in this study from Sintra, we used 88 additional samples (45 females and 43 males) of *M. lusitanicus* captured in Caldas da Rainha, in 2002-2003 (Monarca 2003). Genomic DNA was extracted from tail and ear tissue samples from *M. lusitanicus* specimens using the E.Z.N.A.® Tissue DNA Kit from *Omega Bio-Tek*. Extracted DNA was run by electrophoresis on a 1% agarose gel and quantified in a NanoDrop® ND-1000 UV-Vis Spectrophotometer to ascertain DNA quantity and quality. All samples were diluted to 30 ng/µl when the obtained DNA concentration was higher.

Table 2.1. Characterization of 12 microsatellite loci amplified in *Microtus lusitanicus* DNA samples.

Locus	Primer sequence	Repeat motif	Size range (bp)	Set	Dye
MM1	F: TGCACACATATGCACGGCCA	$(CA)_{17}$	47 - 63	1	FAM
	R: TTTACGTGTGTGTGGTGGGAAC				
MM2	F: TAACCACAACCCCTCCAACTG	$(CA)_{21}$	166 - 196	2	HEX
	R: TCATTTGGAGTTGCTGAGAAC				
MSCRB5	F: GGTTGGTGTTTGCATTTAGG	CA-, ATAC- and ATGT-	104 - 128	1	FAM
	R: CGTCTGGGTTTTACATCTGA				
MSCRB7	F: GTTTTATGTTAGTCTCATCTG	$(AC)_{20}$	88 - 101	2	HEX
	R: AGGCAATCCTGGTGAGTAACA				
MAG006	F: AGGTATGCCCAGCTCAAGC	$(TAA)_5TAG(TAA)_5$	161 - 197	1	FAM
	R: GAGAAGTATTTGGAACCCTG				
MAG025	F: TGGGATAGCCTAGCAGCAAGA	$(CA)_{17}$	147 - 185	1	HEX
	R: GTTTGTAGGGTTAGGTTCTCAGTTG				
MAR003	F: GGAGATACAAGGCCCAAACA	$(TG)_{21}$	136 - 170	2	Atto 565
	R: TGGCATTAGATGACCTGTGG				
MAR012	F: TTGCTCAATTCTCTCATAAAAGG	$(GA)_{23}$	84 - 96	2	FAM
	R: TGTCATGGATTGGGCATACA				
MAR016	F: CATCATCTTCTGGGGCACTG	$(CA)_{19}$	151 - 157	2	FAM
	R: ACGGTCTGTGCAAACCACTT				
MAR063	F: GCCTGGACACAACCAAACTT	$(AC)_{23}$	269 - 315	1	HEX
	R: GGCTATGGGCAGCTCCTG				
MAR076	F: TCACCAGGACCTACTGAGCA	$(AC)_{16}$	106 - 126	1	HEX
	R: GCCAGCTTCATTTCAAGAGG				
MAR080	F: ATGGATCATTCCGCTTCTGT	$(TG)_{12}(CA)_2(TGCG)_3$	189 - 226	2	FAM
	R: AACCTTCAGCCCAAACCATT				

All individuals were genotyped at 12 microsatellite loci, previously described for other *Microtus* species but known to successfully amplify in *M. lusitanicus*: MM1, MM2 (Ishibashi et al. 1999), MSCRB5, MSCRB7 (Ishibashi et al. 1997), MAG006, MAG025 (Jaarola et al. 2007), MARAR3, MAR012, MAR016, MAR063, MAR073 and MAR080 (Walser and Heckel 2008). After referring to the existing literature regarding the 12 selected microsatellites and knowing the range of size fragments obtained for each loci among *M. lusitanicus* populations in Portugal (Bastos-Silveira et al. 2012), two multiplex sets were set up: MM1, MSCRB5, MAG006, MAG025, MAR063 and MAR076 make up Multiplex 1; MM2, MSCRB7, MAR003, MAR012, MAR016 and MAR080 constitute Multiplex 2 (Table 2.1.). Each PCR reaction was run in a total volume of 12,5 µl, containing 0.2 µM of each primer (forward primer of each pair was fluorescently labelled), 6.25 µL of mastermix from Qiagen Multiplex Kit, 2.25 µl of deionized water and 1 µl of template DNA (DNA concentration ≤ 30 ng/ µl). Cycling conditions started with 95°C of initial denaturation for 15 min, followed by 30 cycles of 94°C for 30 s, 59°C for 1.5 min and 72°C for 1 min, with a final extension at 60°C for 30 min. Post PCR products were kept in the dark at 4º until further analysis. Fragment analysis was run commercially in STAB VIDA Lda., with PCR products being separated with capillary electrophoresis on an ABI 3730xl sequencer. Obtained genotypes were classified in PEAK SCANNER 1.0 (Applied Biosystems) against the GS500LIZ 3730 size standard. To control for scoring errors and ensure replication, a random selection of 10% of all individuals were reamplified and rescored. TANDEM (Matschiner and Salzburger 2009) was used to round the obtained values from PEAK SCANNER 1.0 into valid integers corresponding to multiples of the known repetition motif of each microsatellite (see Table 2.1.). CREATE 1.37 (Coombs et al. 2008)

and PGDSpider 2.1.1.5 (Lischer and Excoffier 2012) were used to prepare input files for all software subsequently used in microsatellite analysis. KINGROUP 2.0 (Konovalov et al. 2004) was used to calculate general estimations of diversity: number of alleles per locus, observed heterozygosity (H_O) and expected heterozygosity (H_E), number of homozygotes and heterozygotes per locus. Linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) were tested with GENEPOP 3.1. Vole populations were tested for deviations from equilibrium for all loci. Tests were also run for heterozygosity deficiency with ML-RELATE (Kalinowski et al. 2006). Through the estimation of heterozygosity deficit, we were able to identify null alleles in the data. This is crucial for the analysis of relatedness coefficient and consequently kinship because the results obtained can be highly influenced by the presence of null alleles at certain loci if not considered. With the same software, the relatedness coefficient (*r*) was calculated between all possible pairs of individuals by using the implemented maximum likelihood method and corresponding estimation of kinship. This software allows to identify four types of relationships between individuals: Unrelated (U), Half-Sibling (HS), Full-Sibling (FS) and Parent-Offspring (PO). The use of this software in individual relationship analysis in contrast with others used for the same purpose (for example KINGROUP or PEDIGREE) has two important advantages: most of the estimators used by most authors do not take into account the presence of null alleles in the dataset and their influence on the final results of the coefficients ratio. Also, this software allows to infer four types of family relationships. However, these relationships do not reflect their true intrinsic value, especially the HS (Half-Sibling) pedigree. Given that the relationship estimator uses the probability of an individual sharing zero, one or two alleles with another individual as the basis of their formula, the HS relationship can be attributed to a pair of individuals who may not represent true half-siblings. If we compare two individuals with a HS relationship, both have at each locus 50% probability of sharing no alleles and 50% of sharing only one allele. However, if we consider a relationship between grandparent and grandchild, the same probabilities are obtained but, because the software only considers four types of relationships, the obtained pedigree is also HS. And the same probabilities equally apply to the case of uncle and nephew. The relationship between first cousins (Kalinowski et al., 2006) is the only one not represented in any of the four relationships computed by the software as the associated probability is 75% of not sharing any alleles and 25% of sharing only one, very different from the probabilities associated with U, HS, FS, and PO. Given these constrains, it is not possible to consider that certain relationships obtained correspond to the real biological kinship. During the pedigree classification of each pair, the individuals' relationships with others were considered to help classifying the more complex cases, with the HS being more difficult to separate. To help with the classification, information obtained during field work (sex and age) were also taken into consideration.

To calculate the inbreeding coefficient (*F*) of each individual and each population, the software COANCESTRY 1.0.1.9 (Wang 2011) was used. This also allowed to estimate the relatedness coefficients through six of the most used estimators (Queller and Goodnight 1989; Li et al. 1993; Ritland 1996; Lynch and Ritland 1999; Wang 2002, 2007). There is no consensus in the scientific community as to what is the best estimator, therefore, the additional calculation of all estimators helped to confirm that the relatedness coefficient calculated in ML-RELATE were reliable.

In order to understand how the vole populations analyzed in this study were genetically structured, both microsatellite datasets were submitted to STRUCTURE 2.3.4 with K-values tested from K=1 to K=8. Simulations were run under the "admixture ancestry" model (i.e. each individual may have mixed ancestry, possibly carrying a fraction of its genome from each of the K populations) and the "correlated allele frequencies" model (frequencies in different populations are likely to be similar as a result of common ancestry or migration). Each K was run for 500.000 Monte Carlo Markov Chains (MCMC) iterations after a burn-in period of 100.000 iterations with five independent replicates. ΔK statistics developed by Evanno and colleagues (2005), widely known as the Evanno Method, was used to detect

which K value was the best to fit the number of genetic clusters, graphicly represented with DISTRUCT 1.1 (Rosenberg 2004).

2.6. Ethical note

Capture, transport and maintenance of animals was authorized by the competent Portuguese authority, ICNF (Instituto da Conservação da Natureza e das Florestas), under the license number 615/2018/CAPT. All experimental procedures were approved by the Faculty of Sciences (University of Lisbon) animal welfare body ORBEA – Orgão Regulador do Bem-Estar dos Animais (Statement 4/2017). After the excavation of the burrow system was completed, all animals, including the three individuals born in the laboratory, were released at the place of capture. All animals were released considering a minimum distance between them to minimize the potential impact on established individuals.

3. Results

A total of 68 *M. lusitanicus* specimens were captured at Tapada do Mouco, 56 in the main colony site (J1) and 12 in the adjacent site (J2). Of the 56 individuals captured at the main colony, 15 were captured during February 2018 (over 7 days), 38 in April and May of 2018 (over 10 days) and the remaining 3 were born at the Faculty's animal facility. Captures in J2 occurred during July 2018 (over 8 days). Individuals were captured in 31 of the 120 set traps (25.8%), some capturing up to 4 individuals each (on separate occasions) (Figure 3.10.).

