Trophic niches of rodent communities in agroecosystems of Northeastern Portugal

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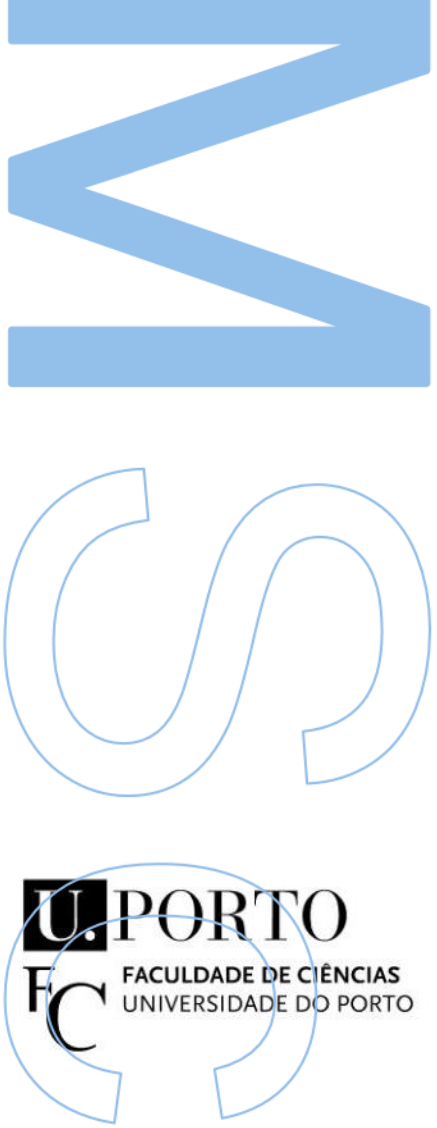

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Luísa Rodrigues

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Resumo

O Nordeste de Portugal é uma região com elevada biodiversidade e possui comunidades importantes de roedores. Os roedores desempenham papéis ecológicos essenciais em agroecossistemas. No entanto, algumas espécies alimentam-se de espécies cultivadas e são percecionadas como pestes. Os olivais, assim como a matriz de terrenos adjacente, proporcionam um leque de habitats para as comunidades de roedores, sustendo até populações de espécies vulneráveis (VU), como o rato de Cabrera (*Microtus cabrerae)* e o rato de água (*Arvicola sapidus)*. Assim, o estudo destas comunidades representa uma oportunidade de conservação. A análise das suas dietas fornece informações novas sobre a ecologia dos roedores; sendo o objetivo principal desta tese a estimativa de nichos tróficos em olivais tradicionais, com implicações na gestão dos mesmos (espécies peste) assim como na conservação de espécies vulneráveis. Foram analisadas as dietas de cinco espécies de roedor *Apodemus sylvaticus, Arvicola sapidus, Microtus cabrerae, Microtus lusitanicus* and *Mus spretus* com amostragem genética não-invasiva (gNIS) e um protocolo de metabarcoding com uso de Sequenciação de Nova Geração (NGS). Os resultados revelaram sobreposição na amplitude e composição de nichos entre as espécies, assim como informações detalhadas sobre a dieta de todas as espécies, ao nível do género de plantas. A maior sobreposição de nichos observada foi de 0.81, entre *A. sapidus* e *M. cabrerae,* relativamente às famílias de plantas consumidas; e de 0.68 entre *M. cabrerae* e *M. lusitanicus,* para os géneros de plantas consumidos. Algum grau de partição de nichos foi detetado, possivelmente para evitar competição nos olivais de Trás-os-Montes. Concluímos que a manutenção de comunidades de roedores ricas em espécies, no contexto de agroecossistemas extensivos, é favorável a *M. cabrerae* e *A.sapidus*, propondo que os olivais sejam vistos como unidades de conservação. Concluimos também que é possível minimizar efeitos de possíveis pestes através da biodiversidade, tanto de plantas como de roedores, sustida pelas práticas de agricultura tradicional.

Palavras-chave:

Pequenos mamíferos, *DNA metabarcoding*, analise de dieta, *Apodemus sylvaticus, Mus spretus, Microtus lusitanicus, Microtus cabrerae, Arvicola sapidus*

Abstract

Northeastern Portugal is highly biodiverse and holds important rodent communities Rodents play key ecological roles in agroecosystems. However, some species feed on plantations and are therefore perceived as pests. Olive groves, an important culture in northeastern Portugal, with their surrounding matrix, provide a range of habitats for rodent communities sustaining both populations of vulnerable species, like the Cabrera vole (*Microtus cabrerae)* and water vole (*Arvicola sapidus)* and potential pests (e.g. *Microtus lusitanicus*). Diet analysis provides valuable new insights into species ecology allowing better understand species interactions within communities. The main objective of this thesis was to estimate the trophic interactions between rodent species within communities in extensive olive groves for management (pest species) and conservation purposes. The diet of five rodent species *Apodemus sylvaticus, Arvicola sapidus, Microtus cabrerae, Microtus lusitanicus* and *Mus spretus* were assessed using genetic non-invasive sampling (gNIS) and a metabarcoding protocol with Next Generation Sequencing (NGS). The results revealed overlaps in both niche width and composition among species, alongside detailed information about the diets of all five species, to the plant genus-level. Highest observed overlap was 0.81, between *A. sapidus* and *M. cabrerae*, regarding plant families consumed; and 0.68, between *M. cabrerae* and *M. lusitanicus* for plant genera consumed. Some degree of niche partitioning was also detected, possibly to avoid competition in olive groves from Northeastern Portugal. We conclude that the maintenance of species-rich rodent communities, in the context of extensive agroecosystems, is suitable for *M. cabrerae* and *A.sapidus*, proposing that olive groves be viewed as conservation units. We also conclude it is possible to minimize impact of potential pests through maintenance of both plant and rodent biodiversity, supported by traditional management practices.

Keywords

Small mammals, DNA metabarcoding, diet analysis, *Apodemus sylvaticus, Mus spretus, Microtus lusitanicus, Microtus cabrerae, Arvicola sapidus*

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List of abbreviations

BOLD	Barcode of Life Data System
DNA	Deoxyribonucleic acid
glm	Generalized linear model
gNIS	Genetic non-invasive sampling
ICNF	Institute of Nature and Forest conservation
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
NCBI	National Center of Biotechnology Information
NE	Northeastern
NGS	Next generation sequencing
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational analysis of variance
ID	Identification
VU	Vulnerable

1.Introduction

1.1.Northeastern Portugal agroecosystems

The northeast of Portugal, specifically the Bragança district, in historical region of Trás-os-Montes, is influenced by both sub-Atlantic and Mediterranean climates. Atlantic humidity arrives from the west and meets cold continental winds from the east and warm dry winds from the south. This creates a continental macroclimate with Mediterranean characteristics; the hypsometry is mountainous and sub-mountainous (Comissão Nacional do Ambiente, 1982), influencing both the macroclimate and several microclimates. The region is located within the Douro River basin (Divisão do Atlas do Ambiente, 1989) and the soil type is mainly granitic, associated with schists and grewakes (Cardoso et al., 1978). The climate and soil characteristics influence the flora, where woody plants include the common oak (*Quercus robur* L.), the Portuguese oak (*Quercus faginea* Lam.), the Pyrenean oak (*Quercus pyrenaica* Willd.), the evergreen oak (*Quercus ilex* L.), the cork oak (*Quercus suber* L.), the chestnut (*Castanea sativa Mill.)* and the pine *(Pinus pinaster* Aiton*)* (do Rosário, 2004). The shrubs are composed of *Genisteae*, gorze (*Ulex L.*) and heather [*Erica* Tourn. Ex L. and *Calluna* Salisb.); perennial grasses include sweet vernal grass (*Anthoxanthum* L.) and clover (*Trifolium* Tourn. Ex L.); herbs such as mint (*Mentha* L.), foxglove (*Digitalis* Tourn. Ex L.), brooklime (*Veronica* L.), St. Benedict's herb (*Geum* L.), broomrape (*Orobanche* L. and *Galium* L.) and graminoids (Poacea) are plentiful.

An abundance of ecological niches makes the Bragança district highly biodiverse (ICNF) and has led to the creation of two protected areas: Parque Natural de Montesinho and Parque Natural do Douro Internacional. Moreover, this region has an important traditional agricultural system: family farming is extensive, and the land is mostly cultivated by small farmers. Common crops include corn, potatoes, cereals, fresh fruit orchards and vineyards. Grapevines (*Vitis* L.) are usually planted in association with almond (*Prunus dulcis* Mill.) and olive trees (*Olea europaea* L.), depending on the predominant climatic influence (Aguiar, 2001; Do Rosário, 2004). Farmers rely on a combination of well-adapted crops best suited to the land, as the region is informally divided into "cold", "warm" and "transitional" areas. They also practice agroforestry with native trees and raise local cattle breeds for meat and milk. The mixed land use and vegetation cover results in a highly heterogeneous landscape (Barão et al., 2022), which promotes high biodiversity in this Mediterranean biodiversity *hotspot* (Farina et al., 2003; Pascual et al., 2011).

Olive groves are one of the most common agroecosystems in northeast of Portugal, as olive production is historically relevant to Trás-os-Montes and characterizes the landscape. Production in the Mediterranean region, namely in southern Iberia, has become increasingly intensive in recent decades (e.g. Silveira et al., 2018), with intensive and super-intensive groves. These are extremely productive, as they contain rows of small trees in high density, which never grow larger than bush size. Average distance between trees in intensive groves is 6m, whereas super-intensive groves may be closer together, forming hedgerows. Olive trees from fast-growing varieties (eg. *Olea europaea L*. var. *Arbequina*) become productive earlier and are harvested at a younger age (Reis, 2014; Silveira et al., 2018). Chemical fertilizers and herbicides, combined with mechanized harvesting, make these groves highly efficient in producing low acid (high quality) olive oil. However, the use of herbicides almost completely eliminates other vegetation, reducing ground cover (Sánchez Martínez et al., 2015; Infante-Amate et al., 2016) and resulting in lower biodiversity across taxa (Rey et al., 2019).

Despite recent pressures, olive production in Trás-os-Montes remains largely traditional and smallholder based (Duarte et al., 2006). Small-sized groves are maintained at low densities, using traditional olive varieties that are genetically diverse. These are grown over decades to reach more mature stages, as olive trees can bear fruit for up to 500 years if properly managed. Chemical fertilizer and herbicide use is low as weeds are mechanically removed, allowing for more ground cover. Ground cover in turn promotes equilibrium, prevents soil erosion and nutrient depletion (Neves et al., 2013), maintains pollinator populations and increases plant diversity (Silveira et al., 2018). Harvesting is labour intensive, with low levels of mechanization, and is often done by hand. The yield is lower and the final acidity of the olive oil is higher, which is considered to be of lower quality. Therefore, traditional groves are not competitive in the global market, but they have a higher environmental and ecological value (Reis, 2014), maintaining high levels of biodiversity (Rey et al., 2019), thus these agrosystems can play a key role in its conservation (Barão et al., 2022).

Agroecosystems have historically been managed to increase food and fibre production, but there has been a gradual shift in perspective. It is now recognized that agricultural landscapes provide ecosystem services to society, as Garbach et al. (2014) describe how efforts have been made in recent decades to characterise, value and manage ecosystems for their potential services. These range from the provision of food, fuel and fibre, climate regulation, flood protection, disease control, waste decomposition and water quality, to maintaining nutrient cycling and primary (plant biomass) production. In

addition, agricultural landscapes provide recreational and cultural benefits for human well-being. Agroecosystems are therefore increasingly viewed as ‘multifunctional’ units, and ecosystem services have become part of the productivity equation, rather than measuring biomass productivity alone.

1.2.Rodent communities in Northeast Portugal

Northeast Portugal is known for its natural values and high biodiversity (ICNF), especially its wildlife. Raptors such as the Montagu's harrier (*Circus pygargus*), little owl (*Athene noctua*), common raven (*Corvus corax*), golden eagle (*Aquila chrysaetos*), griffon vulture (*Gyps fulvus*), western jackdaw (*Coloeus monedula*) and the scops owl (*Otus scops*) roam the area. Reptiles such as the Lataste's viper (*Vipera latastei*), the ladder snake (*Zamenis scalaris*) and the Montpellier snake (*Malpolon monspessulanus*), among other species are common. Mammals include roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), fox (*Vulpes vulpes*), genet (*Genetta genetta*), and the threatened carnivores European wildcat (*Felis silvestris*) and Iberian wolf (*Canis lupus signatus*). As well as the lagomorphs, the European rabbit (*Oryctolagus cuniculus*) and Iberian hare (*Lepus granatensis*).

The small mammal community is also diverse, with several insectivore (Eulipotyphla) species, such as the threatened Pyrenean desman (*Galemys pyrenaicus*) and the greater white-toothed shrew (*Crocidura russula*); and rodents (Rodentia) like the wood mouse (*Apodemus sylvaticus*), water vole (*Arvicola sapidus*), Cabrerae mouse (*Microtus cabrerae)*, Lusitanean pine vole (*Microtus lusitanicus*) and Algerian mouse (*Mus spretus)*(Figure 1). The latter compose rodent communities, which are described as highly diverse, and mainly inhabit agroecosystems (Barão et al., 2022), as seen on figure 1.

Figure 1: Rodent community in north-eastern Portugal: (1) Wood mouse *Apodemus sylvaticus* (Carmo Silva); (2) Water vole *Arvicola sapidus* (Ricardo Pita; (3) Cabrerae vole *Microtus cabrerae* (Soraia Barbosa); (4) Luistanean pine vole *Microtus lusitanicus* (Ana Cerveira); (5)) Algerian mouse *Mus spretus* (Guilherme Dias) [All pictures from Livro Vermelho dos Mamíferos de Portugal Continental (Mathias et al., 2023)]

Rodents represent a duality in agroecosystems. On the one hand, some species cause crop damage and are perceived as pests by farmers. In large numbers, populations become problematic and can lead to economic losses. On the other hand, they play key ecological roles, are essential to trophic webs and support populations of carnivores, birds and snakes. Some are considered keystone species in agroecosystems (Delibes-Mateos et al., 2011; Sullivan & Sullivan, 2021), meaning that loss of their functional role could lead to the loss of other species. Rodents provide services such as seed dispersal (Kunstler et al., 2007), soil aeration, organic matter turnover and mineralisation, which are essential to maintain ecosystem balance and plant productivity. They can even be described as ecosystem engineers (Dickman, 1999), as their presence alters soil composition and shapes soil matrix properties. Ultimately, they influence the surrounding environmental conditions.

Biodiversity is, therefore, a key element in ecosystem function and the provision of ecosystem services, as increased native biodiversity is associated with higher levels of ecosystem services overall (Cardinale et al., 2012; Garbach et al., 2014). In terms of conservation implications, *M. cabrerae* and *A. sapidus* are currently recognized as vulnerable (VU) of extinction in the Mammal Red Book of Continental Portugal (Mathias et al 2023) and the IUCN, respectively. Furthermore, *M. cabrerae* is protected in the European Union by the European Habitats Directive (Council Directive 92/43/EEC, 1992)

as a priority for conservation. The overall decline in natural habitat availability has been attributed to its poor conservation status (Pita et al., 2014), but *M. cabrerae* appears to select groves in our study area (Barão et al., 2022) providing an opportunity for conservation.

1.3.Rodent ecology and behaviour

Rodent communities can be found in most agroecosystems. Especially in extensive olive groves, because they provide a stable food supply, protection from predators and high-quality habitat, including in its adjacent microhabitats: field margins, hedgerows, edges, riparian zones and semi-natural areas. Rodents may choose these over open fields if the habitat is of high-quality and/ or anthropogenic disturbance is minimal (Sullivan & Sullivan, 2021). Namely if left uncut, perennial grasses may have similar characteristics to grassland, mimicking natural habitat.

Within olive groves, species may adopt different strategies: 1) shelter and feed within the grove; 2) select microhabitats where conditions are more favourable for shelter and enter the grove to feed periodically, and 3) shelter and feed mainly within the microhabitats themselves (e.g. feeding on grasses). The frequency with which species feed inside the olive grove, the parts of the plant they consume and the behaviour they use to feed determine their perceived pest status.

The wood mouse *Apodemus sylvaticus* tends to select arable land and can be found in open cultivated fields with tall vegetation (Pollard & Relton, 1970). In open fields, *A. sylvaticus* displays burrowing behaviours (Green, 1979): burrows provide cover from predators and food storage, as their main feeding strategy is to collect abundant seeds and store them for later consumption. *A. sylvaticus* does this with olives, but the behaviour doesn't seem to cause crop damage because the olives are often collected from the ground.

