UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

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Trabalho realizado sob a orientação de:

Professora Doutora Rita Castilho

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2013

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A realização desta tese foi possível graças ao financiamento da Fundação para a Ciência e Tecnologia, com co-financiamento do Fundo Social Europeu, através da bolsa de doutoramento SFRH/BD/30024/2006.

Declaração de autoria de trabalho:

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Cláudia Sofia Patrão Beliz Rosa e Silva

Agradecimentos / Acknowledgments

Em primeiro lugar quero agradecer à Professora Rita Castilho ter aceite orientar o presente trabalho e por tudo o que me ensinou sobre o mundo da filogenia e filogeografia. Obrigada pelas suas sugestões que permitiram melhorar os manuscritos, por passar-me o gosto pela bioinformática e partilhar comigo, de forma generosa, o seu talento nato para a parte gráfica.

To Thierry, thank you for being so kind with your knowledge, sharing the love you have for science in general and slugs in particular, opening my eyes to a new world – malacology. Your humor, your endless lists of PDF's, even really obscure scientific publications - you are truly the PDF God! I will forever remember all those late night really interesting science discussions in Antwerp/Brussels/Thailand/Azores…

Obrigada Alexandra por me teres mostrado o mundo dos cromossomas, descobri uma nova paixão na ciência, numa área que nunca pensei estudar. Obrigada pela tua (enorme) paciência e por me teres apresentado formas novas de olhar para o mundo científico. És uma inspiração pelo modo como consegues balançar todas as valências da tua vida. Obrigada também a todas as pessoas da estação de moluscicultura do IPMA de Tavira: à Joana que me ajudou de forma tão generosa no trabalho de laboratório, à Tila, Sandra e Margarete que tornaram os meus dias na estação tão divertidos.

Thank you Kurt, you were the one that taught me all about "slug hunting" and allozymes. I really enjoyed the time I spent in Universiteit Antwerpen. And, of course, Natalie, that so patiently taught me all about the secrets of the lab!

Um grande bico para Tere, que me ensinaste tudo o que sabias sobre o mundo misterioso dos *Geomalacus* em Portugal, que compreendes o meu entusiasmo sobre este assunto e que tão fundamental foste na identificação morfológica dos especimens que recolhi.

To the members of the Jury for taking time from their busy schedules for reading my thesis and to participate in the defense.

A todas as pessoas que entraram neste jogo da caça à lesma, me enviaram amostras, me telefonaram e mandaram fotos de tudo o que rastejava: a minha tia Ana, Professor Karim (e aos jardineiros incansáveis, o meu shukran bezzef), Neiva, Gonçalo, Davide, Nelson, Licínia, Esti, Mirijam, Paula Cesário, Verónica Mascarenhas e Mª Clotilde Ribeiro o meu MUITO OBRIGADA!

A todos vocês do grupo BEE do CCMAR que me aturaram, ajudaram e inspiraram, fazendo com que estes anos no laboratório fossem do melhor que há! Pati, Davide (vais ser o melhor médico de sempre!), Mirjiam, Onno, Buga, Rita, Catarina, Pablo, Cristina (leia-se Quitina), Tânia Pereira, Sónia Massa, Maria João, Regina (a tua assertividade é uma inspiração), Xana que é uma força da natureza, com um poder de multitasking e organização sem par; Nelson, és o "mai" lindo do lab e, já sabes, o mais arrumadinho, you are the king of the lab; à Licínia (a tua coragem e boa disposição são a tua marca); ao Neiva, o mais despassarado, o protótipo do cientista que nem sabe onde tem as pipetas nem a chave de casa; à Joaninha voa-voa, que voaste para o outro lado do mundo, mas vais estar sempre no meu coração, à Aires, que estás sempre tão perto de mim, mesmo tão longe, tenho saudades tuas, ao GANG da marmita, estivemos na moda, ainda antes do tempo e ao Gonçalo um obrigada muito especial, ao longo destes anos todos foste o “outro”, estiveste comigo no laboratório e no campo, transformando as saídas de amostragem em aventuras para contar aos bisnetos, aturaste de tudo, o mau humor noturno, o sono, a fome, as birras, os momentos de desespero, TUDO. És um “must” OBRIGADA!

À Vanya, que estás sempre lá quando é preciso, que conduzes kms na neve para me apoiares, a minha cicerone na Bélgica! Para sempre tens lugar de destaque no meu coração, uma amiga que vai ficar para toda a vida.

A todos os meus amigos espalhados pelo mundo inteiro, que me apoiaram, que acreditaram (mesmo quando eu já não acreditei) e me incentivaram a não desistir, me ajudaram a seguir em frente, OBRIGADA.

À minha família, que me ajudou ao longo destes anos, que acredita em mim. Ao meu Pai, Avó, Tias e Primas! À minha Mãe que é a melhor do mundo, incansável, sempre pronta a ajudar. Ao Alexandre, que é o meu pilar, é o capitão do barco sempre que eu não estou presente (e nestes últimos anos eu sei que isso acontece muito mais vezes do que seria desejável...), obrigada pelo teu apoio incondicional durante estes últimos meses de escrita ta tese. E aos meus filhos, que são o Sol e a Lua dos meus dias!

“(…) I look at the term species, as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms. The term variety, again, in comparison with mere individual differences, is also applied arbitrarily, and for mere convenience sake.”

Darwin, 1859

Abstract

First described more that 150 years ago, the systematics of the genera *Geomalacus* and *Letourneuxia* (Arionidae, Gastropoda, Pulmonata) is still challenging. The taxonomic classification of arionid species is based on extremely labile characters such as body size or color that depends both on diet and environment, as well as age. Moreover, there is little information on the genetic diversity and population structure of the Iberian slugs that could provide extra clues to disentangle their problematic classification.

The present work uses different analytical tools such as habitat suitability (Ecological Niche Modeling - ENM), cytogenetic analysis and phylogeography to establish the geographical distribution and evolutionary history of these pulmonate slugs.

The potential distribution of the four *Geomalacus* species was modeled using ENM, which allowed the identification of new locations for *G. malagensis*, including a first report in Portugal. Also, it was predicted a much wider distribution for *G. malagensis* and *G. oliveirae* than previously known. Classical cytogenetic analyses were assayed with reproductive and a novel use of somatic tissues (mouth and tentacles) returning the number of chromosomes for the four *Geomalacus* species and *L. numidica* (n = 31, 2n = 62) and the respective karyotypes. *G. malagensis* and *L. numidica* present similar chromosome morphologies and karyotypic formulae, being more similar to each other than the *Geomalacus* among themselves.