Of the 56 individuals captured at J1, 51 (91%) were adults and 5 were juveniles. The sex ratio among adults was balanced (25 females and 26 males; 1:1; $\chi^2 = 0.02$, $p = 0.882$). The mean body weight of adult females was not significantly different from that of males (Student's t-test =1,799 ; $p = 0.08$, $N = 51$; *females*: mean weight = 17.02 g; min-max: 10 - 23.1 g; *males*: mean weight = 16.10 g; min-max: 9.2 – 21.2 g).

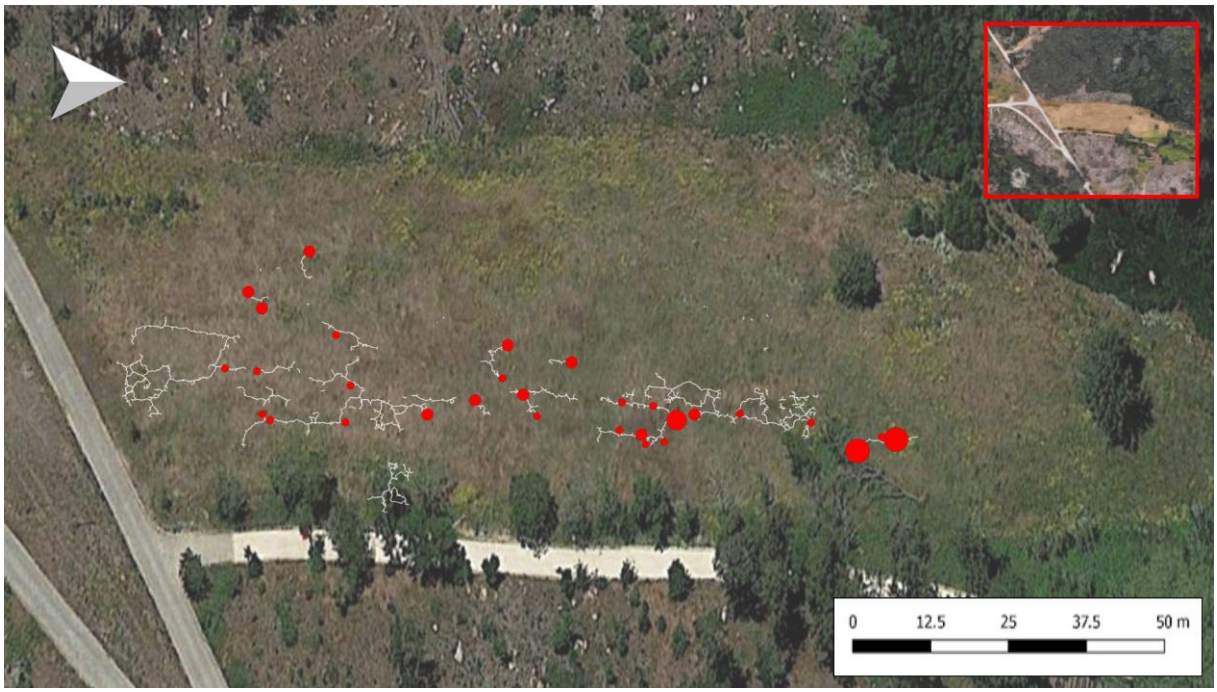

Figure 3.10. Map of the main study area (J1) showing the location of the 31 traps (red circles) where *M. lusitanicus* were captured. Circle size is proportional to the number of captured animals in each trap, 1 to 4 per trap. Satellite view extracted from Google Earth.

3.1. Architecture of the burrow system

A total of 11478 GPS data points were recorded using the DGPS. Of these, 5037 were used to produce a topographic profile of the study area and calculate the depth of the burrow system. The remaining 6441 points corresponded to GPS coordinates of the actual burrow system and were classified as: 1) tunnel, 2) ramification, 3) entrance/exit, 4) nest, and 5) food chamber.

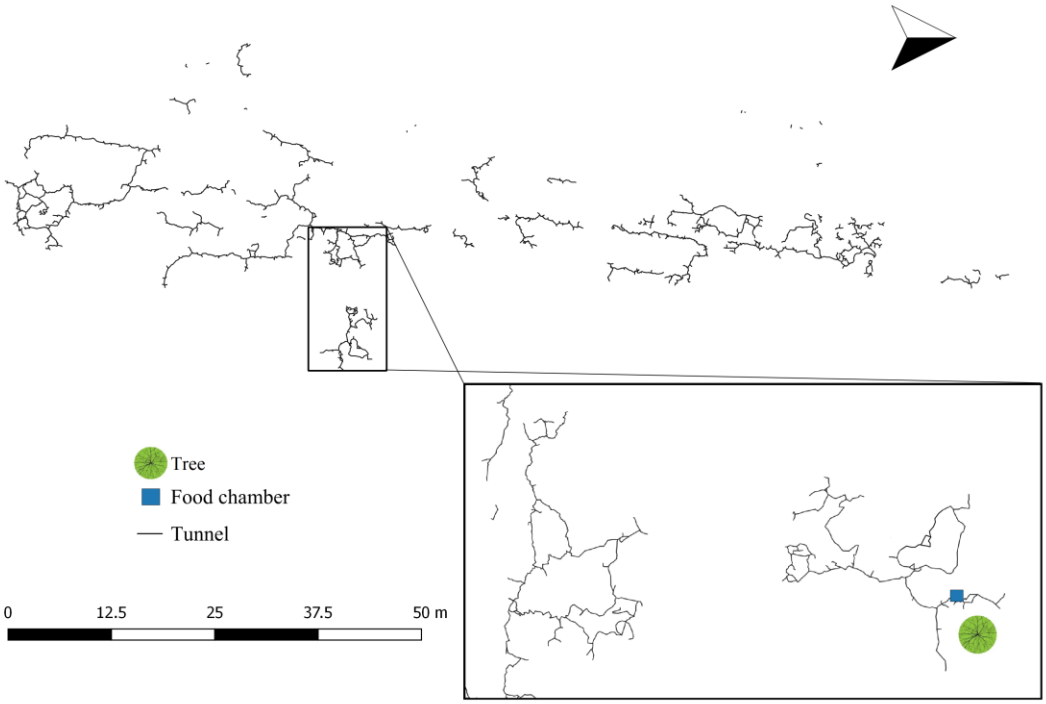

Figure 3.11. Map of *M. lusitanicus* burrow system with a close-up window to the food storage area located near a tree.

The burrow system had a North-South orientation, occupying an area of c. 120 m by 40 m. A total of 606.35 m of tunnels were excavated and mapped, covering an area of 1780.10 m^2 (Figure 3.11. and Table 3.2.). Most of the tunnels comprising the burrow system were superficial (67.17%), having a depth of less than 5 cm. The maximum depth recorded was 52.80 cm, having a mean depth of 11.16 cm (see Table 3.3.). A total of 597 tunnel ramifications and 332 entrances/exits were identified in the burrow system. Surprisingly, only a single food chamber was found in the entire excavated area. The cache was composed by 5 bulbs of Spanish iris, *Iris xiphium*, weighing a total of 42.4 g (Figure 3.12.), located under an oak tree (see Figure 3.11.). No nests were found throughout the excavated portion of the burrow system. Soil hardness was relatively low (mean of 100 psi), indicative of a soft and moist soil, mostly composed of silt, clay and organic matter.

In terms of architecture, the system revealed some heterogeneity, both highly reticulated areas (tunnels with secondary and tertiary branching), as well as relatively simple areas (long and straight tunnels). Regarding the system's architectural indexes, we obtained an Index of linearity of 1.5193 and an Index of circularity of 0.4332. The system's convolution Index, related with the complexity of the system in which higher convolution values relate to more complex areas (higher number of tunnel contortions), was 14.3714 (Table 3.4.).

Figure 3.12. Food cache composed of five bulbs found in a food chamber within *M. lusitanicus* burrow system.

Table 3.2. Architectural parameters of *Microtus lusitanicus* burrow system.

L_{tot} (m)	L_{bf} (m)	MCP (m^2)	N_{pt}	$N_{e/e}$	N_i	N_{food}	N_{nest}
606.35	12.21	1780.1	34	332	597	1	0

L_{tot} total length excavated, L_{bf} length of tunnels with distance <20 cm, *MCP* area of minimum convex polygon to encompass the burrow system, N_{pt} number of principal tunnels, $N_{e/e}$ number of entrances and exits, N_i number of tunnel interceptions, N_{food} number of food chambers and N_{nest} number of nests.

Table 3.3. Principal depth parameters.

D_{mean}	D_{max}	D_{mfood}	%tunnels ≥5 cm	%tunnels <5 cm
11.16±9.47	52.8	14	67.17	32.83

D_{mean} mean depth (excluding depths below 5 cm), D_{max} maximum depth, D_{mfood} mean food chamber depth, %tunnels ≥5 cm total percentage of tunnels with depth ≥5 cm, %tunnels <5 cm total percentage of tunnels with depth <5 cm.

Table 3.4. Index of linearity, Index of circularity and Index of convolution of *Microtus lusitanicus* burrow system.

Index of linearity	Index of circularity	Index of convolution	Index of convolution corrected
1.5193	0.4332	0.3406	14.3714

Index of linearity (Reichman et al. 1982), index of circularity (Romañach and Le Comber 2004), index of convolution (Cameron et al. 1988) and index of convolution corrected (Šumbera et al. 2008).

Fractal dimension and lacunarity were calculated for the entire excavated burrow system. Additionally, given the systems' heterogeneity in terms of tunnel complexity, fractal dimension and lacunarity were also calculated separately for 6 small zones within the system (Figure 3.13. and Table 3.5.) during fieldwork, some zones (1 and 3) were much more ramified than the middle zones (zone 2) and this could bias index calculation. Zone 4 was also analyzed separately because it was the only one where a food chamber was found. The mean F_D (±SD) value of the entire excavated area was 1.2678±0.006. The

highest F_D value obtained was 1.4401±0.0236 and the lowest was 1.2184±0.0148. Lacunarity (λ) varied between a narrower range of values (between 0.3834-0.4600) indicating a lack of information in the 6 evaluated zones. However, the λ value of the total map (0.7414), is higher than the lacunarity range of values obtained for the 6 zones. When the entire burrow system is considered, the values obtained for fractal dimension and lacunarity indicate *M. lusitanicus* system is of moderate complexity and heterogeneity.