The water vole *Arvicola sapidus* tends to select riparian habitats with sloping banks, a soft substrate and water streams. However, although *A. sapidus* is a specialist in habitat selection, it behaves as a generalist: it can occupy habitats with suboptimal conditions, so long as there is moderate woody cover and a high cover of herbaceous plants (Mate et al., 2013), its main dietary component (Garde & Escala, 2000).

The Cabrera vole *Microtus cabrerae* inhabits humid areas with perennial grassland and its distribution is limited by bioclimatic factors (Bencatel et al., 2019), as it avoids

extremes of temperature and humidity (Pita et al., 2014). This species occurs under very specific bioclimatic conditions (Bencatel etl al., 2019) and requires optimal habitat patches to establish its metapopulation (Pita et al., 2014). *M. cabrerae* feeds mainly on leaves, stems and seeds, with a clear preference for grasses (Soriguer & Amat, 1988).

The Lusitanian pile vole *Microtus lusitanicus* is the only species known to cause crop damage. The presence of *M. lusitanicus* is conditioned by soft, moist soils (Bencatel etl al., 2019). The species has burrowing habits and is mostly found underground. The subterranean behaviour allows it to feed on roots and tubers, namely the roots of olive trees. This causes plant death and consequently crop damage (Ponsa et al., 2011; Miñarro et al., 2012). *M. lusitanicus* causes damage in young trees because the roots are more tender and palatable. Therefore, young olive groves are most likely to be affected by *M. lusitanicus* damage.

The Algerian mouse *Mus spretus* inhabits a variety of crops and orchards, preferring sites with tall vegetation and shrubs for cover (Gray et al., 1998). *M spretus* leaves shelters during periods of high activity, where it feeds almost exclusively on seeds and fruits (Baudin et al., 2013). It is also not reported to cause significant damage to olive groves, as it doesn't pick olives directly from the tree.

Species coexistence is likely facilitated by niche partitioning, a process whereby species with similar ecological niches are able to differentially exploit resources in a given area (e.g. Sato et al., 2018). Species can operate under a wider range of conditions, such as habitat or diet, tolerated within their niche width (Carscadden et al., 2020) in order to coexist. Thereby enriching biodiversity at the species level (Kartzinel et al., 2015; Sato et al., 2018). In this context, molecular techniques offer further opportunities for fine-scale, high-resolution dietary assessment in the context of niche partitioning (Sato et al., 2018).

1.4.Applications and implications of DNA metabarcoding

DNA barcoding is the identification of taxa using a standardised DNA region. DNA barcoding *sensu stricto* corresponds to species-level identification (species ID), but some authors also consider DNA barcoding *sensu lato*, i.e. identification at any taxonomic level (Valentini et al., 2009). Identification is possible by amplifying and sequencing barcode regions, usually using next-generation sequencing (NGS) technologies. Sequences are then compared to a reference library, such as the National

Center for Biotechnology Information (NCBI) or the Barcode of Life Data System (BOLD). DNA metabarcoding is another application of barcoding and allows the identification of multiple taxa in a mixed sample (Taberlet et al., 2012a), such as environmental (soil, water, etc…) or dietary samples.

DNA metabarcoding can be used to analyse the composition of animal diets (Pompanon et al., 2012), in this case through faecal pellets. Herbivore diet analysis is traditionally performed using microhistological methods. Such methods require a high level of taxonomic expertise and introduces observer bias, due to repeated misidentification of plant parts (Soininen et al., 2015). Partly digested remains are difficult to identify, plus morphological methods are limited to what is not digested at the time of sampling; therefore lacking resolution (Groen et al., 2022). DNA metabarcoding with NGS provides higher taxonomic resolution, identifies a larger number of taxa and eliminates observer bias (Soininen et al., 2015). This overcomes known limitations of traditional studies (Kartzinel et al., 2015).

Furthermore, traditional diet analysis has undesirable effects on the sampled individuals, and potentially on populations, because techniques are invasive (Groen et al., 2022). Hence, another advantage of DNA metabarcoding lies in the fact that faecal pellet samples are typically collected using genetically non-invasive sampling (gNIS). gNIS offers a number of practical and ethical advantages, as it eliminates the need to handle or trap animals. Moreover, gNIS increases sample size in rodents, as many species have elusive, nocturnal, burrowing habits (Kartzinel et al., 2015) or avoid traps altogether (known as trap-shy) (e.g. Férnandez-Salvador et al., 2001). Finally, gNIS also minimises disturbance, which is particularly important for threatened species, as they are already under pressure and at low numbers.

In terms of the implications of DNA metabarcoding, it relies on gene regions known as barcodes. Valentini et al. (2009) describe the ideal barcode as variable, standardised, phylogenetically informative, extremely robust and short. Since the ideal barcode hasn't been found yet and may vary among different taxa, the choice of markers is always an issue when conducting a metabarcoding study. For plant identification, it is often-used markers like the DNA trnL (UAA) intron (Yoccoz et al., 2012), which is a chloroplast genome marker; and Internal transcribed spacer 2 – ITS2 (Cheng et al., 2016), a generally appropriate nuclear marker for mammal diet studies. Additionally, multiple barcodes are usually used to overcome marker choice issues (da Silva et al., 2019), as

overlap between markers increases the power of the results and the probability of identifying all taxa in the sample (Fahner et al., 2016).

Another implication of DNA metabarcoding is the reference library database, which should be as complete and comprehensive as possible (Taberlet et al., 2012b). Ideally, a regional reference library curated specifically for the study area should always be constructed to improve the coverage and resolution of taxonomic assignment. Previous studies have shown that the use of a geographically restricted database improves trnL and ITS2 assignments in vascular plants (Fahner et al., 2016; Barnes et al., 2022). Ultimately, this will minimise misidentifications and increase the probability of species identification in pellet samples.

2.General objectives

The main objective of this study is to characterise the diet of five rodent species *Apodemus sylvaticus*, *Arvicola sapidus*, *Microtus cabrerae*, *Microtus lusitanicus* and *Mus spretus*, in the olive grove agroecosystems of Northeastern Portugal, using a DNA metabarcoding approach and pellet samples collected in the field. Two specific objectives are proposed: i) to estimate the tropic niche width, diversity and composition, in all five rodent species, as well as the level of overlap between them; ii) to determine the importance of olive groves (*Olea europea L*.) in the diet of rodents. Uncovering the dynamics of small mammal communities can help predict population fluctuations in response to local vegetation composition, thus inform management policies to keep *M. lusitanicus* populations low while maintaining/ promoting suitable conditions for species of conservation concern.

3.Materials and methods

3.1.Study area and sampling

The study was conducted in the Trás-os-Montes, specifically in Bragança district. The area is mostly occupied by agro-sylvopastoral systems managed by small producers, where a total of 10 olive groves and surrounding areas were sampled, in localities displayed in Figure 2.

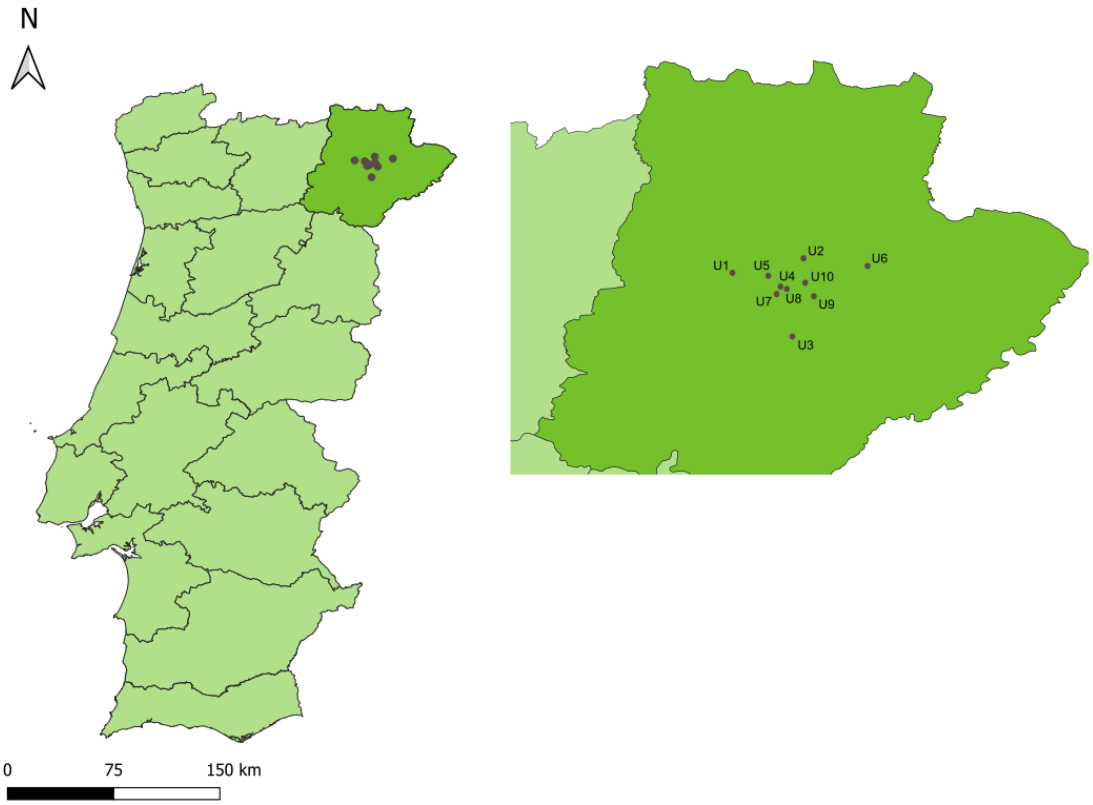

Figure 2: Location of 10 olive grove sites in Bragança, Northeastern Portugal.

We focus on five species *Apodemus sylvaticus*, *Mus spretus*, *Microtus lusitanicus*, *Microtus cabrerae* and *Arvicola sapidus*. All species are distributed within Eurasia (Rigaux et al., 2023). *M. lusitanicus* and *M. cabrerae* are endemic to the Iberian Peninsula (*M. lusitanicus* occurring also in the extreme southwest of France) (Bencatel et al., 2019). *M. lusitanicus* is regarded as a pest in the study area, as it is thought to feed on cultivated species, particularly young tree roots. *M. cabrerae* and *A. sapidus* are currently vulnerable (VU) of extinction (Mathias et al., 2023; Rigaux et al., 2023) and

therefore of conservation concern. *M. cabrerae* is even listed as priority for conservation in European Habitats Directive (Council Directive 92/43/EEC, 1992).

We performed genetic non-invasive sampling (gNIS), as some rodent species have burrowing habits (Kartzinel et al., 2015), are elusive or described as trap-shy, meaning they purposefully avoid traps (Férnandez-Salvador et al., 2001). A total of 10 land mosaics (sampling units) of varying size were defined, composed by a focal olive grove and the surrounding matrix within a 50m buffer (Figure 3). Selected target olive groves were “young” with small trees most susceptible to pest damage. To account for habitat diversity, each buffer included nearing fields and patches within the 50m radius. Approximately 20% of transects were located within the focal olive grove and remaining transects were conducted in surrounding patches, as described by Barão et al. (2022). gNIS was performed by conducting 40 m linear transects in each unit. Transect distribution was stratified and 7-19 transects were sampled, depending on unit size.

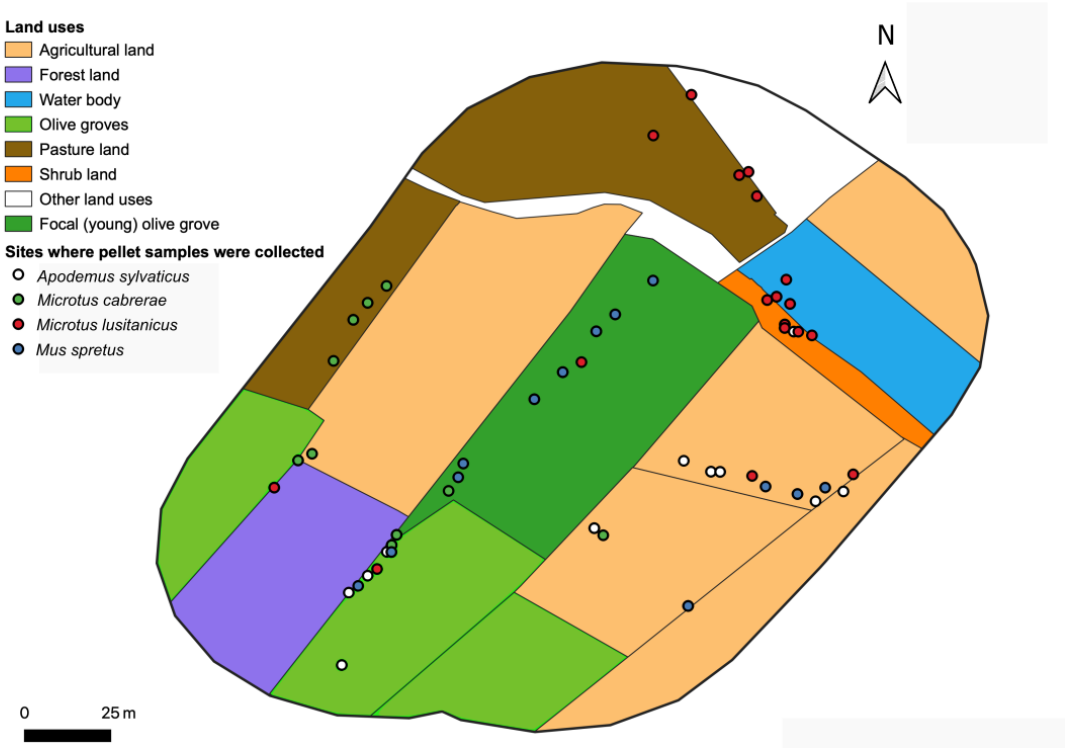

Figure 3: Example of sampling unit with target olive grove and surrounding habitats (different patch coulours) in the 50m buffer. Dots represent the location of pellets that were collected during the transects. Species identification of each pellet is shown by different dot colour.

Units were surveyed for rodents’ presence signs and faecal pellet samples were collected, preferably from small latrines and using sterilized material to avoid cross-

contamination. Pellets were then preserved in ethanol 98% and frozen until laboratory processing as described in Barão et al. (2022). A total of 599 samples were collected across all sampling units, where 528 were selected for pellet diet analysis, as presented in Table 1. Only samples from species with at least 10 detections in a given patch were selected for analyses.

Table 1. Rodent species detected in each sample unit and respective number of pellets collected by site. Bold numbers indicate the pellet samples selected for diet analysis (Total = 528).

Sampling Units	*Apodemus sylvaticus*	*Arvicola sapidus*	*Microtus Cabrerae*	*Microtus lusitanicus*	*Mus spretus*	
U1	9			**15**	2	
U2	**13**		1	**12**	2	
U3	2		**59**	3		
U4	7		**59**			
U5	**35**		2	9	7	
U6	**31**		3		2	
U7	**18**		**71**			
U8	**20**	**49**	**24**		4	
U9	9		**40**		1	
10	**18**	8	**19**	**27**	**18**	
Total sampled	162	57	278	66	36	599
Total selected	135	49	272	54	18	528

A total of 664 plant specimens were also collected to produce a metabarcoding reference library geographically-restricted to the study area. Plant specimens were morphologically

identified by a plant taxonomy expert (Miguel Porto, Flora-on, https://flora-on.pt/equipa.php). In cases where morphological identification was inconclusive, congruent barcode IDs were later used to attribute plant sample ID. (For full list of plant taxa in the study area, see Table S9.)

3.2.DNA extraction and PCR amplification

3.2.1.Faecal pellets

DNA was extracted from collected faeces using a magnetic-based enzymatic protocol, with Agencourt®AMPure®XP beads (Beckman Coulter Company: Beverly, MA, USA) and Qiagen® buffer solutions containing 400 µL ATL solution and 25 µL Proteinase K, as described by Barão et al. (2022). DNA extraction was performed in an isolated room for non-invasive samples to reduce the risk of contamination, and negative controls were included for this step. Rodent species identification was performed by the authors using a barcode of the 12S rRNA gene of mitochondrial DNA and an in-house developed small mammal's reference library for this gene (unpublished). The extracted DNA was then subjected to further analysis for dietary assessment. We used a multi-marker approach to identify plants present in the diet, as the use of multiple barcodes allows for more detailed information and the detection of higher taxa occurrence (da Silva et al., 2019).