We further reconstructed the phylogeny of the genera *Geomalacus* and *Letourneuxia* using partial sequences of the mitochondrial cytochrome oxidase subunit I (COI) and the nuclear ribosomal small subunit (18S rRNA), and applied an independent evolutionary rate method, the indicator vectors correlation, to evaluate the existence of cryptic diversity within species. The five nominal species of *Geomalacus* and *Letourneuxia* comprise 14 well-supported cryptic lineages. *Letourneuxia numidica* was retrieved as a sister group of *G. malagensis*. *G. oliveirae* is paraphyletic with respect to *G. anguiformis*. According to our dating estimates, the most recent common ancestor of *Geomalacus* dates back to the Middle Miocene (end of the Serravallian stage). The major lineage splitting events within *Geomalacus* occurred during the dry periods of the Zanclean stage (5.3-3.6 million years) and some lineages were confined to more humid mountain areas of the Iberian Peninsula, which lead to a highly geographically structured mitochondrial genetic diversity.

The major findings of this are the following: (1) provides updated species distribution maps for the Iberian *Geomalacus* expanding the known geographic distribution of the concerned species, (2) unravels the cryptic diversity within the genera *Geomalacus* and *Letourneuxia*, (3) *Geomalacus oliveirae* is paraphyletic with *G. anguiformis* and (4) *Letourneuxia numidica* is sister group of *G. malagensis*.

Keywords: Gastropoda, *Geomalacus, Letourneuxia*, Iberian Peninsula, habitat suitability, karyotype, phylogeography, biogeography.

Resumo

Apesar de inicialmente descritos há mais de 150 anos, a sistemática dos géneros de lesmas terrestres Ibero-Marroquinas *Geomalacus* e *Letourneuxia* (Arionidae, Gastropoda, Pulmonata), continua a não ser consensual. A classificação taxonómica das espécies da família Arionidae é baseada em caracteres plásticos, tais como o tamanho do corpo e a coloração do tegumento, que dependem não só da dieta e do ambiente mas também da idade dos indivíduos. Para além disso, informação que poderia auxiliar na problemática da classificação destes organismos, como a diversidade genética e estrutura populacional, é bastante escassa. No presente trabalho foram utilizadas diferentes ferramentas de análise tais como modelação de nicho ecológico (ENM), análises de citogenética e filogeografia, para actualizar a distribuição geográfica conhecida e desvendar a história evolutiva destas lesmas terrestres.

A distribuição potencial das quatro espécies de *Geomalacus* (*G. maculosus*, *G. oliveirae*, *G. anguiformis* e *G. malagensis*) foi modelada usando ENM. Os resultados permitiram identificar novas localizações para *G. malagensis*, entre elas o primeiro registo da espécie em Portugal e adicionalmente foi prevista uma distribuição mais alargada para *G. malagensis* e *G. oliveirae* do que a previamente descrita na bibliografia.

Análises de citogenética clássica foram realizadas em tecidos reprodutivos e, inovadoramente, em tecidos somáticos (boca e tentáculos). As quatro espécies de *Geomalacus* e *L. numidica* apresentaram n = 31, 2n = 62 cromossomas tendo sido obtidos os respectivos cariótipos. As espécies *G. malagensis* e *L. numidica* apresentaram fórmulas cariotípicas e morfologias cromossómicas semelhantes, sendo mais próximas entre si do que *G. malagensis* e as restantes espécies do género.

A reconstrução filogenética dos géneros *Geomalacus* e *Letourneuxia*, com sequências parciais da subunidade I do gene mitocondrial citocromo c oxidase e da subunidade ribossomal nuclear 18S. As cinco espécies de *Geomalacus* e *Letourneuxia* apresentaram 14 linhagens crípticas suportadas pelos elevados valores de ‘bootstrap’. *Letourneuxia numidica* apareceu como ‘grupo-irmão’ da espécie *G. malagensis* e *G. oliveirae* revelou-se parafilético em relação a *G. anguiformis*. De acordo com as estimativas, o ancestral comum mais recente de *Geomalacus* remonta ao Mioceno Médio, final do período Serravaliano. Dentro do género *Geomalacus*, a principal divisão de linhagens ocorreu durante as secas do período Zancleano (5.3-3.6 milhões de anos), ficando algumas linhagens restritas a áreas montanhosas mais húmidas da Península Ibérica, o que provocou uma elevada diversidade genética mitocondrial bem estruturada geograficamente.

As principais contribuições deste trabalho são: (1) a actualização dos mapas de distribuição geográfica para as espécies Ibéricas do género *Geomalacus*, (2) a revelação de diversidade críptica dentro dos géneros *Geomalacus* e *Letourneuxia*, (3) a existência de parafilia entre *Geomalacus oliveirae* e *G. anguiformis* e (4) o posicionamento de *Letourneuxia numidica* como ‘grupo-irmão’ de *G. malagensis*.

Palavras-chave: Gastropoda, *Geomalacus, Letourneuxia*, Peninsula Ibérica, ENM, cariótipo, filogeografia, biogeografia.

Table of Contents

Aims and Chapters

The present work aims at understanding the taxonomy and biogeography of the genera *Geomalacus* and *Letourneuxia* (Gastropoda, Pulmonata), with different analytical tools: habitat suitability or ecological niche modeling (ENM), cytogenetic analysis, phylogeography and species delimitation methods, with the purpose of clarifying the taxonomy, geographical distribuition and genetic diversity of this complex and poorly studied genera of the subfamily Arioninae. With this purpose five chapters were developed.

Chapter I provides a general introduction to phylogeography and terrestrial malacofauna of the Iberian Peninsula and explores species concepts and merits of integrative taxonomy. Finally, the current knowledge on the taxonomy, ecology and biology of the species investigated in this study is presented.

In Chapter II the potential distribution of the four *Geomalacus* species was modeled using Ecological Niche Modeling. New populations of *G. malagensis* were uncovered and, for the first time, the presence of this species in Portugal is reported. New potential areas of distribution are presented.

Chapter III deals with chromosome counts and karyotypes of the four *Geomalacus* species and *Letourneuxia numidica*, assessing to which extent cytogenetic data distinguishes the two genera and their subgenera. Different somatic tissues (mouth and both optical and sensory tentacles) were tested to evaluate their suitability for karyological studies. Results show that the different somatic tissues are suitable for cytogenetic studies and that the haploid chromosome number of the genera *Geomalacus* and *Letourneuxia* is n = 31. Also, the karyotypes of *G. malagensis* and *L. numidica* present similar chromosome morphologies and karyotypic formulae.