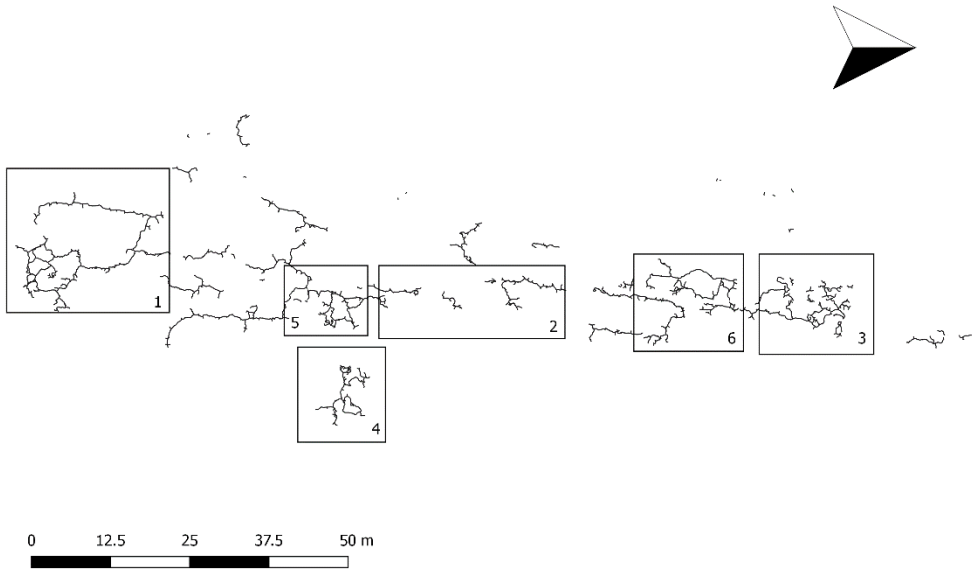

Figure 3.13. Map of *M. lusitanicus* burrow system divided in 6 zones used to calculate fractal dimension (F_D), F_D standard deviation (SD) and lacunarity (λ).

Table 3.5. Fractal dimension (with corresponding standard deviation) and lacunarity of the mapped burrow system and 6 marked zones.

	F_D	SD	λ
Map	1.2678	0.0061	0.7414
Area 1	1.2994	0.0064	0.4048
Area 2	1.2184	0.0148	0.4600
Area 3	1.3349	0.0255	0.4382
Area 4	1.3544	0.0056	0.3834
Area 5	1.4401	0.0236	0.4004
Area 6	1.3706	0.0217	0.4205

3.2. Tapada do Mouco *M. lusitancus* colony (2018)

3.2.1. Genetic diversity

The results based on the analysis of 12 microsatellite loci revealed a high level of variability, with a mean of 6.4 alleles per locus. However, in some loci MAR012 and MAR016, variability was very low in contrast with others MAR003 and MAR080, that presented the highest variability values (see Table 3.5). The average Observed and Expected Heterozygosity was 0.47±0.238 and 0.57±0.251, respectively. Only five loci were found to be in HWE (Hardy-Weinberg Equilibrium, *p*-value<0.05) and tests for heterozygosity deficit were found to be statistically significant for 5 loci (Table 3.6.). These were identified as loci with null alleles during pedigree analysis in ML-RELATE.

Table 3.6. Variation observed in 12 microsatellite loci among *M. lusitanicus* samples from Tapada do Mouco, Portugal.

loci	k	N	Hets	Homs	H(O)	H(E)	pHWE	$Het_{deficit}$
MM1	3	67	20	47	0.299	0.39	0.112	0.0219
MSCRB5	3	65	28	37	0.431	0.39	0.671	0.7024
MAG006	4	67	8	59	0.119	0.12	0.056	0.0995
MAR076	6	66	33	33	0.5	0.56	0.082	0.0802
MAG025	9	67	48	19	0.716	0.79	0.045	0.0181
MAR063	11	67	52	15	0.776	0.88	0.167	0.0083
MAR012	4	67	10	57	0.149	0.47	0	0
MAR016	2	67	11	56	0.164	0.18	0.17	0.1013
MAR080	12	67	50	17	0.746	0.84	0	0.1697
MSCRB7	4	67	29	37	0.439	0.63	0	0.0004
MM2	8	67	51	16	0.761	0.73	0.272	0.7696
MAR003	11	67	39	28	0.582	0.85	0	0

k number of alleles, N number of individuals typed at each locus, Hets observed number of heterozygotes, Homs observed number of homozygotes, H(O) observed heterozygosity, H(E) expected heterozygosity, pHWE *p*-value of deviation from Hardy-Weinberg equilibrium (Guo and Thompson 1992) and $Het_{deficit}$ heterozygosity deficiency.

3.2.2. Pedigree analysis

In both sampling sites, J1 and J2, the inbreeding coefficient (*F*) was very low in both estimators (Table 3.7.). Comparing the mean and variance of both sites with the combination of those two, there is almost no difference between them. Moreover, the correlation between both tests was assessed to understand whether the results were identical for the two different estimators, which was confirmed with a Pearson correlation of 0.85. It was important to identify possible evidence of inbreeding because, in that case, it would not allow the pedigree analysis with ML-RELATE considering that this software does not take this factor into account.

Table 3.7. Inbreeding coefficient of the populations from Tapada do Mouco.

	J1		J2		Overall	
	Ritland	LynchRd	Ritland	LynchRd	Ritland	LynchRd
Mean	0.07985	0.0943	0.08785	0.10317	0.08112	0.09611
Variance	0.02808	0.02847	0.0119	0.01345	0.02506	0.02564

Mean and variance of J1 (N=56) and J2 (N=12) in both Ritland 1996 and Lynch and Ritland 1999.

The software ML-RELATE was able to produce the relatedness coefficients (*r*) of all possible dyads present in the dataset (2278 dyads). Due to scoring difficulties of two microsatellite loci (MM1 and MSCRB7), the software was run without them. A total of 1849 relationships were scored as U (Unrelated, 81.17%), 295 as HS (Half-Sibling, 12.95%), 60 as FS (Full-Sibling, 2.63%) and 74 as PO (Parent-Offspring, 3.25%) (see S1., Annex 1). Mean coefficient of relatedness was 0.081±0.138, where only $r>0$ were taken into consideration (large number of dyads with *r* coefficient=0 influenced the mean relatedness of the dataset).

After analyzing all the dyads and using the field work information as support for the construction of the family trees, it was possible to reconstruct two large families. The first one included a total of 17 identified individuals, with animals from 5 generations (Figure 3.14.). It was possible to identify at least two different litters from the same breeding pair. Four older full-siblings were identified (either from the same litter or from different litters); one female (J1ML32) and three males (J1ML23, J1ML28 and J1ML36) (mean $r_{20/21\text{-litter}}=0.487\pm0.104$ and mean $r_{\text{within litter}}=0.481\pm0.194$). A younger litter, born at the faculty's animal facility, consisted of 3 pups (J1ML54, J1ML55 and J1ML 56) (mean $r_{20/21\text{-}}$

$_{litter}$=0.511±0.021 and mean $r_{within\ litter}$=0.701±0.001). When comparing the *r* values from the two litters, the mean *r* value is high enough to accept the full siblingship between them (mean *r*=0.602±0.2). The individual J1ML20 shares a parent-offspring relationship with female J1ML9 (*r*=0.433) and male J1ML39 shares a full-sibling relationship with the latter (*r*=0.62). J1ML21 shares a parent-offspring relationship with male J1ML2 (*r*=0.5).

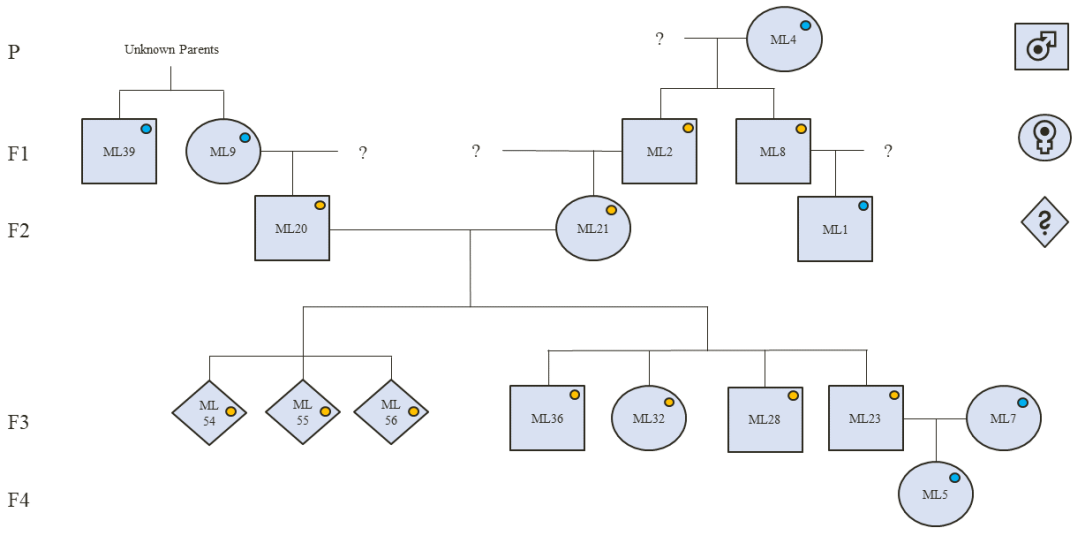

Figure 3.14. Family tree with 17 identified individuals of *M. lusitanicus* from the trapping site J1. Squares represent males, circles represent females and rhombus represent individuals of unknown sex. It was possible to infer 5 generations. Colored dots have correspondence with the results obtained in STRUCTURE (see Figure 3.16.)

The second family group included 14 individuals, distributed across 7 generations; 10 were from the sampling site J1 and 4 from site J2 (Figure 3.15.). It was possible to identify several full-sibling and parent-offspring relationships. The most recent generation was between the pair J1ML15 and J1ML25 that produced two identifiable offspring, J1ML34 ($r_{15\text{-}34}$=0.5 and $r_{25\text{-}34}$=0.5) and J1ML41 ($r_{15\text{-}41}$=0.5 and $r_{25\text{-}41}$=0.5). Between these two, it was possible to identify a full siblingship *r*=0.4. J1ML25 also shared a parent-offspring relation with J1ML24 and a full-sibling with J1ML33 (*r*=0.5 and *r*=0.62 respectively).