Two markers were selected, one from the chloroplast genome – P6 loop of the plastid DNA trnL (UAA) intron (Yoccoz et al., 2012) – and one from the nuclear genome – Internal transcribed spacer 2 (ITS2) (Cheng et al., 2016). To amplify the P6 loop of trnL, we used the primer set trnL-gA49425 and trnL-hB49466 (Taberlet et al., 2007) modified with Illumina overhangs (trnL-gA49425: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGA-GACAGGGGCAATCCTGAGCCAA-3'; trnL-hB49466: 5'-GTCTCGTGGGCTCGGAGA-TGTGTATAAGAGACAGCCATTGAGTCTCTGCACCTATC-3)'. To amplify ITS2, we used the primer pair Uniplant-ITS2-Fw and Uniplant-ITS2-Rv (Moorhouse-Gann et al., 2018), also modified with Illumina overhangs (Uniplant-ITS2-Fw: 5'-TCGTCGGCAGCG-TCAGATGTGTATAAGAGACAGTGTGAATTGCARRATYCMG-3'; Uniplant-ITS2-Rv 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCCGHYTGAYYTGRGGTCDC-3'). Regarding fragment length, trnL fragments amplified with gh primer set are typically very small with 20-80 bp, whereas ITS2 fragments are usually between 250-350 bp. Additionally, a mix of seven Fw and seven Rv primers was used for each marker, using an increasing number of N (unspecified) bases in each primer, to increase base diversity in Miseq sequencing.

For pellet samples, trnL was amplified using 5µL hotstart master mix (Multiplex PCR Kit, QIAGEN), 2µL of trnL-g primer mix (1:10), 2µL of trnL-h primer mix (1:10), and 2.5µL DNA. Amplification was done with an initial denaturation step of 95°C for 15min, 45 cycles of 1min annealing at 55°C, 30s extension at 72°C and a final extension at 72°C for 10 min. ITS2 was amplified using 5µL of hotstart master mix, 0.3µL of ITS2-FW primer mix (1:10), 0.3µL of ITS2-RV primer mix (1:10), 2.4µL of purified water and 2.5µL DNA. A touch-down method was used to increase the specificity of the PCR products: initial denaturation at 95°C for 15min, followed by 5 cycles of 30s (annealing temperature was reduced -0.5°C /every cycle) starting at 56°C, and 30s extension at 72°C; followed by further denaturation at 95°C for 30s, 40 cycles of 30s annealing at 54°C, 30s extension at 72°C and a final extension at 72°C for 10min. Each sample was replicated twice, including PCR negative controls. PCR amplicons were then visualized on a 2% agarose gel.

3.2.2. Plants

Plant DNA was extracted from dry leaf tissue. To break cell walls, ZY Premium zirconium oxide Typ Zy-P 2.7mm-3.3mm beads (Ginzel) were used in Retsch Mill at maximum speed of 25x for at least 10 minutes. Lysis was performed using lysis buffer (2% SDS, 2% PVP 40, 250 mM NaCl, 200 mM Tris HCl, 5mM EDTA, pH8) and pK enzyme. Following centrifugation, binding buffer (2M Guanidine hydrochloride in 95% ethanol) was used for column binding. Membranes were washed twice with 80% ethanol and potassium acetate 3M was used for precipitation.

The same markers and primers used in diet analysis were used to generate DNA barcodes of collected plant specimens. Amplification conditions were the same as described before for pellet samples.

3.3.Library preparation and sequencing

PCR products were indexed following the Illumina MiSeq protocol 16S Metagenomic Sequencing Library Preparation Illumina, 2013) with KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Cape Town, South Africa) and a cleaning step using Agencourt AMPure XP beads (Beckman Coulter), with a 0.8x ration. Then, PCR products were quantified in Epoch spectrophotometer, normalized to 20nM, and pooled per marker. Pools were analysed with Agilent 2200 Tapestation for sizing and quantification of each

library (Padmanaban, 2015). Finally, each pool was quantified by qPCR (KAPA Library Quant Kit qPCR Mix, Bio-Rad iCycler), diluted to 4 nM, and run in NovaSeq 6000 sequencer (Illumina) using a 2x250 bp approach for an expected average of 30,000 paired-end reads per sample and replicate for pellet samples, and 5,000 paired-end reads for plants.

3.4. Bioinformatic processing

Bioinformatic processing of the sequencing reads obtained for the plant reference database and dietary analysis was performed using OBITools (Boyer et al., 2016). Initially, paired-end reads were aligned using the "illuminapairedend" command and discarded if the overlap quality was < 40 (Taberlet et al. 2018). Unaligned sequences were also removed using the 'obigrep' function. Sequences were dereplicated to unique sequences across samples using 'obiuniq', and primer sequences were removed using 'ngsfilter'. Sequences of 20-80 bp for trnL-gh and 250-350 bp for ITS2 were retained and sequences with read counts less than two were removed. Finally, "sumacluster" with 99% similarity was used to clean sequences from PCR or sequencing errors. For the plant reference barcode database, the central cluster was then compared to the reference nucleotide database (NCBI) using the BLASTN algorithm (McGinnis and Madden 2004) to check the accuracy of the morphological identification of the specimens. This inhouse reference nucleotide database of plants from the region was then used as a reference for comparison with the central clusters obtained from the dietary analysis using the BLASTn algorithm (McGinnis and Madden 2004). Unassigned clusters were blasted against the general NCBI database containing plant barcodes from reference specimens worldwide. THE BLASTn algorithm was set to 80% percentage identity (perc_identity), 90% query coverage (qcov_hsp_perc) and a maximum extraction of 20 items per sequence. A complete taxonomy for each BLAST result was obtained using the 'taxonomizr' package in R (https://cran.r-project.org/web/packages/taxonomizr/index. html). A Python script was then used to cluster the taxonomic identifications assigned to each central cluster. For clustering, assignment to the species level was considered to have a percentage identity of at least 99%, while lower identity thresholds were set for assignment to order or lower taxonomic levels (> 80% identity), family (≥ 95%) and genus (≥ 98%). The number of reads of the resulting taxonomic identifications per sample was summed, including the two replicates, and taxa with less than 10 reads per sample were excluded from further analyses.

Statistical analyses were performed using only those taxa with equal or greater than 1% relative reads in each sample (Aizpurua et al., 2018).

3.5. Statistical analysis

Statistical analyses were conducted to estimate the width, diversity, composition and level of overlap of the trophic niche at two taxonomical levels (plant family – derived from trnL-gh – and plant genera – derived from ITS2) in five rodent species. Analyses were conducted using R statistical environment (R Development Core Team, 2023).

To better describe and visualise the plant taxa consumed by each rodent species, frequency of occurrence plots were built for each species using ggplot2 (Wickham, 2016).

Niche width, i.e. total number of taxa consumed by each species, was estimated using rarefaction curves with function iNEXT of the iNEXT package (Hsieh et al., 2016). Sample coverage (SC) was calculated for both datasets, and the diversity estimate of order q (qD) metric informed niche width. Statistical differences among species were considered when 95% confidence intervals of each curve did not overlap.

Diet richness, i.e. number of taxa present in each pellet sample, was calculated and compared among species with a Generalized Linear Mixed Model (GLMM), using glmer function from package lme4 (Bates et al., 2015). The model was set with a negative binomial distribution, using number of plant taxa as independent variable (plant family for trnL data and plant genera for ITS2 data), rodent species as dependent variable, and olive grove unit as random factor. To assess variable significance, an ANOVA was performed with the function Anova of the package car (Fox & Weisberg, 2019) and results were plotted using the function effect of the effects package (Fox, 2003; Fox & Weisberg, 2019). To understand which pair of species differed significantly, multiple comparisons were done for each species-pair with Tukey contrasts using the function summary of the package multcomp (Hothorn et al., 2008).

Diet composition was compared among species using PERMANOVA. For that, a jaccard distance matrix was built using the function vegdist from Vegan (Oksanen et al., 2022). In order to ensure that significant differences among species were not an effect of differences in variance, a beta dispersion test was conducted using betadisper from Vegan, followed by Anova. Resulting centroids and sample points were plotted using a Principal Coordinate Analysis (PCoA). PERMANOVA was done using the function

adonis from Vegan, using the jaccard distance matrix as response variable, rodent species as explanatory variable, and unit as strata in order to minimize the effect of local plant abundance variation between units. Differences among species-pairs were calculated with adonis.pair from EcolUtils (Salazar, 2023). Finally, to identify plant taxa significantly contributing to differences in diet among species pairs, a Similarity percentages (Simper) analysis was conducted with the function simper from Vegan.

Niche-overlap among species was further calculated using the function niche_null_model from EcoSimR (Nicolhas et al., 2015), considering Pianka's index of niche overlap and null model ra3. A niche overlap matrix was then produced and plotted with library corrplot (Wei &Simko, 2021).

4.Results

4.1. Plant reference library

Morphological analysis resulted in the successful identification of 584 out of the 664 plant samples collected: 563 to the species level and to 21 to the genus level. These belonged to a total of 66 families, 32 orders and 1 phylum (Streptophyla). For 80 plants it was not possible to classify due to absence of diagnostic characters. From the 664 plants collected, a total of 608 plant barcodes were obtained for trnL and 616 for ITS2; with an average read number of 7995 for trnL and 440 for ITS2. For 601 samples, both trnL and ITS2 barcodes were obtained simultaneously.

4.2. Dietary analysis

4.2.1 Sequencing data

A total of 524 pellet samples were successfully sequenced for trnL and 525 samples for ITS2. For trnL, the average number of reads was 47,043, with a median of 42,467 and a range of 613-242,189. For ITS2, the average number of reads was 54,177, with a median of 46,625 and a range of 184-265,307. The number of obtained OTUS was 118,759 for trnL and 41,700 for ITS2.

A total of 85 plant families were identified with trnL, of which only 46 families were detected with over 1% of reads in at least one sample. A total of 198 plant genera were identified with ITS2, of which only 153 genera were detected with over 1% of reads in at least one sample.

4.2.2. Frequently consumed plant taxa

Frequently consumed plant families were ranked for each rodent species according to the frequency of occurrence (Figure 4).

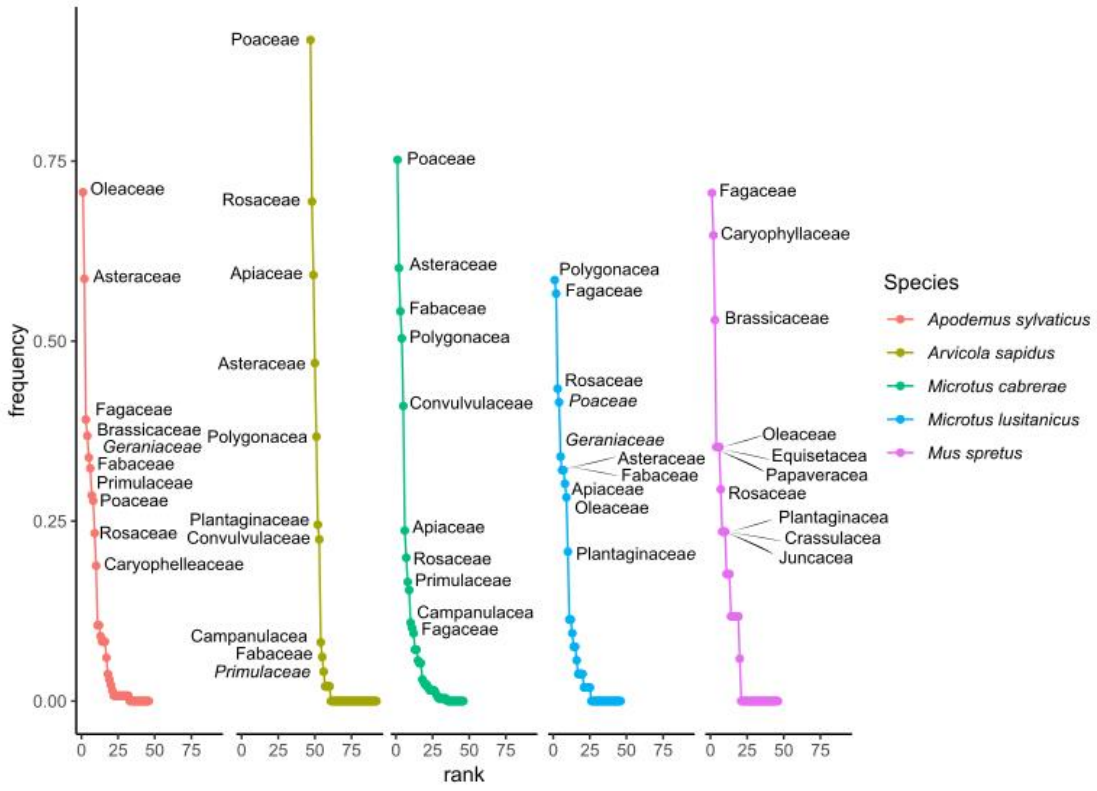

Figure 4: Frequency of occurrence of plant families using trnL data for each rodent species. Top 10 ranked families are indicated.

A. sylvaticus has the highest rank for Oleaceae (0.707), followed by *M. spretus* (0.353) and then *M. lusitanicus* (0.283). *A. sylvaticus* also consumed high amounts of Asteraceae (0.587), a family commonly consumed in the top 10 rank among all other species, except for *M. spretus*. *A. sapidus* consumed mostly Poaceae (0.918), or graminoids. This was followed by Rosaceae (0.694) and Apiaceae (0.592). Poaceae was also the most consumed family by *M. cabrerae* (0.752), which was followed by Asteraceae (0.602). *M. lusitanicus* displayed highest intakes of Polygonaceae (0.585), and then Fagaceae (0.566), which is comprised of woody plants (e.g. oak trees). But also Rosaceae (0.434) and Poaceae (0.415). *M. spretus* also consumed high amounts of woody Fagaceae (0.706), as well as Caryophyllaceae (0.647) and Brassicaceae (0.529). Additionally, most consumed plant genera were ranked according to frequency of occurrence in Figure 5.

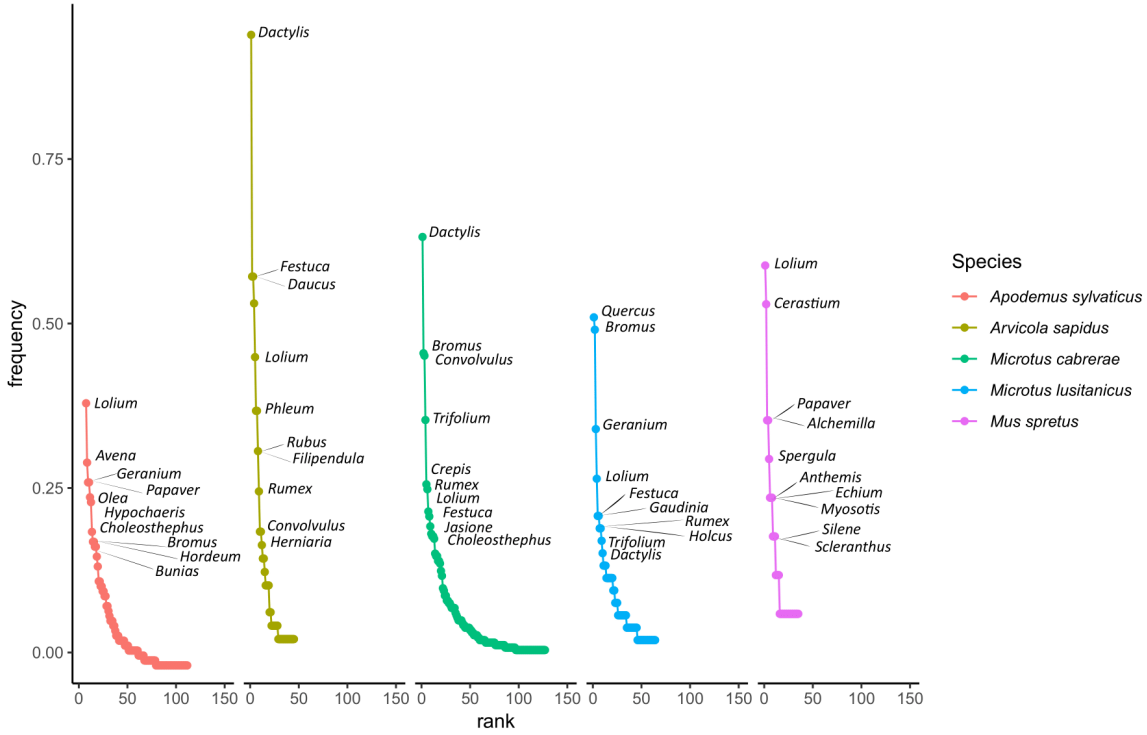

Figure 5: Frequency of occurrence of plant genera using ITS2 data for each rodent species. Top 10 ranked genera are indicated.