In Chapter IV, the phylogeny of *Geomalacus* and *Letourneuxia* across their Iberian-Moroccan distribution was inferred using sequence fragments of the mitochondrial cytochrome *c oxidase* subunit I (COI) and the nuclear ribosomal small subunit (18S rRNA) genes. Evolutionary significant units, i.e., unique reciprocally monophyletic genetic lineages were identified. *Geomalacus oliveirae* returned as a paraphyletic taxon comprising *G. anguiformis*, and five cryptic lineages. The main clades were dated and a biogeographical scenario is offered to explain the genetic structure of *Geomalacus/Letourneuxia*.

Finally, CHAPTER V provides a synthesis of the results of the preceding chapters and highlights the main contributions of this work.

Chapter I

General Introduction

Chapter I. General Introduction

Biogeography, a centenary discipline founded by Alfred Russel Wallace, is the study of the distribution of species and ecosystems through space and time, an essential discipline to understand not only the where and when, but also the “how come” (Lomolino *et al.*, 2010). How come are species distributed across a certain range and were they always there? How come are there shifts in the occupancy of landscape throughout time? How come certain species are absent from some locations, if apparently the optimal biotic conditions are met? When the spatial distribution of genetic variability was added to the geographical distribution of organisms ‘phylogeography’[1] (Avise *et al.*, 1987) was born, opening new possibilities to unravel the natural history of species. Since then three main advents have influenced phylogeography: the use of new molecular marker classes, such as microsatellites and Single Nucleotide Polymorphisms (SNPs), the development of analytical tools and habitat suitability modeling. New analytical tools have been developed to address specific matters, such as historical demography, calibration built-in phylogenies and landscape genetics. New algorithms that integrate the known geographical distribution of species with arrays of environmental variables of that distribution (Franklin, 2010; Guisan & Thuiller, 2005) allow the prediction of easy to interpret past and present geographic distribution maps.

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[1] Biogeography was defined by Avise (1998) as “The field of study concerned with the principles and processes governing the geographical distributions of genealogical lineages, especially those at the intraspecifc level”.

1. Study area: The Iberian Peninsula – endemisms and glacial refugia

Invertebrates show a wide variety of life histories and a tolerance for different temperatures and humidity; therefore studying the genetic variation of invertebrates on a range-wide scale may clarify the recent history of postglacial expansion and the location of refugia (Hewitt, 2001). The way invertebrates react to glacial and interglacial periods received less attention than the vertebrates and the existing studies only cover limited geographical areas referring mostly to insects (Gómez & Lunt, 2007). However, the large majority of phylogeographical studies has been focused on vertebrates, in particular mammals (Taberlet & Bouvet, 1994; Santucci, Emerson & Hewitt, 1998; Igea *et al.*, 2013), birds (Griswold & Baker, 2002; Hampe *et al.*, 2003; Neto *et al.*, 2012), fishes (Weiss *et al.*, 2000; Machordom & Doadrio, 2001; Mesquita *et al.*, 2007; Faria, Weiss & Alexandrino, 2012), reptilians (Fritz *et al.*, 2006; Joger *et al.*, 2007; Velo-Antón, García-París & Cordero Rivera, 2008; Kornilios *et al.*, 2010), amphibians (Martinez-Solano *et al.*, 2006; Reis *et al.*, 2011; Valbuena-Ureña, Amat & Carranza, 2013) and some insects (Cooper & Hewitt, 1993; Lunt, Ibrahim & Hewitt, 1998; Gomez-Zurita, Petitpierre & Juan, 2000; Miraldo *et al.*, 2011).

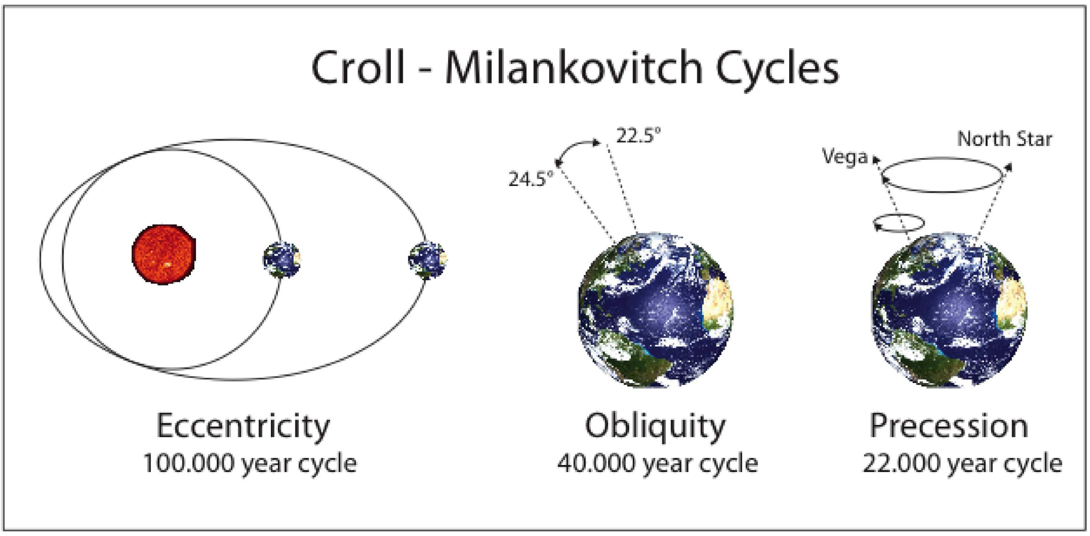

Figure 1.1. Cyclic variation in the orbit of Planet Earth (Croll - Milankovitch cycles)

With the beginning of the Tertiary period (~65.5 Million years ago – MYA), temperatures have changed dramatically, and the most recent global climate event, the last ice age, marked the beginning of the Pleistocene period (2.5 MYA - 10 thousand years ago). The quaternary glaciations had major influence shaping the spatial species/population structure: the cyclic variation in the orbit of Planet Earth (Croll-Milankovitch cycles, Figure 1.1) lasts around 100 thousand years (Muller & MacDonald, 1997) and is responsible for dramatic climate changes – glaciations – producing shifts in population ranges: during ice ages, species distribution becomes reduced and fragmented, and organisms tend to migrate south due to the expansion of ice sheets from the pole covering most of the northern hemisphere (Hewitt, 1996; Thompson & Russell, 2005) (Figure 1.2). During glacial periods, some geographic areas became 'refugia' for different species of fauna and flora. These long cold periods were interrupted by short, warm interglacial periods, lasting around 10 KY each. During these interglacial periods, populations on the edge of the ice sheets tended to migrate North rapidly (some species are known to have migrated between hundreds of meters and 1 km per year, e.g. (Hewitt, 2001), accompanying the rise in global temperatures (Hewitt, 2000; Thompson & Russell, 2005), colonizing the available ice-free land. These successive shifts between glacials and interglacial periods are responsible for a variety of phylogenic patterns in species (Hewitt, 2000). Table 1.1 includes a list of dated paleogeographic and climatic events that preceded the last ice age, in the Iberian Peninsula.