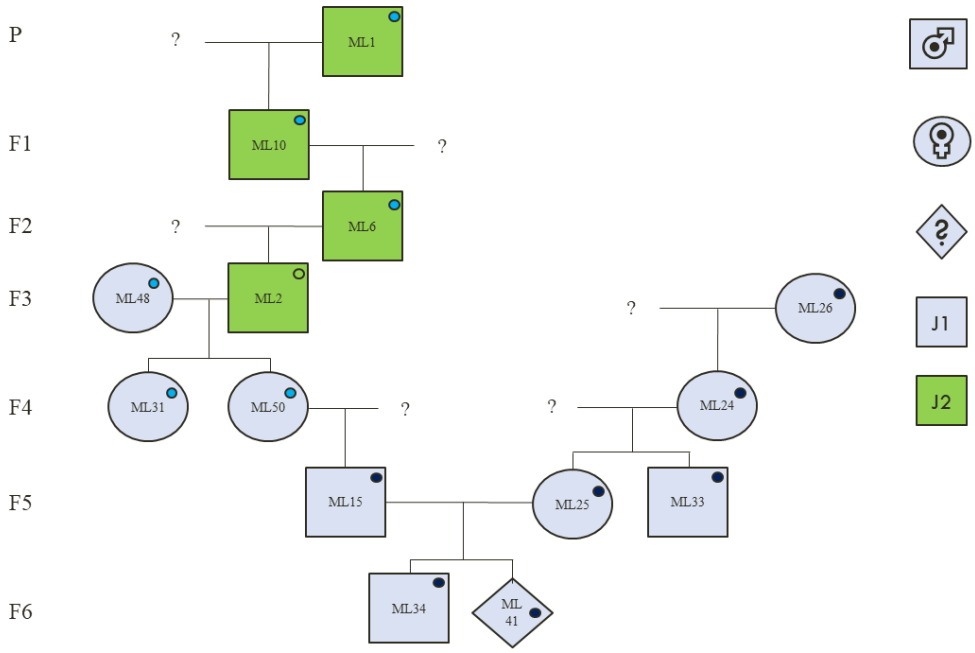

Figure 3.15. Family tree with 14 individuals of *M. lusitanicus* distributed by 7 generations from two sampling sites, J1 and J2, where blue represents the individuals from the main sampling site (J1), and green those captured at the second trapping site (J2). Squares represent males, circles represent females and rhombus represent individuals of unknown sex. Colored dots have correspondence with the results obtained in STRUCTURE (see Figure 3.16.).

Since there is no consensus about the best estimator to calculate the relatedness coefficient, it is important to calculate *r* for each dyad with several different estimators. With COANCESTRY, it was possible to calculate these relatedness coefficients with 6 well known estimators and compared them to ML-Relate *r* results: Queller & Goodnight (1989), Li et al. (1993), Ritland (1996), Lynch & Ritland (1999), Wang (2002) and Wang (2007) (Table 3.8.).

Table 3.8. Mean and variance of seven relatedness estimators.

	Kalinowski	**TrioML**	**Wang**	**LynchLi**	**LynchRd**	**Ritland**	**QuellerGt**
Mean	0.080951	0.06413	-0.0642	-0.05427	-0.01499	-0.0161	-0.01002
Variance	0.018978	0.01366	0.05042	0.06157	0.02714	0.03121	0.04996
J1-J1							
Mean	0.090435	0.07222	-0.04769	-0.04038	-0.00857	-0.012	-0.00602
Variance	0.021811	0.01617	0.05497	0.06553	0.03117	0.03622	0.05458
J2-J2							
Mean	0.168225	0.15347	0.02676	0.04144	0.11295	0.11103	0.12174
Variance	0.04547	0.03648	0.08885	0.09298	0.05462	0.03733	0.06862
J1-J2							
Mean	0.05328	0.03713	-0.11904	-0.11075	-0.0423	-0.03872	-0.04465
Variance	0.008797	0.00466	0.03354	0.04583	0.01344	0.01872	0.03476

Kalinowski (Kalinowski et al. 2006), TrioML (Wang 2007), Wang (Wang 2002), LynchLi ((Li et al. 1993), LynchRd (Lynch and Ritland 1999) and QuellerGt (Queller and Goodnight 1989).

As expected, since each estimator relies on a different equation to calculate the *r* value, there were different mean values between all 7 estimators with TrioML being the closest one to Kalinowski (0.64

and 0.81, respectively). Also of note is the fact the three oldest estimators (LynchRd, QuellerGt and Ritland) had very similar mean values. A positive correlation was found between all estimators (Table 3.9.), with only four resulting in a value below 0.65. When focusing on Kalinowski, we can verify almost all estimators showed a high positive correlation with the first one, with major evidence to TrioML (0.918). These high correlation values indicate that the *r* coefficient of each dyad obtained in all 6 estimators, from COANCESTRY, support the *r* values obtained in ML-Relate.

Table 3.9. Correlation matrix between seven relatedness estimators.

	Kalinowski	**TrioML**	**Wang**	**LynchLi**	**LynchRd**	**Ritland**	**QuellerGt**
Kalinowski	1						
TrioML	0.9180599	1					
Wang	0.7526458	0.7455478	1				
LynchLi	0.7171058	0.7206856	0.9420426	1			
LynchRd	0.8249802	0.8425545	0.8361876	0.7928648	1		
Ritland	0.5751131	0.6095885	0.5744861	0.6227302	0.7210723	1	
QuellerGt	0.7199665	0.743005	0.8692506	0.9328567	0.820158	0.6846142	1

Kalinowski (Kalinowski et al. 2006), TrioML (Wang 2007), Wang (Wang 2002), LynchLi ((Li et al. 1993), LynchRd (Lynch and Ritland 1999) and QuellerGt (Queller and Goodnight 1989).

3.2.3. Sub-population structure

Following the STRUCTURE analysis, K=4 represented the most probable number of genetic clusters (See S2., Annex 1), i.e., the total of 68 individuals, sampled in two different sites (separated by 200m) were distributed into four distinct subpopulations (Figure 3.16.). According to the pedigree analysis, the orange bars represent individuals belonging to the first family group, while the individuals composing the second family group were distributed between the light blue and green clusters. Dark blue represents the remaining individuals that were not associated with any of the two family groups. To better understand the remaining unrelated individuals, a second run in STRUCTURE was performed with the same specifications but only with the individuals from the main sampling site, J1. The genetic structure of J1 population is best represented by K=3 clusters (Figure 3.17.). The orange color remains as the first family and light blue as the second one. The dark blue individuals cannot be considered a third family since most of the relationships shared between them are U (Unrelated).

By superimposing the genetic cluster to which individual voles were allocated and the exact trapping site within the underground burrow system, it was possible to observe that family groups (represented by different colors) were widely distributed throughout the whole excavated gallery system (Figure 3.18.). The family group represented by the orange cluster were more concentrated on the northern end of the burrow system.

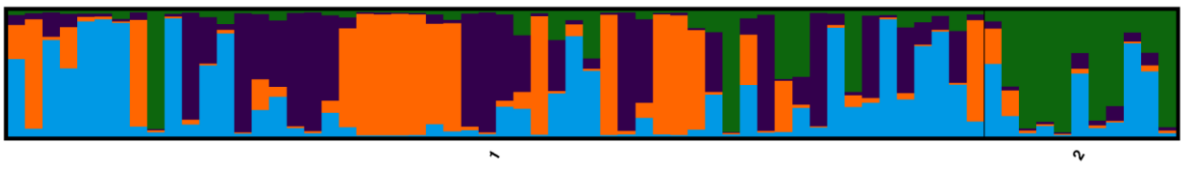

Figure 3.16. Population structure based on 10 microsatellite loci genotypes of 68 *M. lusitanicus* specimens. Each individual is represented by a thin vertical colored line, each color representing an estimated proportion of membership of the genome originating from each of K inferred clusters. '1' refers to the sampling site J1 and '2' refers to sampling site J2.

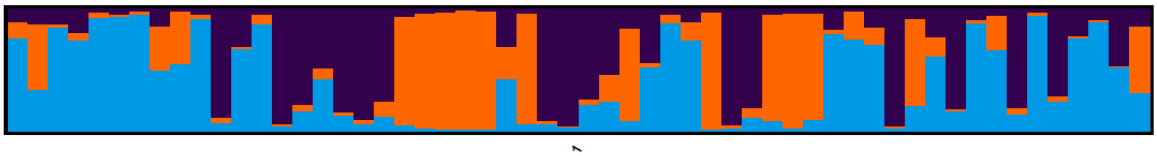

Figure 3.17. Population structure based on 10 microsatellite loci genotypes of 56 *M. lusitanicus* specimens from J1 sampling site. Each individual is represented by a vertical colored line, each color representing an estimated proportion of membership of the genome originating from each of K inferred clusters.