A. sylvaticus revealed highest intakes of *Lolium* (0.406), followed by *Avena (0.316)*, *Geranium* (0.286) and *Papaver (0.286).* Genus *Olea,* associated with olive tree, ranked as the 5th most consumed genus, with a frequency of occurrence of 0.263. *A. sapidus* consumed mostly *Dactylis* (0.939). Then, it consumed *Festuca* and *Daucus* in equal frequency (0.571), followed by *Lolium* (0.531). *Lolium* was consumed at similar frequencies between *A. sylvaticus* and *A. sapidus*, surpassed only by *M. spretus*, with a frequency of 0.588. In both *A. sapidus* and *M. cabrerae*, *Dactylis* was the most consumed genus. *M. cabrerae* consumed *Dactylis* (0.632) followed by *Bromus* (0.455), *Convolvulus* (0.452) and *Trifolium* (0.353), associated with clover. *M. lusitanicus* diet was highest in *Quercus* (0.510), associated with woody plants (e.g. oak trees), followed by consumption of *Bromus* (0.491) which was similar in value to that of *M. cabrerae* (0.455). *M. lusitanicus* also consumed high amounts of *Geranium* (0.340) and *Lolium* (0.264). *M. spretus* consumed highest amounts of *Lolium (*0.588), of which it was the highest consumer among all species. This was followed by consumption of *Cerastium* (0.530), and then *Papaver* and *Alchemilla* at equal frequency (0.353).

Plant taxa consumed by each species across all units are also displayed in Figure 6, illustrating the complex network of interactions within rodent communities in our study

area. Across the whole network, most consumed plant family was Poaceae, and most consumed plant genus was *Lolium*, followed by *Dactylis*.

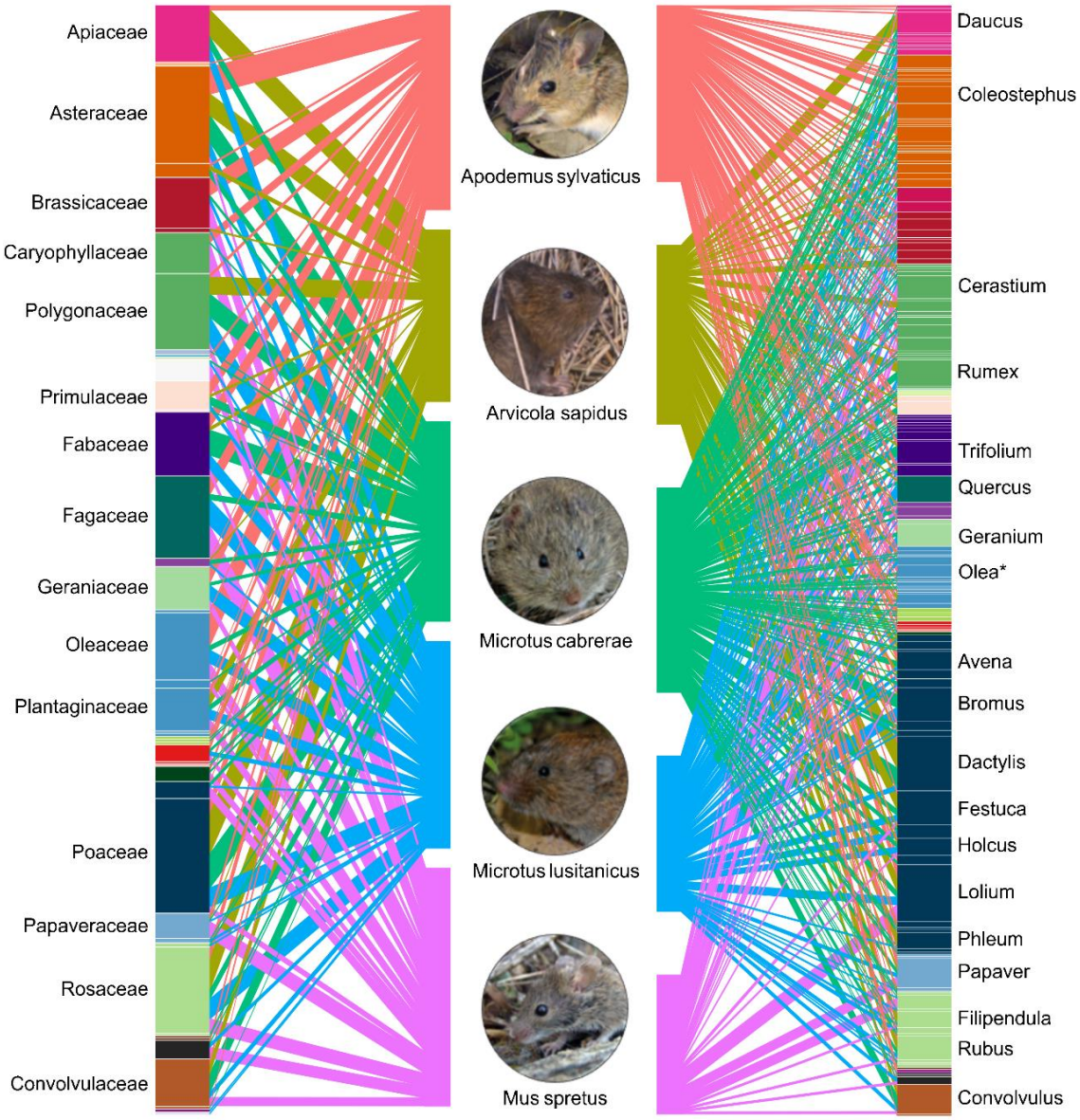

Figure 6: Trophic web of five rodent species in the study area. Consumed families (left) and genera (right), whose sum of the frequency of occurrence of all species are above 50%, are indicated. Genus *Olea* is indicated with an asterisk. Link width represents the frequency of occurrence of each taxa of plants in the diet of rodent species. Plant genera are coloured according to plant family.

4.2.3. Trophic niche width

Considering niche width, rarefaction curves revealed similar levels of niche width among all rodent species regarding both plant families and plant genera (Figure 7 and 8, respectively). Observed sample coverage was higher for trnL, varying between 0.97-0.99 among species, and lower for ITS2 varying between 0.77-0.96.

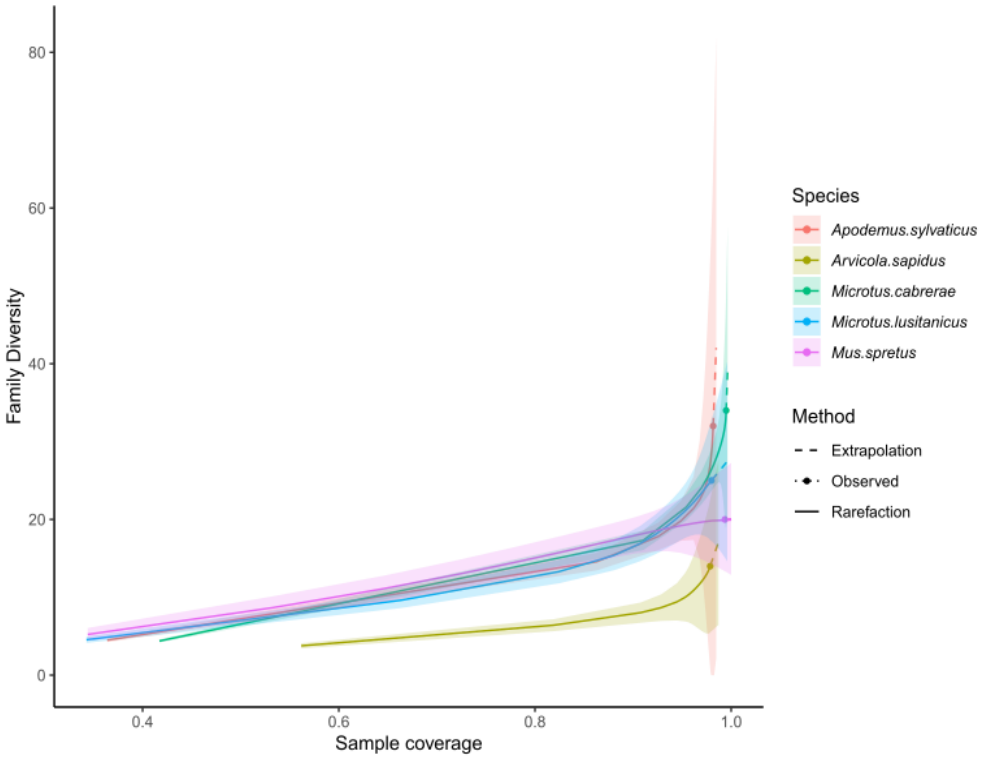

Figure 7: Niche-width rarefaction curves for trnL data for each rodent species. Shaded areas indicate 95% confidence intervals.

A. sylvaticus and *M. cabrerae* were estimated to have the most similar niche widths both with trnl data (diversity estimate of order q, qD=27 and 26, respectively; Appendix Table S1) and with ITS2 (both with qD=46; Appendix Table S2). For both datasets *M. spretus* showed the widest confidence interval; and *A. sapidus* was estimated to consume the lowest number of plant families, as well as plant genera (Figure 8).

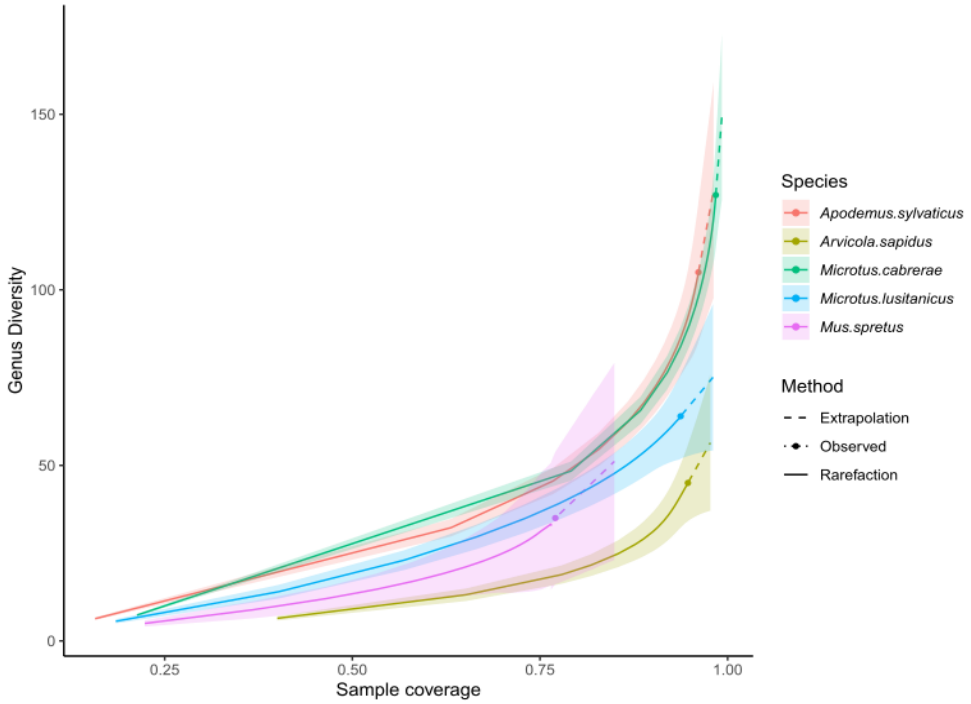

Figure 8: Niche-width rarefaction curves for ITS2 data for each rodent species. Shaded areas indicate 95% confidence intervals.

4.3.4. Trophic niche diversity

Regarding significance of the variable "species", the glm didn't find it to be significant in any species' niche diversity for the trnL data (Table S3). It also wasn't significant in the ITS2 data, except for the case of *M. cabrerae*, relative to *A. sylvaticus* (Table S4). For species effect plots see Supplementary Material (Figures S1 and S2).

Considering diet diversity, the total number of plant families found in each pellet was not significantly different among rodent species ($p = 0.445$). However, the total number of plant genera did differ significantly ($p = 0.03$) with ANOVA. The five species of rodents consumed a similar number of plant families, but a varying number of plant genera.

Multiple comparisons did not identify any specific pair of species whose diet diversity differed significantly in either dataset (Appendix Tables S5 and S6), when considering the number of plant taxa present in each pellet sample.

4.2.5. Trophic niche composition

Beta dispersion tests were significant for both datasets ($P<0.001$ for trnL; $p<0.001$ for ITS2), however, centroids of all species occupied quite distinct positions in PCoAs (Figure 9 and 10). *A. sylvaitcus* and *M. spretus* occupied left centroids whereas *A.*

sapidus and *M. cabrerae* occupied right centroids for trnL data. For ITS2, *M. cabrerae's* was central and *A. sapidus'* was to the left, whereas *A. sylvaticus*, *M. lusitanicus* and *M. spretus* occupied right centroids.

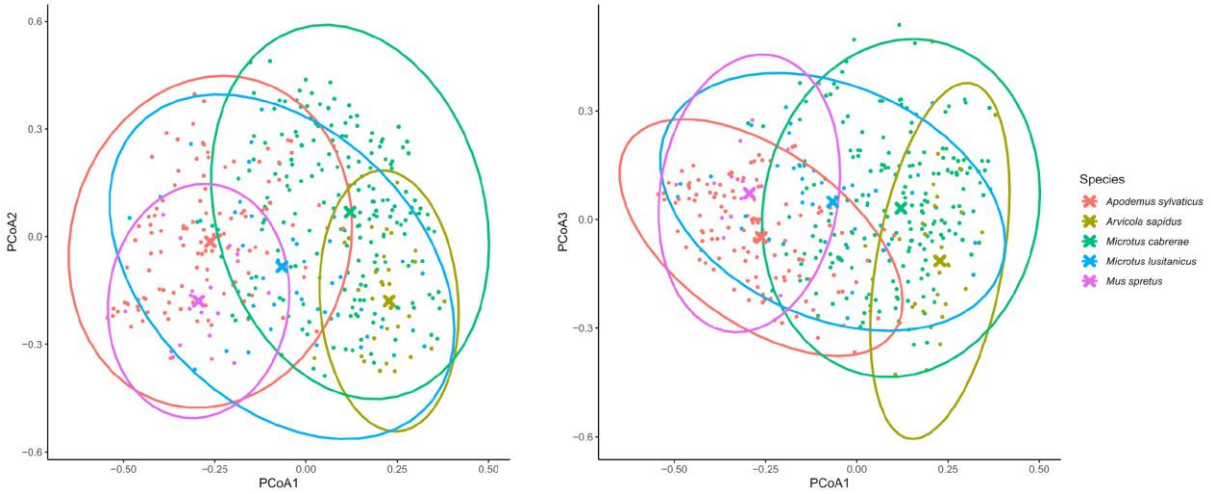

Figure 9: PCoA of diet composition based on trnL data and coloured by rodent species. Each dot represents a different sample. Ellipses represent 95% confidence intervals. Centroids are marked with x.

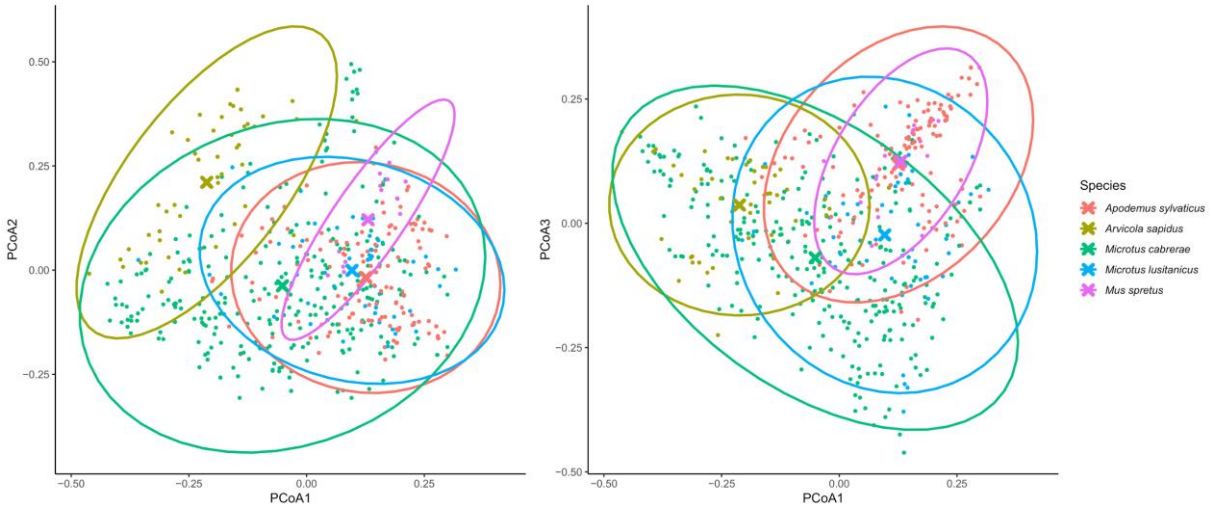

Figure 10: PCoA of diet composition based on ITS2 data and coloured by rodent species\. Each dot represents a different sample. Ellipses represent 95% confidence intervals. Centroids are marked with x.