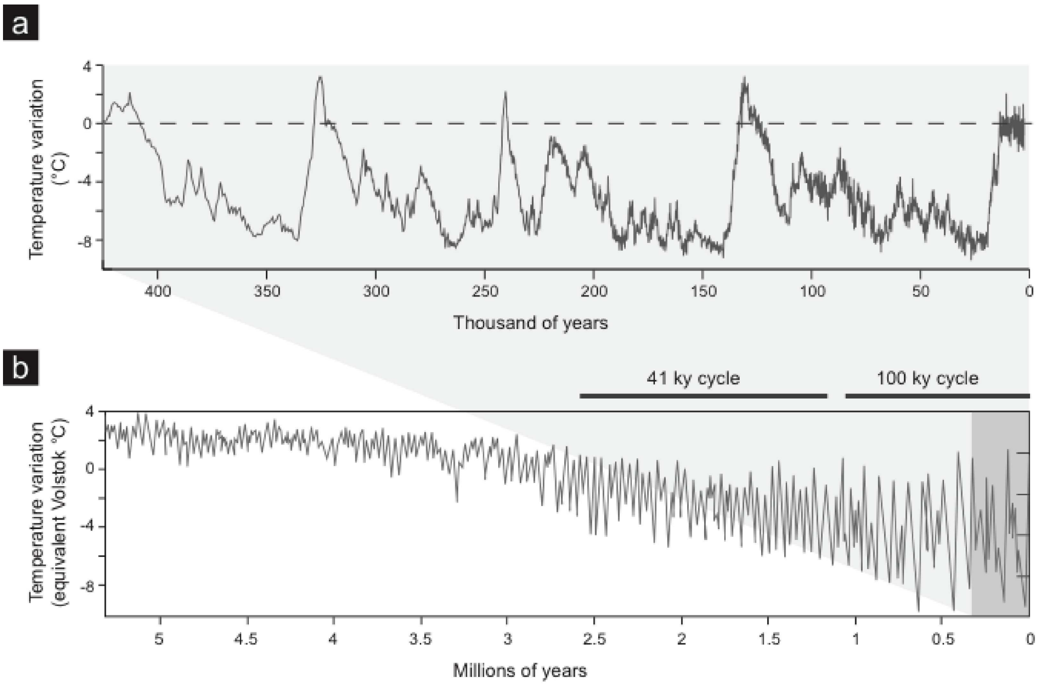

Figure 1.2. Temperature change. a) Reconstructed temperature from the Vostok ice core for the past 420.000 years (Petit *et al.*, 1999) and b) reconstructed temperature by combining measurements from 57 globally distributed deep-sea sediment cores for the past 5 MY (Lisiecki & Raymo, 2005).

The Iberian Peninsula, along with the Italian and Balkan Peninsulas are considered important refugia during glacial periods where species survived during temperature oscillations (Hewitt, 1999)(Figure 1.3). Iberia has a particular geological history that allows the existence of several microhabitats. The physiographic complexity, with several large mountain ranges, that display an east-west orientation (Gómez & Lunt, 2007), is responsible for a multitude of microclimates and habitats, across different altitude ranges, allowing species to occupy different niches, at different altitudes, as climate conditions changes (Hewitt, 1996). Also, due to the unique geographic location, the Iberian Peninsula is under the influence of the North Atlantic Ocean and Mediterranean Sea, inducing diverse climate conditions: from desert to Alpine, Mediterranean to Atlantic. Therefore it is expected that this variety of microclimates is responsible for more than one single refugia in the entire Peninsula (Gómez & Lunt, 2007). In fact, García-Barros (2002) identified 36 areas of high endemism in Iberia and concluded that these were mostly concordant with mountain ranges.

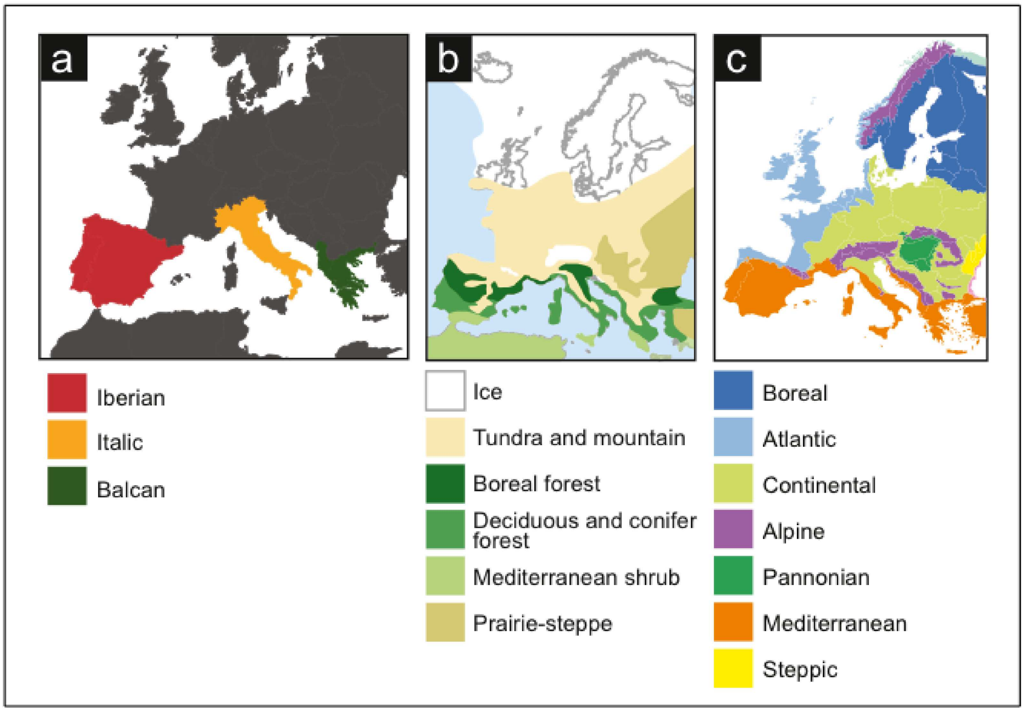

Figure 1.3. Europe and a) The three European Peninsulas; b) Vegetation cover during the Last Glacial Maximum; c) Map of the biogeographic regions of Europe (source: European Environmental Agency).