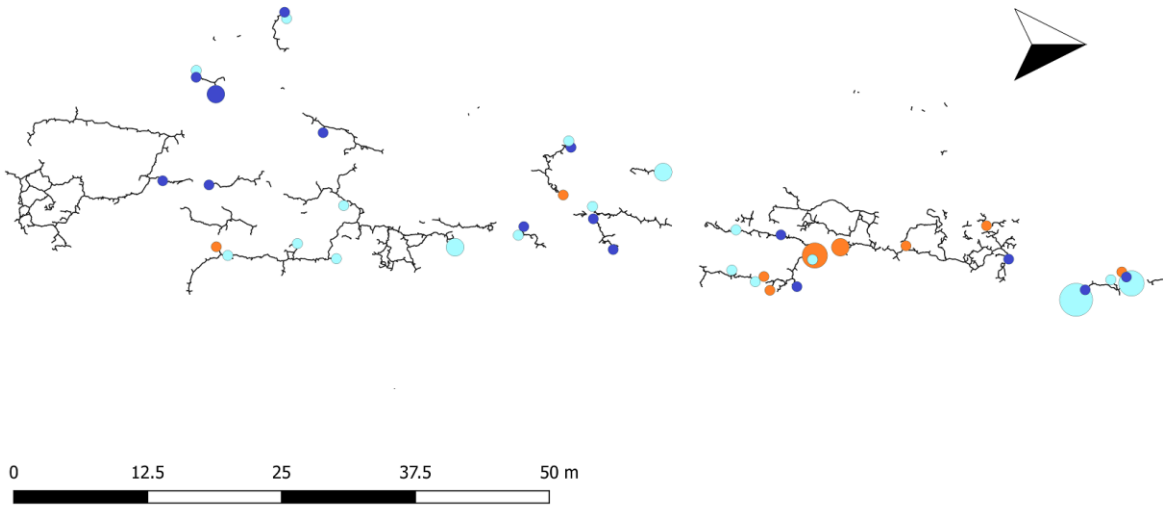

Figure 3.18. Map with the location of the trapped voles according to the genetic clustering obtained with STRUCTURE. Each color corresponds to the genetic cluster to which each individual animal was allocated. Circle size gradient represents the number of animals captured per trap, ranging from 1 to 4 (also see Figure 3.16.).

3.3. Caldas da Rainha *M. lusitanicus* colony (2003)

3.3.1. Genetic diversity

The microsatellite loci showed a high variability with a mean of 7.3 alleles per locus (higher than the colony from Tapada do Mouco). However, like in the first analyzed colony, there where loci than exhibited a low variability (MM1 and MAR016), contrasting with some of the highest variability loci (MAR063 and MAR080). The overall average Observed and Expected Heterozygosity was 0.57±0.199 and 0.63±0.183, respectively. Five loci were found to be in HWE (Hardy-Weinberg equilibrium, *p*-value<0.05) and tests for heterozygosity deficit were found to be statistically significant for 4 loci (Table 3.10.).

Table 3.10. Variation observed in 12 microsatellite loci among *M. lusitanicus* samples from Caldas da Rainha, Portugal.

loci	k	N	Hets	Homs	H(O)	H(E)	pWH	Hetdeficit
MM1	3	172	15	71	0.174	0.408	0	0
MSCRB5	7	174	41	46	0.471	0.381	0.005	0.9943
MAG006	8	176	48	40	0.545	0.73	0	0.0009
MAR076	5	176	40	48	0.455	0.497	0.048	0.3006
MAG025	8	176	69	19	0.784	0.832	0.247	0.138
MAR063	11	176	68	20	0.773	0.781	0.131	0.067
MAR012	4	176	37	51	0.42	0.501	0.221	0.1021
MAR016	3	176	33	55	0.375	0.366	1	0.6061
MAR080	13	176	67	21	0.761	0.876	0.051	0.0401
MSCRB7	5	176	42	46	0.477	0.668	0	0.0153

MM2	10	176	64	24	0.727	0.825	0	0.0005
MAR003	11	176	75	13	0.852	0.814	0.011	0.4999

k number of alleles, N number of individuals typed at each locus, Hets observed number of heterozygotes, Homs observed number of homozygotes, H(O) observed heterozygosity, H(E) expected heterozygosity, pHWE *p*-value of deviation from Hardy-Weinberg equilibrium (Guo and Thompson 1992) and $Het_{deficit}$ heterozygosity deficiency.

3.3.2. Pedigree analysis

A total of 88 Lusitanian pine voles were genotyped following the same laboratory methodologies used with the samples from Tapada do Mouco, as well as the statistical treatment of the results. The inbreeding coefficient (*F*) calculated for the Caldas da Rainha colony was very low according to Ritland and LynchRd estimators (Ritland=0.05465±0.02411, LynchRd=0.0622±0.02589). Again, the correlation between both tests was evaluated to assess if both *F* values in the two estimators were correlated, which was confirmed by a correlation of 0.92.

ML-RELATE produced the relatedness coefficients of all possible dyads in this dataset (3828 dyads). Due to scoring difficulties of two microsatellite loci, MM1 and MSCRB5 were exclude from the subsequent software run. Most of the 3165 relationships were scored as U (Unrelated, 82.68%), 521 as HS (Half-Sibling, 13.61%), 86 as FS (Full-Sibling, 2.25%) and 56 as PO (Parent-Offspring, 1.46%). Taking into consideration only $r>0$, the mean coefficient of relatedness was 0.1522±0.128. It was possible to infer two large family trees and a small one, including a total of 41 in 88 sampled individuals.

Three family groups were detected among the dataset. The first family was composed by a total of 16 individuals across 6 generations (Figure 3.19.). The oldest sampled generation was composed only by IM16 sharing a parent-offspring relationship with 3 individuals: females IF12 (r=0.49) and IF21 (r=0.52) and male PM4 (r=0.5). These three individuals shared between them a full-sibling relationship represented by their mean *r* value of 0.48. All three siblings reproduced and originated descendants, some of them also captured during the 2002-2003 study.

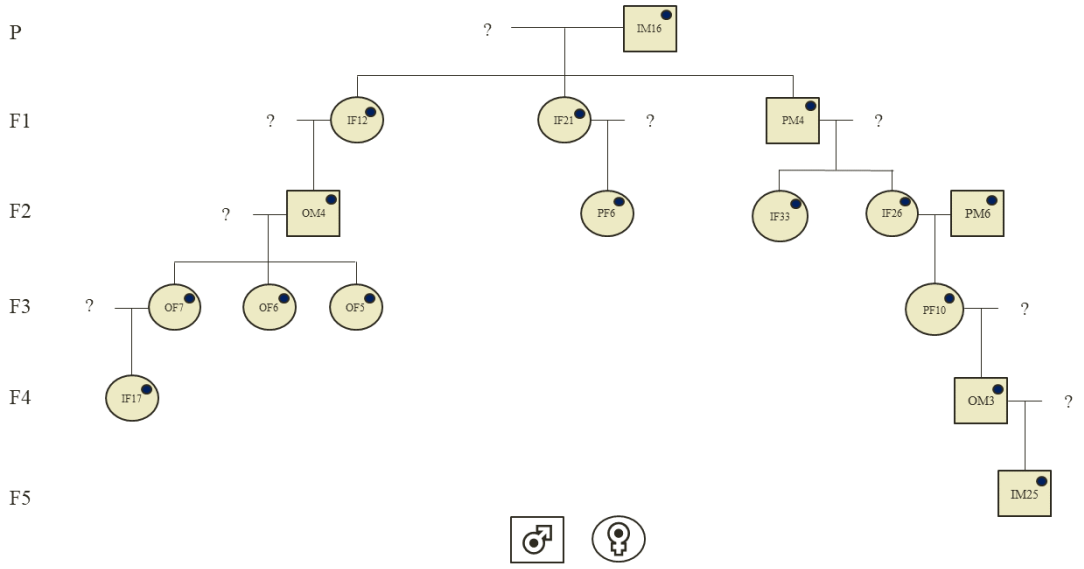

Figure 3.19. Family tree with 16 identified individuals of *M. lusitanicus*. Squares represent males and circles females. It was possible to identify 6 different generations. Colored dots have correspondence with the results obtained in STRUCTURE (see Figure 3.22.).

The second family group was composed by 18 identified individuals from 4 generations (Figure 3.20.). The individual IM8 shared a parent-offspring relationship with females IF8 (r=0.55), IF34 (r=0.5) and

male IM18 (r=0.5). These individuals shared the same mother and father (inferred by a FS relationship between them) since their mean r value is 0.59. IM18 had at least two female offspring, PF2 and PF3 (r=0.54 and 0.5 respectively), the two having a full-sibling relationship (r=0.51). Female IF8 had at least three offspring, two with the male IM5 (two females IF28 and IF29; r=0.5 with IF8 and IM5, $r_{IF29\text{-}IM5}$=0.65) and one offspring from male PM3 (PF4; r=0.5 with both parents). This was the only case of a female breeding with more than one mate, as inferred by the software. The mean r value between the two litters was 0.06, suggesting a half-sibling relationship.

The third identified family tree was considerably smaller than the previous, being composed by only 7 identified individuals over 4 generations (Figure 3.21.).

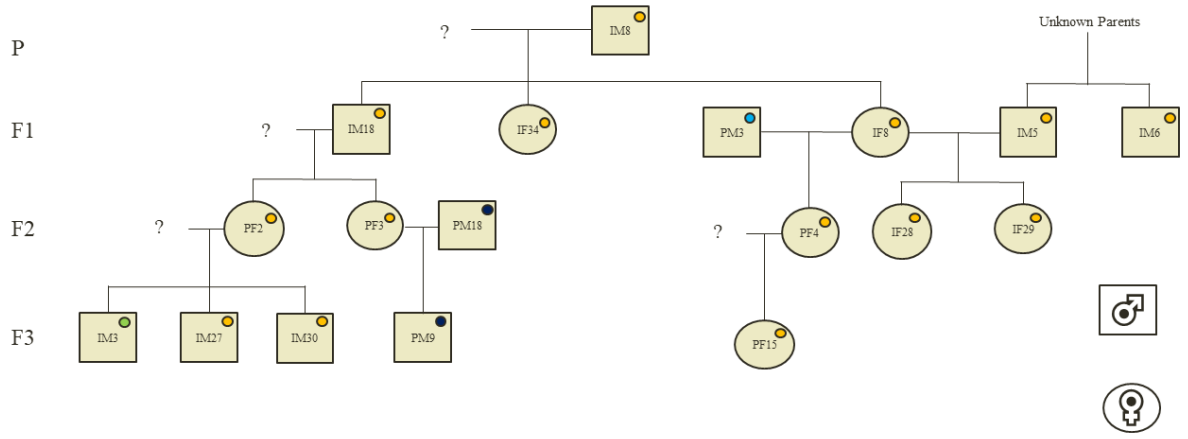

Figure 3.20. Family tree with 18 identified individuals of *M. lusitanicus*. Squares represent males and circles females. It was possible to identify 4 different generations. Colored dots have correspondence with the results obtained in STRUCTURE (see Figure 3.22.).