PERMANOVA revealed that all species' diets were significantly different regarding both plant family and plant genera composition (p = 0.0001, both for trnL and ITS2). Multiple comparisons were significant between all species-pairs (Tables S7. and S8). Simper results produced a detailed comparison of trophic niche interactions between all species-

pairs as displayed in Figure 11. Simper results further showed that 27 familes and 103 genera were significantly contributing to differences among species.

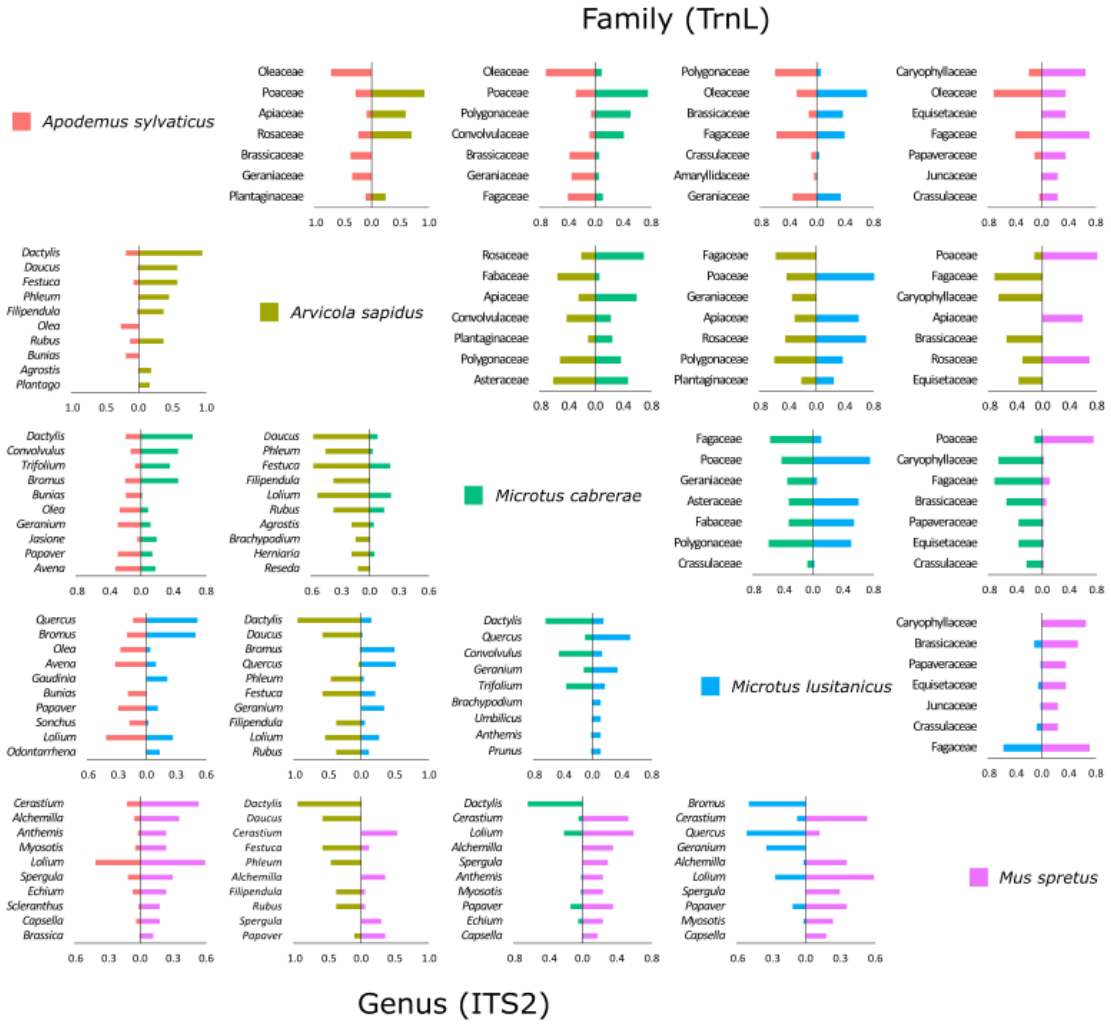

Figure 11: Frequency of occurrence of plant families (trnL, upper triangle) and plant genera (ITS2, lower triangle) that significantly contribute to differences in diet composition among rodent species-pairs based on SIMPER analysis. The seven and 10 most differently consumed plant families and genera, respectively, among each species-pair are displayed.

Family Oleaceae was consumed more by *A. sylvaticus* comparatively to all other species, except when compared with *M. lusitanicus*, according to trnL data. Genus *Olea* was more consumed by *A. sylvaticus*, when compared to *A. sapidus*, *M. cabrerae* and *M. lusitanicus*, as revealed by ITS2 data.

4.2.6. Trophic niche overlap

Niche overlap among most species pairs was significantly higher than expected from null models for both markers (Figure 12). For trnL data, lowest overlap was 0.26, between *A. sapidus* and *M. spretus*; and highest overlap was 0.81 between *A. sapidus* and *M. cabrerae*. For ITS2 data, lowest overlap was 0.27, between *M. cabrerae* and *M. spretus*; and highest overlap was 0.68, between *M. cabrerae* and *M. lusitanicus*.

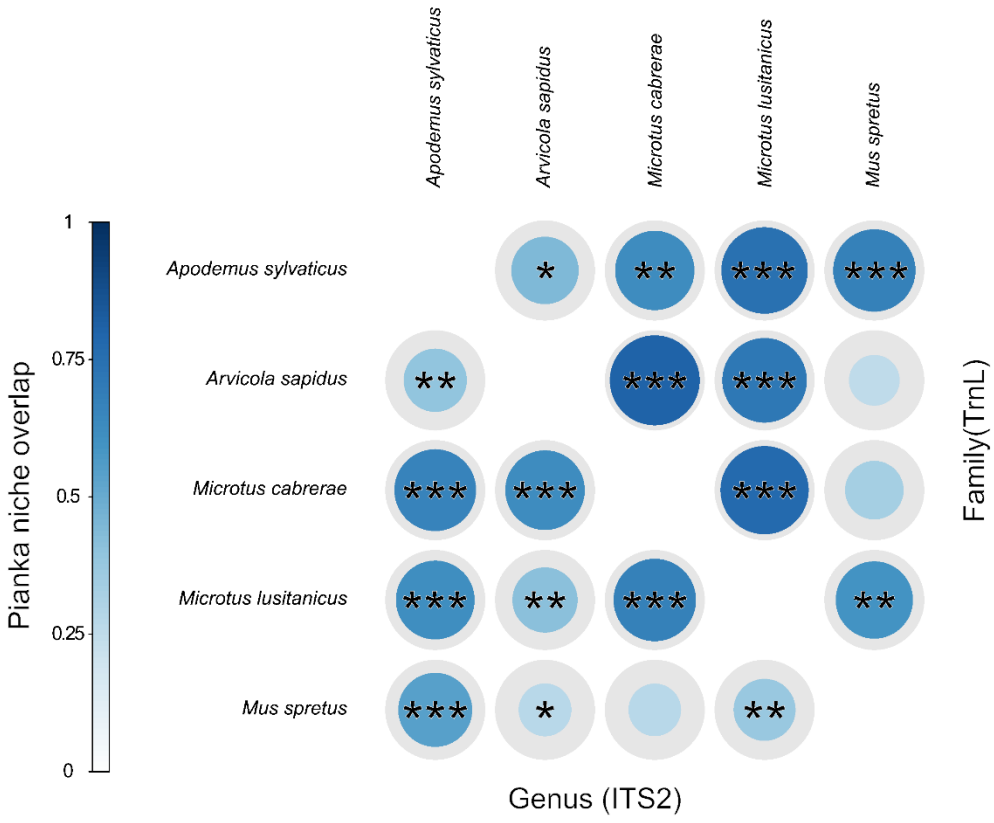

Figure 12: Pianka niche overlap comparison of all species-pairs, using trnL data for plant family niche overlap (upper triangle) and ITS2 data for plant genus overlap (lower triangle), respectively.

5.Discussion

In this thesis, we provide the first trophic niche analysis of 5 rodent species occurring in olive grove agroecosystems in northeastern Portugal, using a genetic non-invasive sampling (gNIS) approach combined with a multi-marker DNA metabarcoding analysis, and a plant DNA reference library constructed specifically for the study area. This work provides new knowledge on the feeding ecology of rodent communities in olive grove agroecosystems, important for informing management policies to keep *M. lusitanicus* populations low while maintaining/ promoting suitable conditions for species of conservation concern *A. sapidus* and *M. cabrerae.*

5.1. Diet analysis with molecular methods

With our non-invasive approach, relying on fecal pellet analysis, we were able to uncover diets of 5 rodent species, 2 of which are threatened, without causing disturbance on populations. The use of molecular methods allowed for detailed diet descriptions of all species, identifying the plant families Asteraceae, Poaceae and Fagaceae as predominant across the diet of multiple species; as well as genera *Lolium* or *Dactylis*.

The threatened *A. sapidus* diet was comprised mostly of graminoids *Dactylis*, *Festuca*, *Lolium* and herbaceous plants like *Daucus*. We also unveiled how threatened *M. cabrerae* similarly consumed graminoids like *Dactylis* and *Bromus*, but also herbaceous plants like *Convolvulus* and *Trifolium* (e.g. clover). The data obtained also allowed us to measure consumption of olive tree, with both trnL and ITS2 data; being Oleaceae and *Olea* detected mainly in species *A. sylvaticus, M. lusitanicus* and *M. spretus*. In the case of the perceived pest species *M. lusitanicus*, we also identified alternative dietary components to olive tree, which are easy to promote in the study area, such as *Bromus, Geranium* or *Lolium*.

Our findings also contribute to the current collective efforts to establish metabarcoding protocols and universal markers in Ecology, particularly Molecular Ecology. Considering the 2 datasets obtained, it is possible to compare results produced by both barcodes, which had a complimentary role. trnL provided mostly information at family level and ITS2 at the genus level, allowing for 2 levels of taxonomic identification in the analysis. This may be explained by barcode length, as TnrL (when amplified with g-h primer set) produces a very small marker, with just 20-80 bp (Yoccoz et al., 2012). Therefore, resolution is lower and species ID is not always possible, whereas ITS2 is around 250-

350 bp (Cheng et al., 2016), allowing for more resolution and more precise identification of taxa.

This is congruent with the findings of Fahner et al. (2016) in a study comparing sequence resolution among taxa evaluated for 4 established DNA markers (*mat*K, *rbc*L, ITS2, and the *trn*L P6 loop), for plant metabarcoding in environmental samples. Although shorter barcodes were initially proposed in the search for a land plant universal marker (Sass et al., 2007), both authors point to full-length barcode regions associated with the internal transcribed spacer (e.g. ITS2) actually outperforming shorter markers (<100bp), such as trnL (Fahner et al., 2016). In our study, ITS2 showed better accuracy (most ID's reached species ID) and ITS2 trophic niche estimates were highly detailed (to the genus level), likely making ITS2 a more suitable plant DNA marker for an analysis on that level.

Rodent conservation may be facilitated by molecular techniques, as they are elusive animals and difficult to sample. Increasing sample size/ scale with metabarcoding studies presents an interesting solution for rodent monitoring: albeit high in cost, it is extremely effective at producing new knowledge to best manage rodent communities. However, results produced by molecular techniques are only as useful as their rightful interpretation – knowledge of rodent ecology and behaviour, as well as very close attention to detail, is key to conduct this type of research and derive robust conclusions.

5.2. Trophic web interactions

Interactions unveiled by our results generally reflect the ecology of each species in the study area, which is tightly linked to their feeding ecology. For instance, PCoA plots for both datasets highlight a higher similarity between trophic niches of dry/ land/ subterranean species (*A. sylvaticus, M. lusitanicus* and *M. spretus*) when compared to the species with humid patch and/or riparian vegetation-related ecology (*A. sapidus* and *M. cabrerae*), which are also both threatened. This could possibly be linked to a higher diversity in availability of dry land plants then of riparian plants, as the study was done in an agroecosystem.

Regarding species of conservation concern, *M. cabrerae* has a generally wider trophic niche than that of observed in *A. sapidus*, revealing a degree of plasticity towards exploring its food options. In terms of overall niche composition, both threatened species' diets overlap, which is congruent with available information indicating that *M. cabrerae* and *A. sapidus* select slightly humid (Bencatel et al., 2019) to humid-riparian patches

(Mate et al., 2013; Garde & Escala, 2000), with a degree of similarity between vegetation structure and composition.

Furthermore, *Dactylis* is noticeably the 1st ranked genus for both threatened species. Therefore, there may be some competition for Dactylis between *A. sapidus* and *M cabrerae*, with frequencies of occurrence of 0.939 and 0.632, respectively. Genus *Dactylis* may be a dietary component playing a key role in trophic interactions between these threatened species.

Additionally, diet overlap estimates using Pianka's index revealed multiple overlapping diets with both datasets, which indicates there's supportive evidence for highly overlapping diets in our study area. However, high potential for resource competition predicted between species may not always translate into actual competition, as discussed by (Soininen et al., 2015) in a DNA metabarcoding diet analysis of two lemming (Rodentia, Cricetidae) species using a similar approach.

If food competition is clearly present, niche partitioning is the strategy adopted by rodents to coexist. With niche partitioning, species with the same ecological niche coexist by exploring resources differentially (eg. Sato et al. 2018). Competing species are driven into different patterns of resource use within a similar niche; thus, facilitating species coexistence within limited resources and enriching biodiversity. As described by Kartzinel et al. (2015) in a similar study, taxonomically fine-grained diet partitioning observed in large mammalian herbivores, suggests how mammalian diversity may be more tightly linked to plant diversity than is currently recognized.

Hence, rodent species coexistence in these communities is likely tied to the high plant diversity associated with extensive agricultural practices (Neves et al., 2013; Silveira et al., 2018), known to increase both agroecosystem ecological value (Reis, 2014) and diversity across all taxa in these systems (Rey et al., 2019). This diversity offers coexisting species a wider potential trophic niche, which can then be explored differently according to specific ecology and habitat preferences. So long as the dietary options are diversified enough to create niche partitioning opportunities, the existence of populations of threatened species may be supported in these diverse rodent communities.

5.3. Rodents in agricultural context

Regarding olive tree consumption, it is firstly important to note how perceived plant taxon consumption may be influenced by plant morphology itself. Molecular methods don't

allow for identification of particular plant parts, simply informing about plant taxa presence in the diet. Hence, there are limitations associated when interpreting consumption. For instance, in woody plants, different parts may be consumed by rodents, from tree roots to fruits and seeds; all of which are identified as the corresponding taxa regardless. Therefore, considering how certain species appear to consume *Olea* VS interpreting these results may be essential in assessing, for instance, actual crop damage inflected upon olive groves.

The only *Olea* plant in the study area was *Olea europaea*, so detection of genus *Olea* with ITS2 always refers to the olive tree in this context. *A. sylvaticus* ranked as highest *Olea* consumer, but this species displays a particular feeding ecology, by gathering small fruits seed and making piles for later consumption. Hence, *A. sylvaticus' Olea* intake is most likely linked to consumption of olive seeds and should be interpreted as such. This could relate to picking olives from the olive tree, but also gathering of fallen (riper) olives, a behaviour which was observed during sampling.