Table 1.1 - Paleogeographic, hydrographic and climatic events in the Iberian Peninsula; time frame in million years ago (MYA).

Time Frame	Event	Reference
200 MYA (Triassic period)	The Tethys Sea roughly covered the eastern half of the Iberian Peninsula	(Montes & Martino, 1987) (Comin & Alonso, 1988)
16 MYA	Formation of large inland saline lakes in Iberia	(Anadón, Cabrera & Roca, 1989)
14 MYA	Emergence of the BeticRiffean Massif	(Weijermars, 1991)
14–13.5 MYA (Middle–Late Badenian transition)	Renewed cooling and reestablishment of the major ice-sheets on Antarctica caused an ca. 11 ° C decrease in average annual temperature,	(Bohme, 2003)
10 MYA	Final structuring of the Neo-Pyrenees	(Oosterbroek & Arntzen, 1992)
5–10 MYA (Miocene period)	Inland saline lakes in the Miocene period	(Montes & Martino, 1987) (Comin & Alonso, 1988)
10–8 MYA	Reopening of the Betic Strait after the marine Transgression of the Upper Tortonian that isolated the Sub-Betic region	(López Martınez, 1989)
8–6 MYA	Fragmentation of the BeticRiffean Massif	(Martinez-Solano *et al.*, 2004)
5.96–5.33 MYA	Opening of the Gibraltar Strait at the end of the Messinian Salinity Crisis	(Duggen *et al.*, 2003)
2.5–1.8 MYA	Emergence of the Iberian hydrogeographic basins	(Calvo *et al.*, 1992)
3.4–0.01 MYA	Periodic ice ages	Review in Hewitt (2011)

2. The species concept problem, taxonomy and evolutionary significant units

Taxonomy, the science that describes and classifies species is the cornerstone of biology. Without a clear classification of the organisms, the work of conservationists becomes impaired, hindering management and conservation policies regarding selection of conservation areas and formulation of biodiversity plans (Agapow *et al.*, 2004). It is crucial to establish taxonomic units that can be managed and handled not only by biologists but also by decision makers (Dayrat, 2005). However, what is actually a species has been debated for centuries. The task of describing and classifying species can become overwhelming, since the definition of species is, in itself, debatable and highly dependent on which species concept is being used. At the present, biologists use more than 25 species concepts, for compilation see Wilkins (2011), and the large majority of studies do not clearly state under what kind of species concept they are considering (Bininda-Emonds, 2011). Since Mayr (1942), authors have addressed this problem, and discussed the implications of using different concepts in different studies (see Wilkins (2003) for a philosophical review) and how this differences can have an impact on results (Agapow *et al.*, 2004).

The taxonomy of many land slugs remains equivocal because it is based almost exclusively on morphological characters. Therefore their traditional classification is based on one of the following species concept: Morphospecies Concept, Phenetic Species Concept or the classical Biological Species Concept. Morphospecies concept is probably one of the oldest, with its origin in Aristotle and Linnaeus. More recently, (Cronquist, 1978) defined the concept as the smallest groups that are consistently and persistently distinct, and distinguishable by ordinary means. The Phenetic Species Concept (PSC) describes species as a set of organisms that look similar to each other and distinct from other sets (Monaghan *et al.*, 2009; Ridley, 1993) and the Biological Species Concept (BSC), made popular by Mayr (1942), consists in interbreeding natural population isolated from other such groups dependent upon endogenous reproductive isolating mechanisms (Mayr, 1970; Mayr & Ashlock, 1991).

Differentiating land slug species based on the internal and external morphology can be challenging since they often lack reliable diagnostic morphological traits. Their body size and color exhibit extreme variability (Backeljau & De Bruyn, 1990) and it is well known that the color of slugs is dependent on several factors, such as available food source, age and environment (Jordaens *et al.*, 2001). Aso, the morphology of their variable reproductive apparatus, which varies according to developmental stage and sexual maturation, often prevents the correct identification of juveniles at the species level (Backeljau & De Bruyn, 1990 and references therein; Backeljau *et al.*, 1996). These challenges compromise the use of the morphospecies and the PSC when describing new species. Also, the BSC, the one most biologist still prefer, present a major problem: it is either impossible, extremely time consuming or just too expensive to test if individuals are actually reproductive isolated (Agapow *et al.*, 2004). Moreover, usually species are identified on a subjective assessment of phenotypic distinctness (Sokal & Crovello, 1970). In organisms where facultative self-fertilization is common, defining species is extremely challenging, since these taxa may produce phenotypically divergent strains due to fixation and linkage disequilibria of alternative alleles in different multilocus genotypes (e.g. (Backeljau *et al.*, 1996; Geenen, Jordaens & Backeljau, 2006; Jordaens *et al.*, 2000). This would result in morphospecies, but not in real different biological species.

A different approach could be the use of the Phylogenetic Species Concept (PSC) that defines species, as a diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent (Cracraft, 1983; Cracraft, 1989) or monophyly (de Queiroz & Donoghue, 1988; de Queiroz & Donoghue, 1990; Donoghue, 1985), in short, a lineage. This concept is easier to apply than the non-phylogenetic species concepts in general, since it can be applied to asexual organisms and allopatric populations (Agapow *et al.*, 2004). Also, by not relying on morphological characters, morphological indistinct species may be found and because it incorporates both the history and phylogeny it is more useful for the classification and preservation of biodiversity (Hibbett & Donoghue, 1996). Obviously this approach also has its shortcomings; there is no clear definition about the amount of variation needed to define a species and this amount of variation is likely to vary among different taxa – which could prompt subspecies or even individual organisms to be classified as species, for instances in cases of uniparental systems where gene flow is restricted e.g. (Amadon & Short, 1992; Coyne & Orr, 2004; Isaac & Purvis, 2004). Obviously the opposite is also possible by overemphasizing potential gene flow between populations, and therefore aggregating different species together (Agapow *et al.*, 2004). Also, since phylogenies are not seldomly based on single genes, the result is actually a phylogeny of a gene, which may not be concordant with the phylogeny of the species due to events like incomplete lineage sorting, gene duplication (or loss) and horizontal gene transfer (Mindell & Meyer, 2001; Slowinski & Page, 1999). This may result in a higher number of species, with more restricted geographic ranges and decreased abundance (Agapow *et al.*, 2004). Therefore, it is better to rely on multiple (e.g. molecular, cytogenetic, ecological, behavioral) data to clarify taxonomic problems, i.e. in the present case that of the genera *Geomalacus* and *Letourneuxia*.