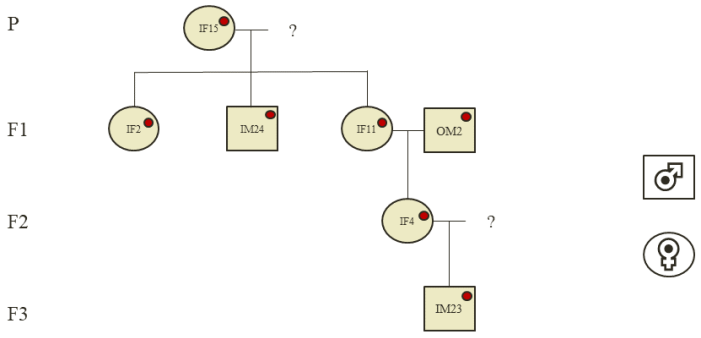

Figure 3.21. Family tree with 7 identified individuals of *M. lusitanicus*. Squares represent males and circles females. It was possible to identify 4 different generations. Colored dots have correspondence with the results obtained in STRUCTURE (see Figure 3.22.).

Again, to validate the r values obtained in ML-RELATE (Kalinowski estimator) and consequent pedigree classification the relatedness was calculated for each dyad, using the same six estimators used in the Tapada do Mouco analysis (Queller & Goodnight (1989), Li et al. (1993), Ritland (1996), Lynch & Ritland (1999), Wang (2002) and Wang (2007)) (Table 3.11.). A positive correlation between all estimators was found, the large majority above 0.7, indicating a high level of correlation between the r values obtained with each estimator (Table 3.12.). All estimators showed a high positive correlation

with the Kalinowski estimator, particularly with TrioML (0.97). These results suggest that the *r* coefficient of each dyad obtained with all estimators supports the *r* values obtained in ML-RELATE. The Ritland estimator showed the lowest set of correlation values, indicating a lower quality on the performance of the *r* values.

Table 3.11. Mean and variance of seven relatedness estimators.

	Kalinowski	TrioML	Wang	LynchLi	LynchRd	Ritland	QuellerGt
Mean	0.0701	0.06051	-0.04556	-0.0402	-0.01149	-0.0121	-0.01132
Variance	0.01418	0.01082	0.04368	0.04972	0.0197	0.02016	0.04269

Kalinowski (Kalinowski et al. 2006), TrioML (Wang 2007), Wang (Wang 2002), LynchLi ((Li et al. 1993), LynchRd (Lynch and Ritland 1999) and QuellerGt (Queller and Goodnight 1989).

Table 3.12. Correlation matrix between seven relatedness estimators in the Caldas da Rainha dataset.

	Kalinowski	TrioML	Wang	LynchLi	LynchRd	Ritland	QuellerGt
Kalinowski	1						
TrioML	0.9697938	1					
Wang	0.7433926	0.7205173	1				
LynchLi	0.7134208	0.6983265	0.9426088	1			
LynchRd	0.8370426	0.8041281	0.7946636	0.7699217	1		
Ritland	0.7103106	0.6929425	0.6656235	0.7190715	0.8381757	1	
QuellerGt	0.7154539	0.7138112	0.8445336	0.9210479	0.78958	0.7739812	1

Kalinowski (Kalinowski et al. 2006), TrioML (Wang 2007), Wang (Wang 2002), LynchLi ((Li et al. 1993), LynchRd (Lynch and Ritland 1999) and QuellerGt (Queller and Goodnight 1989).

3.3.3. Sub-populational structure

According to the results obtained in STRUCTURE, K=5 represented the best number of genetic clusters fitting the dataset's structure (Figure 3.22.). Predominantly dark blue bars represent animals from the first family, orange bars represent the second family and burgundy bars represent the third family, while the remaining colors (light blue and green) represent sampled individuals not related to each other.

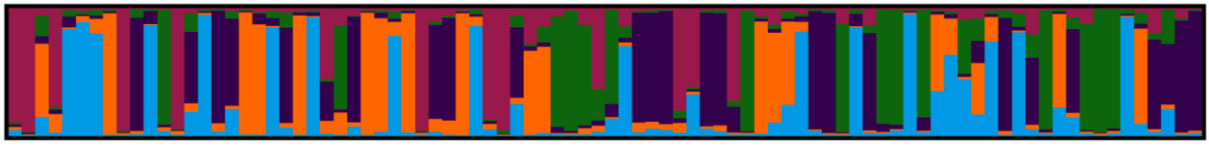

Figure 3.22. Population structure based on 10 microsatellite loci genotypes of 88 *M. lusitanicus* specimens from Caldas da Rainha. Each individual is represented by a vertical coloured bar, each colour representing an estimated proportion of membership of the genome originating from each of K inferred clusters.

4. Discussion

Most studies on subterranean rodents have focused on mole-rats (Bathyergidae), in particular, on the naked mole-rat, concentrating mostly on their ecology, sensory biology, morphological and physiological adaptations to the subterranean life-style (Begall et al. 2007; Šumbera et al. 2011; Šklíba et al. 2012). Studies on their social behavior and mating systems are increasingly more common as molecular markers become available for non-model species and parentage analysis more affordable (Reeve et al. 1990; Zenuto et al. 1999; Burland et al. 2002; Patzenhauerová et al. 2009). Regarding the architecture of burrow systems, the number of available studies is even smaller, with only a few species other than mole rats having their burrows mapped (Begall and Gallardo 2000; Brügger et al. 2010; Patzenhauerová et al. 2013; Rekouti 2018).

The main goal of this study was to better understand the social structure of a *M. lusitanicus'* colony in the context of the species underground burrowing system in a semi-natural habitat. The work previously done was mostly carried out in highly disturbed agricultural systems (mostly fruit tree orchards), where the architecture of subterranean burrows is highly influenced by the above ground context.

4.1. Architecture of the burrow system

The excavation and mapping of subterranean mammals' burrow systems can be a challenging task given their depth and usually considerable extension, especially in areas with hard soil containing stones or tree roots (Šumbera et al. 2012). Although the study area had a soft soil and not many obstacles, such as rocks or trees, it was difficult to excavate the entire burrow system not only for its large extension (more than 100 m long) but also due to unfavorable weather conditions and time constraints during the work. The pasture field, where the main study colony was located, is under the management of 'Parques de Sintra – Monte da Lua', and an unscheduled intervention with heavy machinery to mow the field, prevented the conclusion of the excavations. As such, the map of *M. lusitanicus* burrow system at Tapada do Mouco is a partial representation of the entire underground system.

The burrow system of the Lusitanian pine vole population analyzed in this study presented a similar pattern to the burrow systems previously described for this species (Godinho 1982) (Sezinando 1982) but was considerably larger and displayed greater complexity. As previously documented, the burrow system was composed by two types of tunnels: superficial tunnels - up to 15 cm in depth, mostly used to come to the surface in search for food and as escape routes from predators; and deep tunnels - up to 40 cm in depth, where food storage chambers and nests are located (Godinho 1982; Mira and Mathias 2007).

The burrow system had a linear structure with a North-South orientation, the two extremes being connected by superficial tunnels but also by surface tunnels in the vegetation. By analysing the map, several different areas can be highlighted: (1) the North and South ends showed more reticulated areas, with a high density of tunnels; (2) the central zone exhibited the opposite pattern, showing less reticulation and a lower number of tunnels; (3) there was a positive gradient in terms of tunnel depth from North to South, with the latter zone presenting the deepest tunnels of the mapped system; (4) the zone near the tree (see Figure 3.11.) showed almost the same complexity of tunnels as the North and South zones, although the depth of the tunnels was more similar to that found on the south end of the system.

M. lusitanicus' burrow system had a considerably high number of entrances/exits (N=334) compared to what is described for other *Microtus* voles, such as *M. ochrogaster* (Davis and Kalisz 1992; Mankin and Getz 1994) and *M. gregalis* (Pal'chekh et al. 2003)*;* both these species exhibit considerably less entrances in their systems, from 2 to 22 holes in the former, up to almost 160 in the latter. Additionally, *M. lusitanicus*' burrow system was also considerably longer (c. 100 m long, 606.35 m of total tunnel length) than what was described for this species (not longer than 70 m, Godinho 1982; Mira and Mathias 2007). According to the literature, the number of animals within a burrow system is a decisive factor in its length and complexity (Le Comber et al. 2002; Šumbera et al. 2003). As such, the system's large dimensions could mainly be due to the colony's unusually large size. And although a colony composed by 56 individuals can already be considered large for this species (Godinho 1982), considering the difficulty in capturing fossorial rodents, it is very likely that this is an underestimation of the colony's actual size.

Surprisingly, considering the system's dimensions and the number of individuals captured, only one food chamber and no nests were found throughout the whole excavated area. This low number of food chambers can be possibly explained by the landscape and environmental conditions of the study site.

The habitat surrounding the colony at Tapada do Mouco is mostly composed by pine trees, contrasting with that of the colony's micro-habitat, an open pasture field. Other than being mowed and sown once a year, the pasture field is free from other human intervention. The vegetation cover, present almost all year round, with plants reaching up to 2 m in height during spring, may provide *M. lusitanicus* with an almost constant food supply. Such high food supply may decrease the need to storage food in underground galleries, similarly to what is described for coruro colonies (*Spalacopus cyanus*) living in more densely vegetated areas (Begall and Gallardo 2000). Moreover, this low food chamber count is also consistent with what is described for other fossorial species, such as *M. ochrogaster* and *M. arvalis* (Davis and Kalisz 1992; Brügger et al. 2010).

According to the literature, nest chambers are usually found in the deepest parts of underground systems (Mackin-Rogalska et al. 1986). Nests are usually connected to several tunnels for easier access to voles, leading to a higher complexity of the tunnel network in the nests' proximity. Although some of the exposed zones had such characteristics, no nests were found throughout the entire excavated area. Most of the exposed burrow system was very shallow, thus suggesting that existing nests were not exposed during the field work.