When looking at the trnL dataset, olive intake is also associated with *M. lusitanicus* and *M. spretus*: both species ranked consumption of family Oleaceae, with 9th for *M. lusitanicus* and 4th for *M. spretus*. In both cases, the species also ranked high for Fagaceae, associated with woody plants (e. g. oak trees). This may indicate that consumption is likely occurring in the form of tree roots, probably of younger trees, where the roots are more tender and palatable. However, it is important to note that neither *M. lusitanicus* nor *M. spretus* had genus *Olea* in their top 10 consumed genera; which indicates that their diets are comprised of many other plant genera, consumed in higher amounts than *Olea europaea.*

Furthermore, considering rodent conservation implications in agricultural contexts, Delibes-Mateos et al. (2011) describe the paradox in native small mammals between their role as keystone species vs perceived role as pests. Mostly, pest status is assumed because the species abounds within native range and may produce damage in agriculture. It's important to recognize that where agriculture is now practiced, is historically the species' native range. The authors further point to how persecution of perceived pests led to loss of ecosystem functions provided, highlighting understated importance of these species.

Hence, conservation of native voles like *M. lusitanicus* is equally important. Not just from an ecological or conservational perspective, but also from an agricultural standpoint. Agroecosystems rely on biodiversity to ensure optimal performance: Garbach et al.

(2014) even describe biodiversity as a 'biological insurance' for agroecosystem resilience in the event of abrupt environmental change. Such insurance is of utmost importance in the current context and challenges we face, regarding ecosystem resilience and food security; as appointed by UN targets for 2030: (2.3) to double the agricultural productivity and incomes of small-scale food producers (…) and (2.4) to ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, that help maintain ecosystems, that strengthen capacity for adaptation to climate change, extreme weather, drought, flooding and other disasters and that progressively improve land and soil quality (UN General assembly, 2015).

Furthermore, it is likely due to small-scale management that rodent species can coexist so easily in these agricultural contexts, including the threatened voles. If plant diversity would be reduced drastically, as a result of ecosystem services loss and/or deliberate system intensification; niche partitioning opportunities could be reduced, and competition could increase. In a scenario of highly homogenous agroecosystems, featuring very low ground cover and elimination of surrounding weed/ grassy patches, it would be more likely for species to come into direct competition over plant genera that are overlapping in their diets. This leads us to consider how traditional olive groves could be regarded as potential conservation units. Which is useful as Portugal is largely comprised of agroecosystems, many of which are olive groves or systems with similar functional structure.

New insights on rodent diets can help inform guidelines towards both a) preserving suitable conditions for species conservation and b) managing vole populations within native range. For instance, promotion of highly consumed plant taxa may favour *M. cabrerae* and *A. sapidus'* populations; contributing to improve their conservation status. We recommend promotion of plant genera that are consumed by one species and not the other, for instance *Festuca* + *Daucus* for A. sapidus and *Bromus* + *Convolvulus* for *M. cabrerae*, or other combinations that would divert them from competing directly over genus *Dactylis*.

In the case of *M. lusitanicus* (regarded as pest), such detailed knowledge of most consumed plant genera may aid in the need to divert rodents from feeding inside olive groves. It is possible to increase other food options valued by *M. lusitanicus,* such as *Bromus, Geranium* or *Lolium*, in an effort to avoid crop damage in support of small-scale producers.

Our results offer a detailed list of significantly consumed genera, but also point to one consistent notion: the maintenance of high plant diversity, through practice of extensive agriculture, helps maintain a species-rich rodent community. Traditional land use, in combination with continuous historical management practices, promotes the very diversity that results in opportunities for rodent species to explore differently.

5.4. Future perspectives

Regarding future research, one possibility would be to follow a similar statistical analysis but including habitat of origin for each sample. Habitats of origin, which may be classified in a number of categories, such tall grass patch, hedge, among others, may be incorporated as a variable. This could be interesting to compare with the work of Barão et al. (2022), which used a similar classification of habitats for their Occupancy model study in the same area. Additionally, it could be interesting to integrate local plant taxa abundance data with the dietary analysis, where the plant taxa present within each unit would be taken into direct account when producing results. This may even inform on rodent dietary preference (present plant taxa *vs* consumed ones).

Moreover, other factors influencing plant availability could be considered: for instance, seasonality. This study was based on a single sampling effort in late Spring/ early Summer, where a higher number of plant taxa would be in bloom. Yet dynamics regarding Fall/Winter could be equally interesting to explore as they may reveal other aspects of dietary overlap and possible niche partitioning.

Another perspective could be to look at not only the rodent communities, but perceive them more so as small mammal communities (which would include both Rodentia and Eulypotiphla). Although Rodentia are considered mostly herbivore, there could be a degree of dietary overlap with insectivore Eulypotiphla, considering rodents can occasionally feed on food items other than plants. Additionally, all small mammals display a degree of similarity in behaviours and ecological roles; hence interactions between Rodentia and Eulipotyphla are likely to occur on multiple levels, which could include trophic web interactions.

6.Conclusion

Diet descriptions obtained were highly detailed for all 5 species of the rodent communities. We ranked top 10 consumed plant families, as well as plant genera, and identified prevalent plant taxa across multiple diets. Asteraceae, Poaceae and Fagaceae were highly consumed families; as well as genera *Lolium* and *Dactylis*. We revealed that *A. sapidus* diet is comprised mostly of *Dactylis*, *Festuca*, *Daucus* and *Lolium*; and that *M. cabrerae* also consumed mostly *Dactylis*, *Bromus*, *Convolvulus* and *Trifolium*. We measured consumption of olive tree with both trnL and ITS2 data, presenting Oleaceae and *Olea* intake. We also point to how *Microtus lusitanicus* consumed easy to promote alternatives to olive tree, such as *Bromus, Geranium* or *Lolium*. We conclude that rodent diets are highly overlapping in our study area, yet species may be avoiding competition by exploring different resources available in olive groves. Our results have a wide variety of applications ranging from agroecosystem management to mammalian conservation. Trás-os-Montes' mosaic landscapes provide a range of dietary opportunities for rodent communities; which play seemingly small, yet critical ecological roles, towards agroecosystem equilibrium and productivity; acting as "biological insurance" for olive grove resilience in the years to come. In turn, these systems provide suitable habitat for species of under conservation concern, despite their overlapping diets. We also conclude there is an olive consumption, which leads us to consider how olive groves could be regarded as potential conservation units; proving useful considering Portugal is largely comprised of agroecosystems. In summary, we suggest that it is possible to minimize impact of potential pests through maintenance of both plant and rodent biodiversity, supported by traditional management practices.

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8. Supplementary material

Appendix I - Gamma diversity analysis

Table S1. Estimated richness at level=0.9786 for trnL data. LCL= lower confidence level and UCL = upper confidence level for qD.

Assemblage	T	Method	Order.q	SC	qD	qD.LCL	**qD.UCL**
A. sylvaticus	76.842	Rarefaction	0	0.979	27.063	8.090	46.035
A. sapidus	47.312	Rarefaction	0	0.978	13.862	6.096	21.628
M. cabrerae	62.736	Rarefaction	0	0.979	26.055	22.982	29.128
M. lusitanicus	50.977	Rarefaction	0	0.979	24.806	14.554	35.059
M. spretus	14.794	Rarefaction	0	0.979	19.807	15.958	23.656

Table S2. Estimated richness at level=0.7704 for ITS2 data. LCL= lower confidence level and UCL = upper confidence level for qD.

Assemblage	T	Method	Order.q	SC	qD	qD.LCL	**qD.UCL**
A. sylvaticus	15.243	Rarefaction	0	0.770	45.930	42.638	49.223
A. sapidus	5.681	Rarefaction	0	0.770	18.373	15.701	21.045
M. cabrerae	13.279	Rarefaction	0	0.770	45.541	43.301	47.782
M. lusitanicus	14.605	Rarefaction	0	0.770	38.628	33.909	31.774
M. spretus	15.379	Rarefaction	0	0.765	33.075	14.839	16.991

Appendix II – Alfa diversity analysis

Table S3. Variable (= species) significance test relative to *A. sylvaticus* using a glm for trnL data. Std Error = standard deviation error.

(Intercept)	Estimate	Std Error	Z value	Pr (>|z|)
A. sapidus	-0.108	0.120	-0.901	0.368
M. cabrerae	-0.013	0.059	-0.215	0.830
M. lusitanicus	0.024	0.082	0.296	0.767
M. spretus	0.175	0.122	1.440	0.150

Table S4. Variable (= species) significance test relative to *A. sylvaticus* using a glm for ITS2 data. Std Error = standard deviation error. Significant Pr (p) values are marked in bold.

(Intercept)	Estimate	Std Error	Z value	Pr (>|z|)
A. sapidus	0.122	0.106	1.150	0.250
M. cabrerae	0.128	0.064	2.014	**0.044**
M. lusitanicus	-0.091	0.090	-1.002	0.316
M. spretus	-0.231	0.137	.1.679	0.093

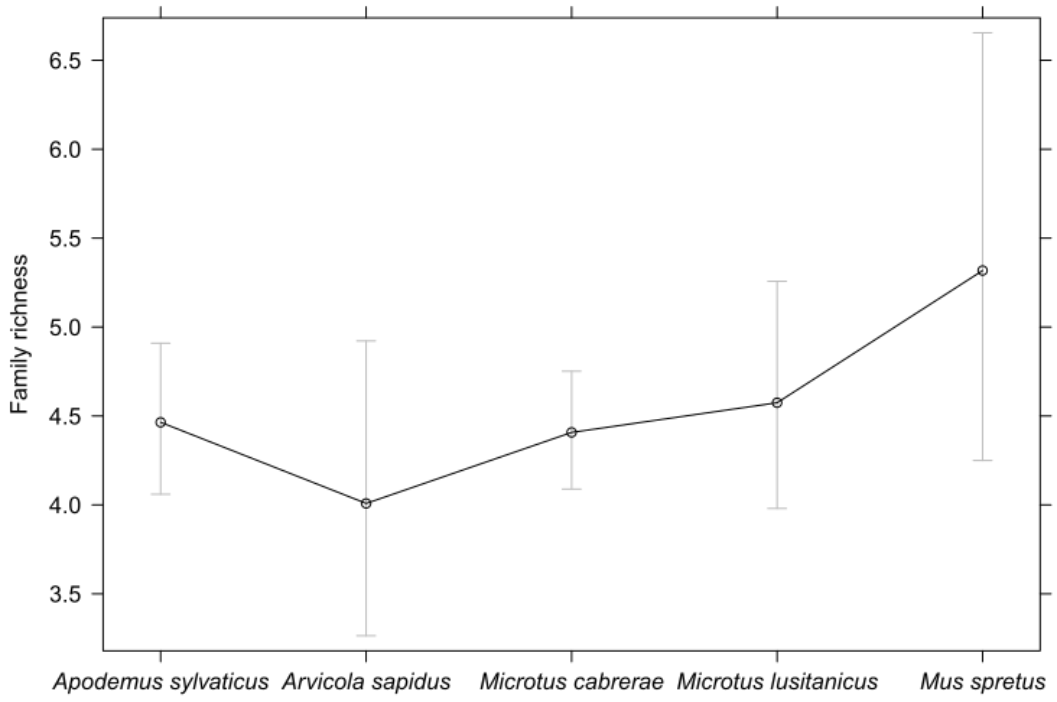

Figure S1: Species effect plot for trnL data.

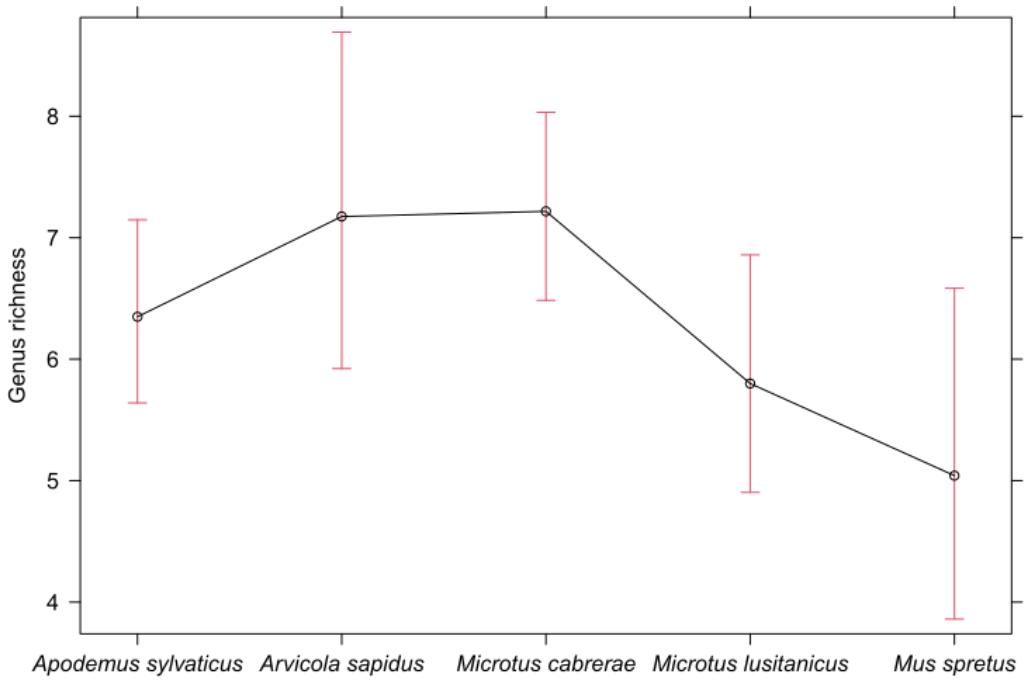

Figure S2: Species effect plot for ITS2 data.

Table S5. Multiple comparison Tukey contrasts between species pairs using trnL data.

Linear Hypotheses	Estimate	Std Error	Z value	Pr (>|z|)
A. sapidus – A. sylvaticus == 0	-0.108	0.120	-0.901	0.887
M. cabrerae – A. sylvaticus == 0	-0.013	0.060	-0.215	0.999
M. lusitanicus – A. Sylvaticus == 0	0.024	0.082	0.296	0.998
M. spretus – A. sylvaticus == 0	0.175	0.122	1.440	0.579
M. cabrerae – A. sapidus == 0	0.095	0.104	0.912	0.882
M. lusitanicus – A. sapidus == 0	0.132	0.124	1.062	0.811
M. spretus – A. sapidus == 0	0.283	0.150	1.884	0.304
M. lusitanicus – M. cabrerae == 0	0.037	0.079	0.483	0.989
M. spretus – M. cabrerae == 0	0.188	0.118	1.592	0.478
M. spretus – M. lusitanicus == 0	0.151	0.126	1.200	0.733

Table S6. Multiple comparison Tukey contrasts between species pairs using ITS2 data.

Linear Hypotheses	Estimate	Std Error	Z value	Pr (>|z|)
A. sapidus – A. sylvaticus == 0	0.122	0.106	1.150	0.762
M. cabrerae – A. sylvaticus == 0	0.128	0.064	2.014	0.240
M. lusitanicus – A. Sylvaticus == 0	-0.091	0.090	-1.002	0.841
M. spretus – A. sylvaticus == 0	-0.231	0.137	-1.679	0.422
M. cabrerae – A. sapidus == 0	0.006	0.091	0.065	1.000
M. lusitanicus – A. sapidus == 0	-0.213	0.123	-1.733	0.380
M. spretus – A. sapidus == 0	-0.353	0.161	-2.195	0.167
M. lusitanicus – M. cabrerae == 0	-0.219	0.091	-2.395	0.106
M. spretus – M. cabrerae == 0	-0.359	0.137	-2.635	0.059
M. spretus – M. lusitanicus == 0	-0.140	0.140	-1.002	0.841

Appendix lll - Beta diversity analysis

Table S7. PERMANOVA model multiple comparison pairs using trnL data. Significant p values are in bold.

Linear Hypotheses	SumofSqs	MeanSqs	F.Model	R2	P value	P value corrected
A. sapidus – A. sylvaticus == 0	10.603	10.603	38.094	0.175	0.000	**0.000**
M. cabrerae – A. sylvaticus == 0	14.972	14.972	52.106	0.116	0.000	**0.000**
M. lusitanicus – A. Sylvaticus == 0	14.971	14.972	12.143	0.062	0.000	**0.000**
M. spretus – A. sylvaticus == 0	1.876	1.876	6.046	0.039	0.000	**0.000**
M. cabrerae – A. sapidus == 0	4.816	4.816	18.341	0.055	0.000	**0.000**
M. lusitanicus – A. sapidus == 0	4.238	4.238	16.125	0.139	0.000	**0.000**
M. spretus – A. sapidus == 0	4.863	4.863	21.621	0.253	0.000	**0.000**
M. lusitanicus – M. cabrerae == 0	4.242	4.242	14.900	0.045	0.000	**0.000**
M. spretus – M. cabrerae == 0	4.662	4.662	16.717	0.056	0.000	**0.000**
M. spretus – M. lusitanicus == 0	1.955	1.955	6.014	0.082	0.000	**0.000**

Table S8. PERMANOVA model multiple comparison pairs using ITS2 data. Significant p values are in bold.