Recently the idea of integrative taxonomy has been proposed as a holistic tool that relies on a multitude of independent data (i.e. morphological, molecular, ecological, behavioral) allowing more accurate classifications (Dayrat, 2005; Yeates *et al.*, 2011). This approach has been used in terrestrial molluscs, where independent data sources were used to identify cryptic species (Jordaens *et al.*, 2010; Pinceel *et al.*, 2004) and to explore the species level status of land snails (Prévot *et al.*, 2013). Still, a question remains, is species level enough to represent lineage variation? The concept of ESU - evolutionary significant unit, was first catapulted into the lexicon of ecologist and evolutionary biologists by Ryder(1986) and was, at the time, used mainly for purposes of conservation, since species level fell shy of representing all genetic diversity that should be under conservation effort (Avise, 1989). As Moritz (1994) stated, "the recognition of ESUs is primarily relevant to long-term management issues, that is, defining conservation priorities and setting strategy”. This concept should not clash, but instead, complement the traditional “species” definition, since, in some species concepts, ESU and “species” are actually the same thing. Blaxter (2004) proposes the terminology MOTU (molecular operational taxonomic units), that falls within the phylogenetic (or lineage) species concept, to identify clades of organisms that can be perceived as “species”. To conclude, it is at least as important to describe and name species, and the relationship between them, as it is to understand how many “units” or “lineages” those species represent.

3. The terrestrial Arionids of the Iberian Peninsula

The nineteenth century was prolific in scientific work on terrestrial slugs in Portugal and Spain (Mabille, 1867; Morelet, 1845; Morelet, 1877; Pollonera, 1890b; Pollonera, 1890a; da Silva e Castro, 1873; da Silva e Castro, 1887; Simroth, 1888; Simroth, 1891; Simroth, 1893; Nobre, 1889), but very few authors (Haas, 1929; Nobre, 1930; Nobre, 1941) revisited this subject during the twentieth century, though in Spain it suffered less hiatus than in Portugal. It was only in the final of the 1970's that several studies advanced on the taxonomy and distribution of terrestrial slugs in Portugal (Castillejo, 1981; Castillejo, Rodríguez & Outeiro, 1987; Castillejo, Rodríguez & Outeiro, 1989; Seixas, 1976; 1977; 1978). An exhaustive listing of slug species present in Portugal and a classification based on internal morphology (reproductive organs and ligula) was produced in 1989 by Rodriguez. In the last decade of the past century, three books with all the species of slugs present in the Iberian Peninsula with available distribution maps were published (Castillejo & Rodriguez, 1991; Castillejo, 1997; Castillejo, 1998), as well as several morphology studies on arionids (e.g. Castillejo, 1992; Castillejo, 1993; Castillejo, Garrido & Iglesias, 1994; Garrido, Castillejo & Iglesias, 1994; Garrido, Castillejo & Iglesias, 1995).

Though the taxonomy of terrestrial slugs is well documented (but not resolved), there is only paper dealing with the phylogeny and phylogeography of Iberian *Arion sp.* (Quinteiro *et al.*, 2005). In contrast, there is a myriad of publications attempting biogeographic scenarios of Iberian amphibians e.g. (Alexandrino *et al.*, 2000; Alexandrino *et al.*, 2007; Altaba, 1997; Martinez-Solano *et al.*, 2004; Martinez-Solano *et al.*, 2006; Teixeira, Ferrand & Arntzen, 2001). While biologically completely different from terrestrial gastropods, amphibians have also low dispersal abilities (Staub, Brown & Wake, 1995) and are highly affected by habitat loss and fragmentation (Cushman, 2006), therefore it is possible that the events that shaped speciation in this group may also be responsible for shaping speciation and present day distribution of terrestrial slugs.

The terrestrial malacofauna of the Iberian Peninsula is extremely rich. The subfamily Arioninae comprises 40-60 species worldwide (this uncertainty is a result of the multitude of cryptic species) of which it is estimated that up to 2/3 are present in Iberia (Backeljau, personal communication). This geographic area includes several endemic species (Castillejo, 1998) making Iberia a hot-spot for arionid diversity. The arionid species richness in Iberia may be associated with (1) the relative climatic stability in this area from the Paleogene to the present, even during the Pleistocene climatic events; contrasting to the much more intense changes occurring at that same time in Europe, strongly affecting terrestrial slugs (Quinteiro *et al.*, 2005), (2) the multitude of micro-refugia during the Pliocene-Pleistocene with changing topography and climate, from the mountains to the valleys, from desert to Alpine and Mediterranean to Atlantic climate (Gómez & Lunt, 2007). Since slugs are organisms with low active dispersal ability and sensitive to several biotic parameters such as vegetation, humidity and temperature (Quinteiro *et al.*, 2005), the geographical barriers existing in Iberia (mountain ranges, river basins, variable climate conditions) are favorable to the isolation and lack of connectivity between sites and populations, thus favoring speciation events. Major events that appear to be responsible for speciation in *Arion* in the Iberian Peninsula include the uplift of the Pyrenees in the late Miocene (10-7 MYA), the Messinian salinity crisis and related glaciations (5.3 MYA) and the emergence of the Iberia hydrogeographic basins (2.5-1.8 MYA) (Quinteiro *et al.*, 2005).

For *Arion*, Quinteiro (2005) found three distinct clades (“Iberian endemics”, “Atlantic” and “Continental-Mediterranean”). The divergence of “Atlantic” and “Continental-Mediterranean” was dated to the Pliocene–Pleistocene boundary, with subsequent speciation events during the Pleistocene, while the endemics with distribution centered in the Pyrenean mountain range arose in the Pliocene and survived through the Pleistocene in geographically confined small populations. These results, based on mitochondrial ND1 gene and nuclear internal transcribed spacer 1 (ITS1) suggest that the evolutionary history of these *Arion* species was shaped by the Pliocene–Pleistocene climate events and the topography of southern Europe that prompt repeated cycles of isolation and expansions limited by geographic barriers (Quinteiro *et al.*, 2005). However, Quinteiro et al., (2005) major findings are based in clades that have no support, and the “Iberian endemics” is actually a paraphyletic group.