Most studies of burrow architecture usually rely on low-tech methods, such as measuring tape or strings of known length, as measuring tools. Although being low cost tools, they are highly time consuming and have a huge associated error. The DGPS system used can thus be extremely advantageous, not only because it substantially reduces mapping time but also because of the significant gain in accuracy. The DGPS has an associated error of c.10 cm. This is particularly important when mapping the burrow system of a small species such as *M. lusitanicus* that only excavates comparatively small and shallow tunnels of up to 40 cm in depth. Within such a small range of values, the used geolocation device should have the lowest possible error. As such, even though the DGPS has a precision of c. 10 cm, a perfectly admissible error in the *x* and *y* axis, the depth values obtained may not be fully representative of *M. lusitanicus*' underground systems.

The obtained depth values (after being corrected in QGIS software) for *M. lusitianicus*' burrow system were, in general, very similar to those previously described for this species, however, as mentioned above, the values obtained should be treated with caution. Even though it was only possible to excavate a single burrow system, the results obtained were compared with those of other species, namely other subterranean African mole-rats (Le Comber et al. 2002; Šumbera et al. 2012) and with the fossorial Thomas's pine vole, *M. thomasi* (Rekouti 2018) (Table 4.13.).

Table 4.13. Fractal dimension of underground systems from different subterranean rodents.

Species	Sociality	Fractal dimension	Location	Reference
Heterocephalus glaber	Eusocial	1.47	Mtito Andei, Kenya	Brett 1991
Cryptomys hottentotus	Social	1.40-1.62	Darling, South Africa	Spinks 1998
Heterocephalus glaber	Eusocial	1.34	Lerata, Kenya	Jarvis 1985
Fukomys darlingi	Social	1.33	Goromonzi, Zimbabwe	Le Comber et al. 2002
Cryptomys hottentotus	Social	1.27-1.42	Steinkopf, South Africa	Spinks 1998
Microtus lusitanicus	Social	1.21-144	Sintra, Portugal	this study
Heliophobius argenteocinereus	Solitary	1.26-1.33	Athi Plains, Kenya	Jarvis and Sale 1971
Cryptomys hottentotus	Social	1.23-1.49	Sir Lowry's Pass, South Africa	Spinks 1998

Fukomys mechowii	Social	1.23	Chingola, Zambia	Le Comber et al. 2002
Bathyergus suillus	Solitary	1.22-1.45	Pella, South Africa	Davies and Jarvis 1986
Georychus capensis	Solitary	1.20-1.41	Darling, South Africa	Le Comber et al. 2002
Microtus thomasi	Social	1.18-1.48	Greece	Rekouti 2018

Adaptation from Le Comber (2002) with additional information for *M. thomasi* (Rekouti 2018) and *M. lusitanicus* (this study).

Under the scope of this study, fractal dimension is a measure of to what extent the burrow occupies the surrounding area, with higher values indicating a more efficient exploration of the area (Thomas et al. 2009). According to Le Comber and colleagues (2002), the number of individuals inhabiting the system is positively correlated with the complexity of the burrow system; the higher the number of individuals, the higher the chances of exploring the space more thoroughly and thus have a higher fractal dimension value. The same pattern has been described for *M. ochrogaster*, with burrow systems built by a communal group being more complex than those built by a female-male pair (Mankin and Getz 1994).

Additionally, if only social species are considered, burrows in mesic habitats (all but *C. hottentotus* and *H. glaber* in Table 4.13.) should have a higher fractal dimension than burrows in arid habitats. Although the F_D value of *M. lusitanicus* burrow system in Sintra fits within the range of calculated values for other rodent species, given that *M. lusitanicus* is a social species, living in an agricultural mesic habitat, a higher fractal dimension might have been expected. Several factors can possibly have contributed to this, namely: (1) the impossibility to excavate and map the entire burrow system, the F_D obtained only referring to part of the underground system and thus, not fully reflecting its complexity; (2) the heterogeneity of the map, with some areas having lower F_D values (1.2184) and others having higher values (1.4401); (3) loss of some underground connections due to the collapse of the structure as it was walked on; even if these were only a few small tunnels, when calculating the fractal dimension this represents a loss of relevant information, possibly resulting in a lower F_D value for the whole system.

According to Cameron and colleagues (1988), the Index of convolution (14.3714, see Table 3.4.) is an estimator of the complexity of the burrow system, were low values relate to less convoluted areas. Comparing the results obtained in this study with those in the literature (6.0-23.4, Šklíba et al. 2012), the Index of convolution in this study was moderate. However, considering that this index is influenced by the system's heterogeneity, the value obtained might be an underestimation caused by the impossibility to map the burrow in its totality. The lacunarity (λ) of the burrow system was also calculated. Although this coefficient is not frequently used, it gained some relevance in this study given the high level of heterogeneity observed. Lacunarity is used to explain how much information is missing during image analysis, ranging from 0 - when no additional information is added, and 2 - when large amounts of information are added. The value obtained for the entire map was 0.74, which was not surprising considering that only part of the system was excavated. Moreover, the indexes of linearity and circularity (1.5193 and 0.4332 respectively, see Table 3.4.) indicate an underground system more linear and less circular.

According to Romañach and Le Comber (2004), the burrows with higher F_D were the longest and more circular. The values obtained in this work suggest that, even though most of the tunnels were exposed, revealing a complex underground tunnel system, there were areas that needed to be further explored, which most probably resulted in a lack of information in some sections of the system. Nevertheless, the values of fractal dimension obtained for the Lusitanian pine vole colony indicate that its burrow system is similarly complex and heterogenous to those built by mole-rats, also exhibiting areas with different levels of complexity.

Regarding the tunnel depth values obtained in this study, these are also consistent with those described for other fossorial *Microtus* species. Studies on *M. arvalis*, in Switzerland, and *M. montanus*, in Idaho USA, recorded mean tunnel depths of 12.6 cm (min-max: 2-44 cm) and 23 cm (min-max: 22-55 cm), respectively (Reynolds and Wakkinen 1987; Brügger et al. 2010).

Finally, it cannot be dismissed that this is certainly a dynamic system, a representation of a *M. lusitanicus* underground system in one point in time, possibly changing according to numerous biotic and abiotic factors. Nevertheless, based on the results of this study, it was possible to confirm that this *M. lusitanicus* burrow system shares multiple characteristics with those of other fossorial and subterranean species occurring in completely different geographical settings and influenced by distinct climatic conditions.

4.2. Pedigree status and colony genetic structure

As previously reported for *M. lusitanicus* (Ventura et al. 2010), no sexual dimorphism was found, males and females displaying similar weight ranges. Both colonies, Tapada do Mouco and Caldas da Rainha, displayed a 1:1 sex-ratio. According to some authors (Heske and Ostfeld 1990; Ostfeld and Heske 1993), the degree of sexual dimorphism is sufficient to determine the mating system in arvicoline species. As such, the results here obtained as well as in previous work (Ventura et al. 2010), seem to indicate *M. lusitanicus* has a monogamous mating system. However, as pointed by Ventura and colleagues (2010), lack of information regarding the population's social behavior or the animals' relatedness (determined through a parentage analysis), did not allow to safely predict if the population studied had a facultative or obligate monogamy. Or for instance, if extra-pair mating occurred in this population - impossible to ascertain without genetic analysis of the individuals. Furthermore, although a monogamous mating system is common among *Microtus* species, it is also known to vary between populations (Waterman 2007). For instance, *M. pinetorum* has a monogamous mating system but cooperative polyandry also seems to occur in some circumstances (FitzGerald and Madison 1983).

The analysis of both datasets (Tapada do Mouco and Caldas da Rainha), supported a monogamous mating system in *M. lusitanicus*. Among the two studied locations, two family groups were found in Tapada do Mouco (Figure 3.14. and Figure 3.15.) and three in Caldas da Rainha (Figure 3.19., Figure 3.20. and Figure 3.21.). In total, four of the five family groups were composed by a similar number of individuals and had a balanced sex-ratio. Reproduction between siblings was not detected in any of the inferred families, suggesting that *M. lusitanicus* avoids breeding with kin. This is further supported by the fact that breeding pairs within families were always constituted by two unrelated individuals. In all families, it was possible to identify plural breeding, with several females within a family group breeding simultaneously. Moreover, in Tapada do Mouco (see Figure 3.14.), it was possible to identify a breeding pair (J1ML20 e J1ML21) with at least two litters. One litter with 3 siblings born in the lab, and 4 older full siblings detected in the main colony, originating from, at least, one other litter. Considering that *M. lusitanicus* litter size varies from 1 to 5 pups, most commonly 2 or 3 (Mira and Mathias 2007),this implies that the same pair mated and generated at least two different offspring generations, an argument in favor of a monogamous mating system.

Among all the inferred family groups in both studied colonies, only one polygamous mating was detected. In Caldas da Rainha colony, a female mated with two different males, producing progeny with both. However, the sampling circumstances under which the individuals from this colony were obtained could have had impact in this result. Lusitanian pine voles were trapped during three consecutive seasons: Autumn, Winter and Spring of the following year (2002-2003). The female and the two males in question were captured (and removed from the population) in different seasons, which could mean that the female had mated with the second male after the removal of her first partner from the colony. Yet, at the time of capture, the female had already had one litter from each male. Thus, the extra-pair

mating detected in our analysis cannot be due to the absence of one of the males in the colony. A more plausible hypothesis is related to the intrinsic characteristics of the habitat, an orchard with a high level of anthropogenic disturbance. As mentioned above, monogamy is a common mating system within the *Microtus* genus but is not the only one (Waterman 2007). In fact, it is suggested that when forming large family groups, there is a higher chance of deviation from the common monogamous mating system (Marfori et al. 1997). Under agricultural settings, *M. lusitanicus* can become pests, reaching high population densities (Vinhas 1993; Ventura et al. 2010). As such, even though an event of punctual polygamy was detected, results suggest that, in general, and under a more natural setting, *M. lusitanicus* has a monogamous mating system.

In Caldas da Rainha, individuals of the three families were unrelated even though they shared the same burrow system. In Tapada do Mouco, the same situation occurred, no genetic relation being found between the members of the two families, further supporting the idea that several family groups can be found within a single burrow system (Godinho 1982; Mira and Mathias 2007; Duarte et al. 2015). Although the results obtained in this work seem to support this hypothesis, alternative hypotheses should be considered: (1) family members that could link families were not captured and thus, were not included in the analysis leading to two different family groups; (2) older generations connecting different families were lost considering the animals' lifespan. Monitoring and sampling *M. lusitanicus*' colonies over time should help clarifying this. Large colonies like those analyzed in this study represent a bigger challenge in terms of trapping/sampling effort as, in order to produce the most accurate picture, virtually all animals from the colony should be captured.