Linear Hypotheses	SumofSqs	MeanSqs	F.Model	R2	P value	P value corrected
A. sapidus – A. sylvaticus == 0	7.558	7.558	19.677	0.099	0.001	**0.001**
M. cabrerae – A. sylvaticus == 0	6.673	6.673	16.655	0.040	0.001	**0.001**
M. lusitanicus – A. Sylvaticus == 0	2.900	2.900	6.964	0.036	0.001	**0.001**
M. spretus – A. sylvaticus == 0	1.649	1.649	3.953	0.026	0.001	**0.001**
M. cabrerae – A. sapidus == 0	5.586	5.586	14.956	0.046	0.001	**0.001**
M. lusitanicus – A. sapidus == 0	5.355	5.355	15.536	0.135	0.001	**0.001**
M. spretus – A. sapidus == 0	3.463	3.463	11.316	0.150	0.001	**0.001**
M. lusitanicus – M. cabrerae == 0	3.288	3.288	8.380	0.027	0.001	**0.001**
M. spretus – M. cabrerae == 0	3.030	3.030	7.775	0.027	0.001	**0.001**
M. spretus – M. lusitanicus == 0	2.061	2.061	5.178	0.071	0.001	**0.001**

Appendix IV- Plant library

Table S9. Plant taxa present in the study area, including common names in English and Portuguese.

scientific name	English name	Portuguese name
***Adenocarpus complicatus* (L.) J.Gay**	-	codesso-do-interior
***Aegilops triuncialis* L.**	barbed goatgrass	trigo-de-perdiz
***Agrimonia eupatoria* L.**	common agrimony	agrimónia
***Agrostemma githago* L.**	common corn-cockle	axenuz
***Agrostis castellana* Boiss. & Reut.**	highland bent	barbas-de-raposa
***Aira caryophyllea* L.**	silver hairgrass	-
Airopsis tenella (Cav.) Asch. & Graebn.	-	-
***Alchemilla arvensis* (L.) Scop.**	parsley-piert	falsa-salsa
***Alchemilla australis* (Rydb.) Bomble**	slender parsley-piert	falsa-salsa-do-sul
***Allium pallens* L.**	pale garlic	alho-paniculado
***Allium vineale* L.**	wild garlic	alho-das-vinhas
***Alyssum serpyllifolium* Desf.**	thyme-leaved alison	tomelos
***Amaranthus hybridus* L.**	green amaranth	bredo
***Anacamptis coriophora* (L.) R.M.Bateman, Pridgeon & M.W.Chase**	bug orchid	erva-perceveja
***Anagallis arvensis* L.**	scarlet pimpernel	morrião
***Anarrhinum bellidifolium* (L.) Willd.**	-	samacalo
***Anarrhinum duriminium* (Brot.) Pers.**	-	samacalo-peludo
***Anchusa arvensis* (L.) M.Bieb.**	small bugloss	língua-de-vaca
***Andryala integrifolia* L.**	-	tripa-de-ovelha
***Anthemis arvensis* L.**	corn chamomile	falsa-camomila
***Anthemis cotula* L.**	stinking chamomile	macela-fétida
***Anthriscus caucalis* M.Bieb.**	burr chervil	antriscos
***Anthyllis vulneraria* L.**	kidney vetch	vulnerária-amarela
***Antirrhinum rothmaleri* (Pinto da Silva) Amich, Bernardos & García-Barrius**	snapdragon	bocas-de-lobo-de-trás-os-montes
***Apium nodiflorum* (L.) Lag.**	fool's watercress	rabaça
***Arenaria montana* L.**	mountain sandwort	arísaro
***Arenaria serpyllifolia* L.**	thyme-leaf sandwort	dente-de-cão
***Aristolochia paucinervis* (Pomel)**	birthworth	estrelamim
***Armeria* sp.**	lady's cushion	-
***Asparagus acutifolius* L.**	wild asparagus	espargo-bravo-menor
***Asplenium adiantum-nigrum* L.**	black spleenwort	feto-negro

***Asplenium trichomanes* L.**	maidenhair spleenwort	avencão
Asterolinon linum-stellatum	-	falso-linho-estrelado
***Astragalus cymbaecarpos* Brot.**	milkvetch	saveirinho
***Avena barbata* Link**	slender wild oat	aveia-barbada
***Avena fatua* L.**	common wild oat	aveia-brava
***Avena sterilis* L.**	animated oat	aveão
***Bartsia trixago* L.**	Mediterranean Linseed	flor-de-ouro
***Bellis perennis* L.**	daisy	margarida
***Bellis sylvestris* Cirillo**	southern daisy	margarida-do-monte
***Brachypodium distachyon* (L.) P.Beauv.**	purple false brome	braquipódio-de-duas-espigas
***Brachypodium phoenicoides* (L.) Roem. & Schult.**	thinleaf false brome	braquipódio-avermelhado
***Brachypodium sylvaticum* (Huds.) P.Beauv.**	slender false brome	braquipódio-bravo
***Brassica barrelieri* (L.) Janka**	-	labresto
***Briza maxima* L.**	big quaking grass	bole-bole-maior,
***Briza minor* L.**	little quaking grass	bole-bole-menor
***Bromus diandrus* Roth**	great brome	fura-capa
***Bromus sterilis* L.**	barren brome	-
***Bryonia dioica* Jacq.**	white bryony	briónia-branca
***Bunias erucago* L.**	corn rocket	grizandra
***Bupleurum gerardii* All.**	-	bupleuro-de-gerard
***Callitriche stagnalis* Scop.**	pond water-starwort	lentilhas-da-água
***Campanula lusitanica* L.**	Lusitanian bellflower	campainhas
***Campanula rapunculus* L.**	rampion bellflower	campainhas-rabanete
***Capsella bursa-pastoris* (L.) Medik.**	shepherd's purse	bolsa-de-pastor
***Carduus tenuiflorus* Curtis**	slender-flower thistle	cardo-azul
***Carduus carpetanus* Boiss. & Reut.**	-	-
***Carduus pycnocephalus* L.**	cardo-italiano	Italian thistle
***Carex flacca* Schreb.**	blue sedge	carriço-mole
***Carex spicata* Huds.**	spiked sedge	-
***Carlina corymbosa* L.**	cardo-amarelo	clustered carline thistle
***Carlina racemosa* L.**	-	cardo-asnil
***Carthamus lanatus* L.**	woolly distaff thistle	Cardo-beija-mão
***Carum verticillatum* (L.) W.D.J.Koch**	Whorled Caraway	alcaravia
***Centaurea calcitrapa* L.**	purple star thistle	cardo-estrelado
***Centaurea jacea* L.**	brown knapweed	-
***Centaurea ornata* Willd.**	-	cardazol

***Centaurea solstitialis* L.**	yellow star-thistle	-
***Centaurium grandiflorum* (Pers.) Ronniger**	-	-
***Centranthus calcitrapae* (L.) Dufr.**	annual valerian	calcitrapa
***Cerastium brachypetalum* Desp. ex Pers.**	gray mouse-ear chickweed	-
***Cerastium glomeratum* Thuill.**	sticky mouse-ear chickweed	orelha-de-rato
***Chamaemelum nobile* (L.) All.**	garden chamomile	Camomila-de-Paris
***Chelidonium majus* L.**	greater celandine	erva-andorinha
***Chenopodium album* L.**	lamb's quarters	ansarina-branca
***Chenopodium murale* L.**	nettle-leaved goosefoot	pé-de-ganso
***Chondrilla juncea* L.**	rush skeletonweed	leituga-branca
***Chrysantemum segetum* L.**	corn marigold	pampilho-das-searas
***Cichorium intybus* L.**	Common chicory	chicória
Cirsium arvense	creeping thistle (L.) Scop.	cardo-das-vinhas
***Cistus ladanifer* L.**	gum rockrose	esteva
***Cistus psilosepalus* Sweet**	-	sanganho
***Cladanthus mixtus* (Linnaeus) Chevallier**	Cladanthus mixtus	margaça-das-searas
***Clematis* sp.**	-	clematite
***Clinopodium vulgare* L.**	wild basil	clinopódio
Cnicus benedictus	St. Benedict's thistle	cardo-santo
***Coincya monensis* (L.) Greuter & Burdet**	alpine wallflower	saramago-de-bico-curvo
***Coleostephus myconis* (L.) Rchb.f.**	corn marigold	malmequer
***Conium maculatum* L.**	hemlock	cicuta
***Conopodium subcarneum* (Boiss. & Reut.) Boiss. & Reut.**	-	-
***Convolvulus arvensis* L.**	field bindweed	corriola-campestre
***Coronilla repanda* (Poir.) Guss.**	-	pascoinhas
***Corrigiola telephiifolia* Pourr.**	strapwort	erva-pombinha-áfila
***Corynephorus canescens* (L.) P.Beauv.**	grey hair-grass	erva-pichoneira
***Crassula alata* (Viv.) A.Berger**	three-parted Crassula	-
***Crataegus monogyna* Jacq.**	common hawthorn	branca-espinha
***Crepis capillaris* (L.) Wallr.**	smooth hawksbeard	almeirão-branco
***Crepis foetida* L.**	stinking hawksbeard	-
Crepis vesicaria (Thuill.) Thell.	beaked hawk's-beard	almeiroa
***Crucianella angustifolia* L.**	crosswort	granza
***Cucubalus baccifer* L.**	berry-bearing catchfly	erva-cuco
***Cynodon* sp.**	dog's tooth grass	erva-gramilheira

***Cynosurus cristatus* L.**	crested dog's-tail	rabo-de-cão-empenachado
***Cynosurus echinatus* L.**	bristly dogstail grass	rabo-de-cão-eriçado
***Cyperus longus* L.**	sweet cyperus	junça-de-cheiro
***Cytisus multiflorus* (L'Hér.) Sweet**	white broom	giesta-branca
***Cytisus scoparius* (L.) Link**	common broom	giesta-amarela
***Cytisus striatus* (Hill) Rothm.**	hairy-fruited broom	giesta-negral
***Dactylis glomerata* L.**	cock's-foot	pé-de-galo
***Daphne gnidium* L.**	flax-leaved daphne	trovisco
***Datura stramonium* L.**	thorn apple	castanheiro-do-diabo
***Daucus carota* L.**	wild carrot	cenoura-brava
***Daucus durieua* Lange**	-	cenoura-duriense
***Deschampsia* sp.**	hair grass	feno
***Dianthus laricifolius* Boiss. & Reut.**	-	cravina-transmontana
***Dianthus lusitanus* Brot.**	-	cravina-brava
***Digitalis thapsi* L.**	mullein foxglove	aboleira
Dipsacus fullonum L.	wild teasel	cardo-penteador-do-norte
***Dorycnium pentaphyllum* Scop.**	badassi	erva-mata-pulgas
***Echinochloa* sp.**	barnyard grass	-
***Echium plantagineum* L.**	purple viper's-bugloss	língua-de-vaca
***Echium vulgare* L.**	viper's bugloss	soagem-do-nordeste
***Eleocharis palustris* (L.) Roem. & Schult.**	marsh spike-rush	Junco-marreco
***Epilobium brachycarpum* C. Presl.**	tall willowherb	epilóbio-esguio*
***Epipactis helleborine* (L.) Crantz**	broad-leaved helleborine	heleborina-comum
***Equisetum arvense* L.**	field horsetail	cavalinha
***Eragrotstis minor* Host**	little lovegrass	-
***Erica scoparia* L.**	green heather	urze-das-vassouras
***Erodium botrys* (Cav.) Bertol.**	longbeak stork's bill	bico-de-cegonha
***Erodium cicutarium* (L.) L'Hér.**	common stork's-bill	bico-de-cegonha-das-areias
***Erodium moschatum* (L.) L'Hér.**	musk stork's-bill	bico-de-cegonha-mosqueado
***Eryngium campestre* L.**	Watling Street thistle	cardo-corredor
***Erysidum linifolium* (Pourr. ex Pers.) J.Gay**	wallflower	goivo-roxo-dos-montes
***Euphorbia exigua* L.**	dwarf spurge	ésula-menor
***Euphorbia falcata* L.**	sickle spurge	leiteira-das-três-quilhas
***Euphorbia oxyphylla* Boiss.**	-	leitarega
***Euphorbia segetalis* L.**	grainfield spurge	alforva-brava
***Fallopia convolvulus* (L.) Á.Löve**	black-bindweed	erva-feijoeira

***Festuca murallis* Kunth**	fescue	-
***Filago* sp.**	cottonroses	erva-dos-moinhos
***Filipendula vulgaris* Moench**	dropwort	floristas
***Foeniculum vulgare* Mill.**	funcho	fiolho
***Fraxinus angustifolia* Vahl**	narrow-leaved ash	freixo-comum
***Fumaria bastardii* Boreau**	bastard's fumitory	erva-muleirinha
***Fumaria reuteri* Boiss.**	Martin's Ramping-fumitory	fumária-arvense
***Galium aparine* L.**	cleavers	amor-de-hortelão
***Galium mollugo* L.**	hedge bedstraw	aspérula
***Galium palustre* L.**	common marsh bedstraw	raspa-língua
***Galium papillosum* Lapeyr.**	-	-
***Galium parisiense* L.**	wall bedstraw	solda
***Galium verum* L.**	yellow bedstraw	erva-coalheira
***Gallium mollugo* L.**	hedge bedstraw	solda-branca
***Gamochaeta purpurea* (L.) Cabrera**	purple cudweed	macela-da-folha-fina
***Gastridium ventricosum* (Gouan) Schinz & Thell.**	nit-grass	gastrídio-bojudo
***Gaudinia fragilis* (L.) P.Beauv.**	-	argençana-dos-pastores
***Genista anglica* L.**	petty whin	aliaga
***Geranium columbinum* L.**	long-stalked crane's-bill	bico-de-pomba-maior
***Geranium dissectum* L.**	cut-leaved crane's-bill	bico-de-pomba
***Geranium lucidum* L.**	shining cranesbill	gerânio
***Geranium molle* L.**	dove's-foot crane's-bill	bico-de-pomba-menor
***Geranium purpureum* Vill.**	little-robin	erva-de-são-roberto
***Geranium pyrenaicum* (Samp.) S.Ortiz**	mountain cranesbill	gerânio-de-folha-grande
Geranium rotundifolium	roundleaf geranium	gerânio-peludo
***Geum sylvaticum* Pourr.**	-	erva-benta-dos-bosques
***Geum urbanum* L.**	herb bennet	erva-benta
***Glyceria declinata* Bréb.**	waxy mannagrass	azevém-baboso
***Halimium umbellatum* (L.) Spach**	false sun-rose	erva-sargacinha
***Hedypnois cretica* (L.) Dum.-Courset**	cretanweed	alface-de-Creta
***Helichrysum stoechas* (L.) Moench**	Mediterranean strawflower	marcenilha
***Helictochloa* sp.**	-	-
***Heliotropium europaeum* L.**	potato weed	erva-das-verrugas
***Herniaria glabra* L.**	smooth rupturewort	herniária-glabra
***Hispidella hispanica* Barnades ex Lam.**	-	-