The questions regarding the geographical and temporal origin of the Arionids are controversial. The family Arionidae may have originated in the Iberian Peninsula, since most of the endemic Arionidae species occur in this region (and North Africa) and all of the genus-level taxa are represented in this location (Likharev & Wiktor, 1980). It is possible that during the Quaternary the Iberian Peninsula might have been the distribution center from where the arionids radiated to the vast areas of the Palearctic (Wiktor & Norris, 1991). A Gondwana origin was also suggested for the family Arionidae based on morphological structure of the internal shell and retractor muscle (Sirgel, 1986). Putative fossil records of arionids, *Craterarion pachyostracon* (California, USA) and *Geomalacus indifferens* (Germany) (Tracey, Todd & Erwin, 1993) suggest a possible first appearance of the family Arionidae in the Lower Miocene (23.3–16.3 MYA) and their presence in the Upper Miocene (10.4–5.2 MYA) (Quinteiro *et al.*, 2005). However, the arionid fossil record is highly debatable, since questions remain about the phylogenetic relationship between North American, African and European Arionidae (Sirgel, 1986). It is possible that the North American arionids like *Ariolimax*, *Prophysaon*, *Hemphillia* are in fact, not arionids and therefore the affinities between *Arion* and *Craterarion* are purely speculative (Backeljau, personal communication). Regarding the existence of a fossil of *Geomalacus* from Germany (Tracey *et al.*, 1993), it is quite probable that this involves an erroneous identification. For example, also Mabille (1867) described several *Geomalacus* species, which afterwards were not at all *Geomalacus*. Consequently in the present thesis, the term “arionids” refers only to the subfamily Arioninae, with two genera present in Iberia: *Arion* and *Geomalacus*, a third genus *Letourneuxia* present in North Africa and a fourth but poorly known genus *Ariunculus.*

4. The genera *Geomalacus* and *Letourneuxia*: current taxonomy, distribution and ecology.

4.1 The genus *Geomalacus*

Geomalacus Allman, 1843, presently comprises four species grouped into two subgenera: *G.* (*Arrudia*) *anguiformis* (Morelet, 1845), *G.* (*A.*) *oliveirae* Simroth, 1891, *G.* (*A.*) *malagensis* Wiktor & Norris, 1991 and *G.* (*Geomalacus*) *maculosus* Allman, 1843. The first three species are endemic to the Iberian Peninsula, whereas *G. maculosus* is also found in southwestern Ireland.

Geomalacus description originated from specimens obtained in Carogh Lake in County Kerry, Ireland (*G. maculosus*, Allman, 1843). The genus would include long-bodied slugs presenting large and pale spots, a pneumostome located on the lower third of the mantle and the genital orifice positioned between the mantle and lower right tentacle, a small caudal mucus pore and a solid and flat limacella (Germain, 1930). Two subgenera, *Geomalacus* with typical pale-spot slugs and *Arrudia*, for species presenting dark lateral bands instead of spots and genital apparatus resembling *Arion* were further introduced (Pollonera, 1890b). The type species *G. maculosus* was included in the subgenus *Geomalacus* and *G. anguiformis* and *G. oliveirae* in the subgenus *Arrudia* (Pollonera, 1890b).

Although *G. maculosus* is easily identified by its unique color pattern, *G. anguiformis* and *G. oliveirae* are very similar in their external morphology, showing only subtle differences in their reproductive organs (Rodriguez *et al.*, 1993). Often, these two species have been identified solely from the geographical origin of specimens (Castillejo & Rodriguez, 1991). But this is not the only problem in the taxonomy of *Geomalacus*. When Wiktor & Norris (1991) originally described *G. malagensis*, the set of existing features for the classification of Arionidae provided by Hesse (1926) was “considered unsatisfactory” (Wiktor & Norris, 1991), since this species could have been easily classified within any of the three genera (*Arion*, *Geomalacus* or *Letourneuxia*) of this family. Therefore, Wiktor & Norris (1991) proposed additional diagnostic characters, and the new species was included in the genus *Geomalacus*, subgenus *Arrudia*.

Geomalacus maculosus Allman, 1843

Lectotype: Castlemaine, Co Kerry, Ireland (deposited in the National Museum of Ireland, Dublin).
Geomalacus andrewsi Mabille, 1867
Letourneuxia lusitana da Silva e Castro, 1873
Limax lusitanus Morelet, 1877
Geomalacus lusitanus Pollonera, 1890
Geomalacus grandis Simroth, 1893
Geomalacus grandis Simroth, 1893 *sensu* Castillejo, 1891

For nearly a century, doubts were raised as to the existence of two large and very similar *Geomalacus* species in the Iberial Peninsula: *G. maculosus* was initially described based on Irish specimens (Allman, 1843), and for Iberia, fifty years latter, *G. grandis* was described based on individuals collected in Serra da Estrela (Portugal) (Simroth, 1893). Though several specimens from northern Spain and Portugal were described as *G. maculosus,* with the reinstatement of *G. grandis* as a separate species by Castillejo (1981) it was assumed that the Iberian species would be *G. grandis*, distinct form *G. maculosus* (Platts & Speight, 1988). Externally these two species would be very similar, but *G. grandis* would reach larger size than the Irish species and internally minor differences were observed in the reproductive system, though & Speight (1988) argued that the observed differences were an artifact derived from the ambiguous drawings provided by Quick (1960) that Castillejo (1981) used as a base to distinguish among the two species. When comparing the drawings provided by Castillejo (1981), with individuals collected at Padron (Herbon) locality where Castillejo (1981) found *G. grandis*, Platts & Speight (1988) noticed the similarity between the reproductive systems of both species. Based on these observations and preliminary allozyme electrophoresis, *G. grandis* was considered as a putative synonym of *G. maculosus* (Platts & Speight, 1988). Rodriguez *et al*. (1993) conducted a thorough revision of the material studied by Simroth (1893) and Iberian specimens, and concluded that both species were synonyms. Hitherto, this problem was not yet further explored with DNA sequence data.

Geomalacus maculosus is a large slug (up to 120 mm), grayish, brownish or greenish color and with white or yellow spots in the mantle with yellow mucus. Crimson-colored individuals were also reported when raised in captivity (Oldham, 1942). Juveniles have two lateral dark lines that become less evident with age. This species is easily distinguished from other *Geomalacus* species, not only because of the differences in size and unique color pattern, but also because it displays a peculiar behavior of curling into a ball when disturbed (Platts & Speight, 1988). The internal shell (limacella) is long with irregular outline, a clear nucleus and slightly visible growth lines. *Geomalacus maculosus* has its genital orifice below and behind the right upper tentacle (as in other arionids), and has a distinctive reproductive system: a long atrium with longitudinal, internal grooves, a long atriopenis, with a slight dilation around the atrium and with internal circular grooves. A short free oviduct with internal overlapping longitudinal grooves, resembling a ligula. Round bursa copulatrix, with short pedunculus, where the long retractor muscle is anchored. The retractor muscle inserts in the posterior end of the body, above the hermaphrodite gland. The spiral epiphallus is long and has internal papillae that externally resemble longitudinal grooves (Rodriguez *et al.*, 1993) (Figure 1.4).