Surprisingly, a migration event was detected in Tapada do Mouco, that despite involving a short-distance, would have been difficult to detect otherwise (Figure 3.15). A female and male of a breeding pair were captured in two distinct locations c. 200 m apart (J1 and J2, respectively; see Figure 2.5.). As inferred by the family tree, the male originated from a family line of individuals exclusively found in J2, while his descendancy of three generations was captured in J1. This could be interpreted as an event of opportunistic mating, considering that the parental male did not remain in the same colony of the parental female (or vice-versa), but instead returned to its original colony. However, when overlapping the results obtained with STRUCTURE with those obtained from the relatedness analysis, it is possible to infer that both members of the breeding pair (J2ML2 and J1ML48) belong to the same genetic cluster (see Figure 3.16.), the cluster to which all J2 individuals belong. To corroborate this, both their offspring, captured in J1 (J1ML31 and J1ML50), also belong to the same genetic cluster of their parents, characteristic of the J2 colony. As such, it is also possible that the parental female was originally from J2 but migrated to J1 while pregnant. Although the distance in question is not substantial (~200 m in a straight line), it involves covering this distance at the surface, including the crossing of a small road (see Figure 2.5.). Regardless these potential explanations, this result should be interpreted with caution as there was a time lapse of 2 months between trapping sessions in J1 and J2. Within these two months, the excavation of the burrow system took place, as well as the intervention on the pasture field (J1) from PSML, highly disturbing the colony.

4.3. Limitations

As already mentioned, unfavorable weather conditions and an unplanned intervention in the field site with heavy machinery were two main factors limiting the complete excavation of the entire burrow system. The Autumn/Winter of 2017 was atypically dry, highly influencing the lack of presence signs of *M. lusitanicus*. Heavy rain followed, flooding the selected field site for several weeks, preventing any field work. At the end of May, the PSML management made an intervention over the study area, limiting the time for excavation and mapping of the entire burrow system.

The DGPS device is easy to handle and can produce very reliable results in a 2D perspective (*x* and *y* axis) having a very low associated error (c. 10 cm). However, to obtain a 3D perspective (adding the depth, *z* axis), the associated error can be problematic when considering small depth values as those encountered in this study (up to 20%). Although the depth values obtained are in tune with the literature, this level of error should be considered in future work involving underground systems.

Due to time constraints, it was not possible to analyze other *M. lusitanicus* burrow systems (as replicates) in order to assess the similarities and differences of burrow architecture in colonies inhabiting natural areas with those presenting different levels of disturbance (semi-disturbed such as Tapada do Mouco or highly disturbed such as Caldas da Rainha).

Regarding the microsatellite analysis some limitations can also be highlighted. Inference of relatedness between individuals is always limited by the number of amplified loci, their level of polymorphism and the quality of the obtained genotypes. Although a total of 12 loci were genotyped, the final datasets only included data from 10 loci due to scoring difficulties of 2 of them. The presence of null alleles, despite not controllable, can also negatively influence the results and the confidence in the relatedness coefficient values between pairs of individuals.

Nonetheless, despite all limitations, the information gathered in this work on *M. lusitanicus* is especially relevant considering that not much was known on this species' burrow architecture, mating system or social organization.

4.4. Thesis framework in the Conservation Biology MSc

Microtus lusitanicus is a fossorial rodent commonly found in the North and centre of the Iberian Peninsula and the South of France. It has a "Least Concern" status (according to the IUCN and the Portuguese Red Book of Vertebrates), having no specific conservation requirements. Despite its abundance, its fossorial habits have made it a poorly studied species. Most recent work has focused on its ecology but not much is known about its behavior or social organization in natural conditions. Although no threats are currently known, this species' small distribution and specialized fossorial habits might make it more susceptible to global change. A greater investment in the study of this species is therefore advisable.

The techniques used in his thesis, namely microsatellite genotyping for pedigree reconstruction and DGPS mapping, can be used in many different contexts and species worldwide, independently of their conservation status. Microsatellite analysis is known as an important tool in the conservation of endangered species helping in the identification and profiling of individuals and aiding in the decision of which individuals to use in captive breeding and/or reintroduction programmes. Also, for wild populations, it can be a valuable tool for the assessment of the social structure of a colony/population/family group, and the assessment of how broad or limited in space conservation measures should be taken.

Regarding the DGPS mapping, this technique has revealed itself as a substantial upgrade compared with the current techniques used to map burrow systems, being easily applied to study the burrow architecture of fossorial and subterranean species. Its precision (c. 10 cm), far greater than that of a regular GPS (c. 15 m), allows the user to obtain more precise data, not only in a 2-dimension system but also to gather 3-dimensional information.

4.5. Final remarks

- Mapping the underground architecture of *M. lusitanicus* burrow system with the Differential Global Positioning System (DGPS) enabled the production of an accurate 2D map. Since the common

GPS has a precision of ± 15 m and the DGPS has an associated error of ± 10 cm, the results obtained allowed a more realistic representation of the burrow map.

- The mapped burrow system exhibited a linear north-south structure with a total excavated length of 606.35 m. Thirty-four main tunnels, 332 entrances/exits and 1 food chamber were also identified, but no nests were found in the overall excavated area. Mean tunnel depth was 11.16±9.47 cm reaching a maximum of 52.8 cm.
- The index of linearity obtained was 1.5193 and the circularity was 0.4332, indicative of a linear architecture. Linear systems tend to have lower depths, which was verified in this study. The corrected index of convolution was 14.3714, indicative of a moderate convoluted system. The fractal dimension (F_D) of the final map was 1.2678 which correlates to the obtained index of convolution, further indicating a system of moderate complexity. The lacunarity (λ) was 0.7414, indicative of a heterogenous map, with complexity varying throughout the system. However, these values could differ if the whole underground tunnel system had been mapped.
- The analysis of relatedness between the 56 individuals from Tapada do Mouco's colony resulted in the inference of two family trees (Figure 3.14. and Figure 3.15.). In both cases, no mating between siblings or close relatives was detected. Multiple litters, at least two (of a single breeding pair) were detected, which can be indicative of serial monogamy. Short distance (~200 m) migration was also spotted between both trapping sites.
- In Caldas da Rainha, three family groups were inferred (Figure 3.19., Figure 3.20. and Figure 3.21.). Mating between siblings or close relatives was also not detected. However, there was no support for serial monogamy in this colony, since it was not possible to identify more than one litter per pair (at most, three siblings were detected per mating pair, that can either originate from one or multiple litters). A single extra-pair breeding event was detected, a female mating with two males.
- The overall genetic results obtained in this study support that *M. lusitanicus* adopts a monogamous mating system, although occasional extra-pair mating can occur. Further studies, involving the follow up of colonies over time would certainly provide a better understanding of this species' mating system and of how it may vary between populations.

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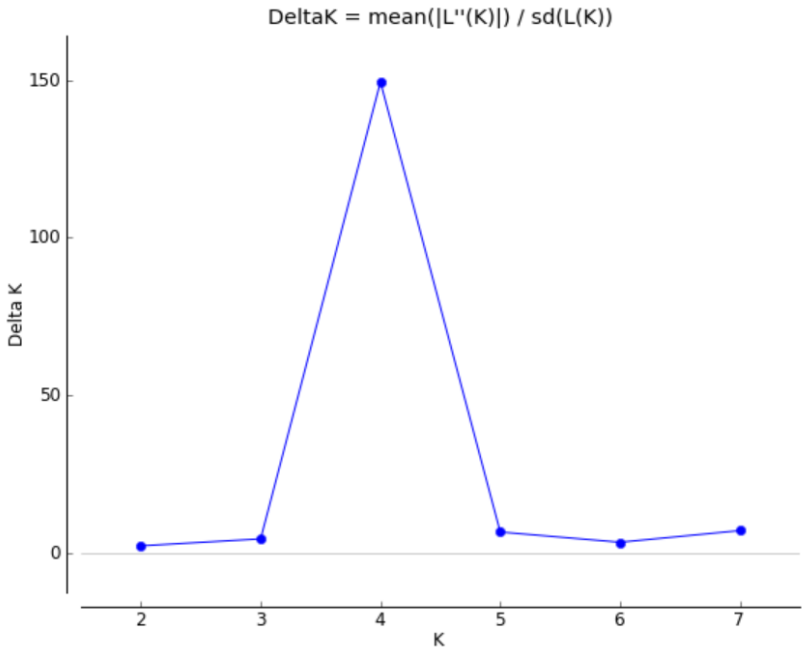
Annex 1

ID_1	ID_2	*r*	Kinship
J1ML2	J1ML21	0.5	PO
J1ML2	J1ML55	0.5	PO
J1ML2	J1ML4	0.4509	PO
J1ML2	J1ML23	0.3868	HS
J1ML2	J1ML54	0.3255	HS
J1ML2	J1ML20	0.0386	U
J1ML20	J1ML23	0.5704	PO
J1ML20	J1ML4	0	U
J1ML20	J1ML21	0	U
J1ML21	J1ML55	0.5	PO
J1ML21	J1ML23	0.4	PO
J1ML21	J1ML4	0	U
J1MLL55	J1ML4	0	U
J1ML56	J1ML4	0	U
J1ML23	J1ML55	0.5	PO
J1ML23	J1ML4	0.0157	U
J1ML4	J1ML55	0	U
J1ML55	J1ML56	0.7006	FS
J1ML55	J1ML20	0.5	PO
J1ML56	J1ML21	0.5	PO
J1ML56	J1ML23	0.7491	FS

S1. Extracted table from ML-RELATED results. ID 1 first individual, ID 2, second individuals, *r* relatedness coefficient and Kinship type of relationship. FS Full-Sibling, PO Parent-Offspring, HS Half-Sibling, and U Unrelated.



S2. Evanno method presented in graphic. As the graph shows, there is a peak at K=4, which means there is enough differences to admit that this population has a genetic structure of 4 subpopulations.