***Holcus lanatus* L.**	tufted grass	erva-lanar
***Holcus mollis* L.**	creeping soft grass	erva-molar
***Hordeum geniculatum* All.**	wall Barley	-
***Hordeum murinum* L.**	false barley	cevada-das-lebres
***Humulus lupulus* L.**	common hop	pé-de-galo
***Hymenocarpos cornicina* (L.) Vis.**	-	vulnerária-do-nordeste
***Hymenocarpos lotoides* (L.) Vis.**	-	vulnerária-reta
***Hypericum humifusum* L.**	erva-das-mil-folhinhas	hipericão-rasteiro
***Hypericum linarlifolium* Vahl**	-	hipericão-estriado
***Hypericum montanum* L.**	-	-
***Hypericum perforatum* L.**	St. John's wort	Erva-de-são-joão
***Hypochaeris radicata* L.**	Hairy cat's ear	leituga
***Isoetes* sp.**	quillworts	-
***Jasione montana* L.**	blue bonnets	botão-azul
***Juglans regia* L.**	English walnut	nogueira
Juncus conglomeratus	jointleaf	junco-glomerato
***Juncus inflexus* L.**	hard rush	junco-curvado
***Juniperus oxycedrus* L.**	cade	zimbro
***Lactuca serriola* L.**	prickly lettuce	alface-brava
***Lactuca viminea* (L.) J.Presl & C.Presl**	pliant lettuce	-
***Lamium amplexicaule* L.**	common henbit	urtiga-branca
***Lamium maculatum* L.**	spotted dead-nettle	chuchas
***Lathyrus angulatus* L.**	angled pea	cizirão-de-folha-estreita
***Lathyrus hirsutus* L.**	Caley pea	cizirão-eriçado
***Lathyrus pratensis* L.**	meadow vetchling	chícharo-dos-prados
***Lathyrus sphaericus* Retz.**	grass pea	cizirão-redondo
***Leontodon taraxacoides* (Vill.) Mérat**	rough hawkbit	leituga-dos-montes
***Lepidium heterophyllum* Benth.**	Smith's Cress	lepídio
***Leucanthemopsis pulverulenta* (Lag.) Heywood**	-	margarida-pulverenta
***Ligustrum vulgare* L.**	common privet	alfenheiro
***Linaria amethystea* (Lam.) Hoffmanns. & Link**	-	-
***Linaria saxatilis* (L.) Chaz.**	rock toadflax	linária-das-rochas
Linaria spartea (L.) Chaz.	ballast toadflax	ansarina-dos-campos
***Linum bienne* Mill.**	pale flax	linho-bravo
***Logfia gallica* (L.) Coss. & Germ.**	daggerleaf cottonrose	erva-dos-moinhos
***Logfia minima* (Sm.) Dumort.**	little false cotton-rose	-
***Lolium arundinaceum* (Schreb.) Darbysh.**	tall fescue	erva-corneira
***Lolium rigidum* Gaudin**	annual ryegrass	azevém
Lonicera etrusca Santi	Etruscan honeysuckle	madressilva

***Lotus corniculatus* L.**	bird's-foot trefoil	cornichão
***Lotus pedunculatus* Cav.**	big trefoil	erva-coelheira
***Lupinus angustifolius* L.**	narrowleaf lupin	tremoceiro-bravo
***Luzula foresteri* (Sm.) DC.**	southern wood-rush	-
***Lythrum hyssopifolia* L.**	hyssop loosestrife	salgueirinho
***Magydaris panacifolia* (Vahl) Lange**	-	bruco-de-folha-larga
***Malus sylvestris* (L.) Mill.**	crab apple	macieira-brava
Malva nicaeensis All.	bull mallow	malva-pequena
***Malva parviflora* L.**	cheeseweed	malva-de-flor-pequena
***Malva sylvestris* L.**	common mallow	malva-maior
Malva tournefortiana L.	-	malva-de-folha-recortada
***Margotia gummifera* (Desf.) Lange**	-	bruco-fétido
***Medicago arabica* (L.) Huds.**	spotted medick	luzerna-negra
***Medicago sativa* L.**	alfafa	luzerna
***Melica ciliata* L.**	hairy melic grass	mélica-ciliada
***Mentha suaveolens* Ehrh.**	apple mint	hortelã-brava
***Mibora minima* (L.) Desv.**	early sand grass	-
***Micropyrum tenellum* (L.) Link**	-	-
***Misopates orontium* (L.) Raf.**	Weasel's Snout	focinho-de-rato
***Moehringia pentandra* Gay**	-	arenária-de-cinco-estames
Montia fontana L.	water blinks	Meruje
***Muscari comosum* (L.) Mill.**	tassel hyacinth	jacinto-de-topete
***Myosotis discolor* Pers.**	forget-me-not	não-me-esqueças
***Nasturtium officinale* R. Brown**	watercress	Agrião
***Odontitella virgata* (Link) Rothm.**	-	mata-pulga
***Oenanthe crocata* L.**	hemlock water dropwort	Rabaças
***Olea europaea* L.**	common olive	oliveira-brava
***Ononis spinosa* L.**	spiny restharrow	unha-gata
***Ophioglossum vulgatum* L.**	adder's-tongue	língua-de-cobra
***Origanum vulgare* L.**	oregano	Orégãos
***Ornithogalum bourgaeanum* Jord. & Fourr.**	-	leite-de-galinha
***Ornithopus compressus* L.**	yellow serradela	serradela-amarela
***Orobanche calendulae* Pomel**	-	erva-toira-barbuda
***Orobanche minor* Sm.**	common broomrape	erva-toira-menor
***Orobanche rapum-genistae* Thuill.**	greater broomrape	erva-toira-maior
***Osyris alba* L.**	white osyris	cássia-branca
***Paeonia broteri* Boiss. & Reut.**	-	rosa-albardeira
***Pallenis spinosa* (L.) Cass.**	spiny starwort	pampilho-espinhoso
***Papaver argemone* L.**	long pricklyhead poppy	papoila-longa-peluda

***Papaver rhoeas* L.**	common poppy	papoila-comum
***Parentucellia latifolia* (L.) Caruel**	red tarweed	-
***Parietaria judaica* L.**	pellitory of the wall	alfavaca-de-cobra
***Pentaglottis sempervirens* (L.) L.H.Bailey**	green alkanet	olhos-de-gato
Persicaria sp.	fleeceflowers	-
***Petrorhagia nanteuilii* (Burnat) P.W.Ball & Heywood**	childing pink	cravina-vulgar
***Phalaris* sp.**	-	-
***Phillyrea angustifolia* L.**	narrow-leaved mock privet	Lentisco
Phleum pratense L.	timothy grass	Timóteo
Picnomon acarna (L.) Cass.	soldier thistle	cardo-espinhoso-das-pastagens
***Picris echioides* L.**	bristly Oxtongue	raspa-saias
***Pilosella officinarum* Vaill.**	mouse-ear hawkweed	pilosela-das-boticas
***Pilosella pseudopilosella* (Ten.) Soják**	-	-
***Pimpinella villosa* Schousb**	-	pimpinela
***Pistacia terebinthus* L.**	turpentine tree	cornalheira
***Plantago coronopus* L.**	buck's-horn plantain	diabelha
***Plantago lanceolata* L.**	ribwort plantain	corrijó
***Plantago subulata* L.**	haircap moss	-
***Poa bulbosa* L.**	bulbous bluegrass	-
***Poa trivialis* L.**	rough bluegrass	relvão
***Polycarpon tetraphyllum* (L.) L.**	four-leaved allseed	saboneteira
Polygala vulgaris L.	common milkwort	erva-leiteira
***Polygonum* sp.**	knotweeds	-
Portulaca oleracea L.	purslane	beldroega
Potentilla reptans L.	creeping cinquefoil	cinco-em-rama
Prunella laciniata (L.) L.	cutleaf self heal	-
***Prunus dulcis* (Mill.) D.A.Webb**	almond	amendoeira
***Prunus insititia* L.**	damsom plum	abrunheiro-bravo
Prunus mahaleb L.	mahaleb cherry	Ginjerineira
***Pteridium aquilinum* (L.) Kuhn**	bracken fern	feto-dos-montes
***Pulicaria paludosa* Link**	Spanish false fleabean	erva-pulgeuira
***Quercus faginea* Lam.**	Portuguese oak	carvalho-cerquinho
***Quercus pyrenaica* Willd.**	Pyrenean oak	carvalho-negral
***Quercus rotundifolia* Lam.**	holm oak	Azinheira
***Ranunculus arvensis* L.**	corn buttercup	ranúnculo-dos-campos
***Ranunculus bulbosus* L.**	bulbous buttercup	ranúnculo-bolboso
***Ranunculus ficaria* L.**	fig buttercup	celidónia-menor
***Ranunculus gramineus* L.**	grass buttercup	ranúnculo-elegante

***Ranunculus muricatus* L.**	spinyfruit buttercup	Burgalhó
***Ranunculus parviflorus* L.**	smallflower buttercup	ranúnculo-de-flor-pequena
***Ranunculus repens* L.**	creeping buttercup	botão-de-oiro
***Raphanus raphanistrum* L.**	wild radish	saramago
***Reseda luteola* L.**	dyer's rocket	lírio-dos-tintureiros
***Reseda virgata* Boiss. & Reut.**	yellow mignonete	reseda-cavalar
***Retama sphaerocarpa* (L.) Boiss.**	yellow broom	piorno-amarelo
***Rosa corymbifera* Borkh.**	dog rose	rosa-brava
***Rubia peregrina* L.**	common wild madder	ruiva-brava
***Rubus lainzii* H.E.Weber**	-	silva-de-lainz
***Rubus ulmifolius* Schott**	elmleaf blackberry	silva-comum
***Rubus vestitus* Weihe**	European blackberry	-
Rumex acetosella (Murb.) Murb.	sheep sorrel	Azedinhas
***Rumex bucephalophorus* L.**	ruby-dock	azeda-de-cão
***Rumex conglomeratus* Murray**	sharp dock	Labaça
***Rumex crispus* L.**	curly dock	labaça-crespa
***Rumex induratus* Boiss. & Reut.**	-	azedão
***Rumex intermedius* DC.**	-	labaça-intermédia
***Ruscus aculeatus* L.**	butcher's broom	gilbardeira
***Ruta montana* (L.) L.**	mountain rue	arrudão
***Salix atrocinerea* Brot.**	grey willow	borrazeira
***Salvia verbenaca* L.**	wild sage	salva-dos-caminhos
***Sanguisorba minor* Scop.**	salad burnet	pimpinela-menor
***Sanguisorba verrucosa* (Link ex G.Don) Ces.**	-	pimpinela-comum
***Santolina semidentata* Hoffmanns. & Link**	-	abrótano
***Saponaria officinalis* L.**	common soapwort	saboeira
***Saxifraga dichotoma* Willd.**	-	-
***Scandix pecten-veneris* L.**	shepherd's-needle	erva-agulha
***Scilla verna* Huds.**	spring squill	cila-do-verão
***Scirpoides holoschoenus* (L.) Soják**	roundhead bulrush	bunho
***Scleranthus annuus* L.**	annual knawel	erva-dura
***Scorzonera angustifolia* L.**	-	escorcioneira-de-folha-estreita
***Scrophularia scorodonia* L.**	balm-leaved figwort	japão
***Sedum amplexicaule* DC.**	-	favária
***Sedum arenarium* Brot.**	-	-
***Sedum forsterianum* Sm.**	rock stonecrop	arroz-das-paredes
***Sedum hirsutum* All.**	hairy stonecrop	uva-de-gato
***Senecio jacobaea* L.**	ragwort	erva-de-são-tiago
***Serapias lingua* L.**	tongue-orchid	erva-língua

***Serapias parviflora* Parl.**	small-flowered tongue-orchid	erva-língua-menor
***Seseli montanum* (Samp.) M.Laínz**	-	cominho-dos-montes
***Setaria* sp.**	-	-
***Sherardia arvensis* L.**	blue field-madder	granza-dos-campos
***Silene gallica* L.**	common catchfly	nariz-de-zorra
***Silene inaperta* L.**	common catchfly	silene-que-nunca-abre
***Silene portensis* L.**	-	silene-da-manhã
***Silene scabriflora* Brot.**	-	silene-áspera
***Silene vulgaris* (Moench) Garcke**	bladder campion	erva-traqueira
***Silybum marianum* (L.) Gaertn.**	milk thistle	cardo-de-santa-maria
***Sisymbrium officinale* (L.) Scop.**	hedge mustard	rinchão
***Smyrnium olusatrum* L.**	alexanders	aipo-dos-cavalos
***Solanum chenopoidioides* Lam.**	whitetip nightshade	-
***Solanum dulcamara* L.**	bittersweet nightshade	doce-amarga
***Solanum nigrum* L.**	black nightshade	erva-moira
***Sonchus asper* (L.) Hill**	prickly sowthistle	serralha-áspera
***Sonchus oleraceus* L.**	common sowthistle	serralha-branca
***Spergula arvensis* L.**	corn spurry	cassamelo
***Spergularia purpurea* (Pers.) G.Don**	purple sandspurry	sapinho-roxo
***Stellaria graminea* L.**	common starwort	estrelária-herbácea
***Stellaria holostea* L.**	greater stitchwort	estrelária-de-folha-estreita
***Stipa gigantea* Link**	giant needle grass	aveia-de-oiro
***Taeniatherum caput-medusae* (L.) Nevski**	medusahead	cabeça-de-medusa
***Tamus communis* L.**	black bryony	uva-de-cão
***Taraxacum* sp.**	dandelions	dente-de-leão
***Teucrium scorodonia* L.**	wood sage	salva-bastarda
***Thalictrum speciosissimum* L.**	-	ruibarbo-dos-pobres
***Thapsia villosa* L.**	villous deadly carrot	canavoura
***Thesium humifusum* DC.**	bastard-toadflax	-
***Thymus mastichina* L.**	mastic thyme	bela-luz
***Thymus zygis* L.**	Spanish thyme	sal-purinho
***Tolpis barbata* (L.) Gaertn.**	European Umbrella Milkwort	olho-de-mocho
***Tolpis umbellata* Bertol.**	-	olho-de-mocho-menor
***Tordylium maximum* L.**	Hartwort	-
***Torilis africana* Spreng.**	spreading hedgeparsley	-

***Torilis arvensis* (Huds.) Link**	common hedge parsley	salsinha-dos-campos
***Tragopogon dubius* Scop.**	yellow salsify	-
***Tribulus terrestris* L.**	puncture vine	abrolhos
***Trifolium angustifolium* L.**	narrowleaf crimson clover	trevo-massaroco
***Trifolium arvense* L.**	hare's-foot clover	trevo-dos-campos
***Trifolium bocconei* Savi**	twin-headed clover	trevo-das-pastagens
***Trifolium campestre* Schreb.**	field-clover	trevo-amarelo
***Trifolium cernuum* Brot.**	drooping-flower clover	trevo-de-flores-caídas
***Trifolium cherleri* L.**	southern clover	trevo-entaçado
***Trifolium dubium* Sibth.**	suckling clover	trevinho
***Trifolium glomeratum* L.**	clustered clover	trevo-aglomerado
***Trifolium hirtum* All.**	rose clover	trevo-rosa
***Trifolium pratense* L.**	red clover	trevo-dos-prados
***Trifolium repens* L.**	white clover	trevo-branco
***Trifolium resupinatum* L.**	Persian clover	trevo-da-pérsia
***Trifolium scabrum* L.**	rough clover	trevo-áspero
***Trifolium squamosum* L.**	sea clover	trevo-escamoso
***Trifolium strictum* L.**	upright clover	trevo-direito
***Trifolium subterraneum* L.**	subterranean clover	trevo-subterrâneo
***Trifolium sylvaticum* Gérard ex Loisel.**	-	trevo-dos-bosques
***Trofilum striatum* L.**	knotted-clover	trevo-estriado
***Tuberaria guttata* (L.) Fourr.**	spotted rock-rose	erva-das-túberas
***Umbilicus rupestris* (Salisb.) Dandy**	navelwort	umbigo-de-vénus
***Urospermum picroides* (L.) F.W.Schmidt**	prickly goldenfleece	leituga-de-burro
***Urtica dioica* L.**	stinging nettle	urtiga-maior
***Valerianella coronata* (L.) DC.**	crowned cornsalad	alface-brava-coroada
***Valerianella locusta* (L.) Laterr.**	common cornsalad	alface-de-coelho
***Velezia rigida* L.**	-	cravinho-rígido
***Verbascum pulverulentum* Vill.**	hoary mullein	cássimo
***Verbascum virgatum* Stokes**	Twiggy mullein	blatária-maior
***Verbena officinalis* L.**	common vervain	aljabão
***Veronica anagallis-aquatica* L.**	water speedwell	morrião-da-água
***Veronica arvensis* L.**	wall speedwell	verónica-dos-campos
***Veronica polita* Fr.**	gray field speedwell	-
***Veronica truphyllos* L.**	-	-
***Vicia angustifolia* L.**	narrow-leaved vetch	ervilhaca-miúda
***Vicia hirsuta* (L.) Gray**	tiny vetch	ervilhaca-dos-lameiros
***Vicia lathyroides* L.**	spring-vetch	ervilhaca-dos-arrelvados

***Vicia lutea* L.**	yellow-vetch	ervilhaca-amarela
Vicia parviflora Cav.	slender-vetch	ervilhaca-dos-brejos
***Vicia sativa* L.**	common vetch	ervilhaca-comum
***Vicia villosa* Roth**	hairy vetch	ervilhaca-vilosa
***Viola kitaibeliana* Schult.**	dwarf violet	amor-perfeito
***Xanthium spinosum* L.**	spiny cocklebur	pica-três