Geomalacus maculosus is a crepuscular and nocturnal slug found near houses and gardens, mostly on stony walls or rocks. It can also be found in barks of oak (*Quercus* sp.), chestnut (*Castanea* sp.) or pine (*Pinus pinaster*) trees (Rodriguez *et al.*, 1993). This is a facultative self-fertilising species that appears to reach maturity at an age of around two years (Oldham, 1942). It is a slug with long longevity, in captivity specimens lived up to six and a half years and it is frequent to find very large specimens in the wild (Oldham, 1942).

Geomalacus maculosus is common in the Altantic part of the Iberian Peninsula: north of Portugal from Mondego River and Serra da Estrela to North of Spain, in Galicia, Asturias and Santander (Rodriguez *et al.*, 1993) and appears to be a rare species in the Southwest of Ireland (Platts & Speight, 1988) (Figure 1.4). For this reason, *G. maculosus* is legally protected under the Habitats Directive 92/43/CEE and Bern Convention Appendix II, and is listed as “vulnerable” in the Invertebrate Red List of Spain (Castillejo & Iglesias-Pineiro, 2011c).

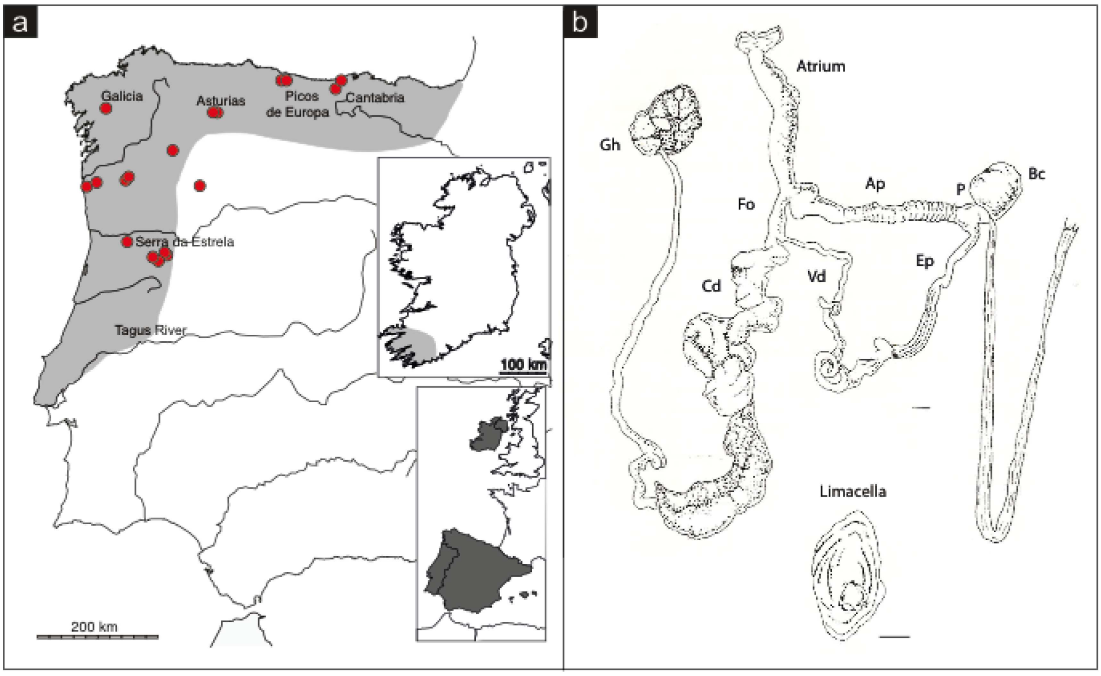

Figure 1.4. *Geomalacus maculosus* (a) nnown distribution in the Iberian Peninsula and Ireland, inferred from Platts & Speight (1988) and Castillejo, (1998). Red circles indicate samples from the present study (b) *Geomalacus maculosus* reproductive system: Ep – epiphallus; Bc – bursa copulatrix; P – pedunculus of the bursa copulatrix; Vd – vas deferens; Fo – free oviduct; Cd – Common duct; Gh – glandula hermaphrodita; Ap – atriopenis. Scale bar represents 1 mm. Illustration adapted from (Rodriguez *et al.*, 1993).

Geomalacus anguiformis (Morelet, 1845)

Limax anguiformis Morelet, 1845

Limax squammatinus Morelet, 1845

Geomalacus anguiforme Mabille, 1867

Geomalacus (Arrudia) anguiformis Pollonera, 1890

Type locality: Serra de Monchique, Portugal

Limax anguiformis was initially described by Morelet from specimens from Serra de Monchique, *Limax squammatinus* and *Limax viridis* were based on specimens from Serra do Caldeirão (Morelet, 1845). Later, Pollonera (1890b) based in the internal morphology considered *L. squammantinus* a juvenile form of *L. anguiformis*, thus synonymizing both species. Simroth (1891), moved *L. anguiformis* to the genus *Geomalacus* based on the fact that the head is only visible when the tentacles are extended, the slow movement, inability to contract and texture of the mantle, and considered *L. viridis* a juvenile form of *G. anguiformis*. Rodriguez *et al*., (1993) questioned this synonymy based on their observations, since a small keel was described for *L. viridis* and this is not a characteristic of the Arionidae. Wiktor & Parejo (1989) re-described *G. anguiformis*, based on specimens form Robledo del Mazo (Toledo, Spain) but without examining individuals form the type locality. Rodriguez *et al*. (1993), however, compared specimens form the type locality and with those from Toledo and concluded that Wiktor & Parejo (1989) described *G. oliveirae* instead of *G. anguiformis*.

Geomalacus anguiformis is a medium-sized slug (60-70 mm) with a variable color pattern and yellow mucus. It can be blackish-blue or brown with whitish tubercules and four almost black or brown dorsal lines. The body margins of adults tend to become lighter to almost yellow (Rodriguez *et al.*, 1993). The oval limacella has light growth lines. The species has a large and cylindrical genital atrium (covered externally by a glandular mass and 7–9 internal grooves), a short cylindrical to spherical diverticulum with small internal grooves, an oval bursa copulatrix oval, with a very long pedunculus, entering together with the epiphallus in the atriopenis. Presents a long bursa retractor muscle connected at the lower third of the duct. The free oviduct is shorter than the genital atrium, but the tubular, dilated epiphallus is 10–15 times larger than the free oviduct. Internally, the epiphallus shows grooves. The vas deferens is about half the length of the epiphallus, with indistinct transition (Rodriguez *et al.*, 1993)(Figure 1.5).

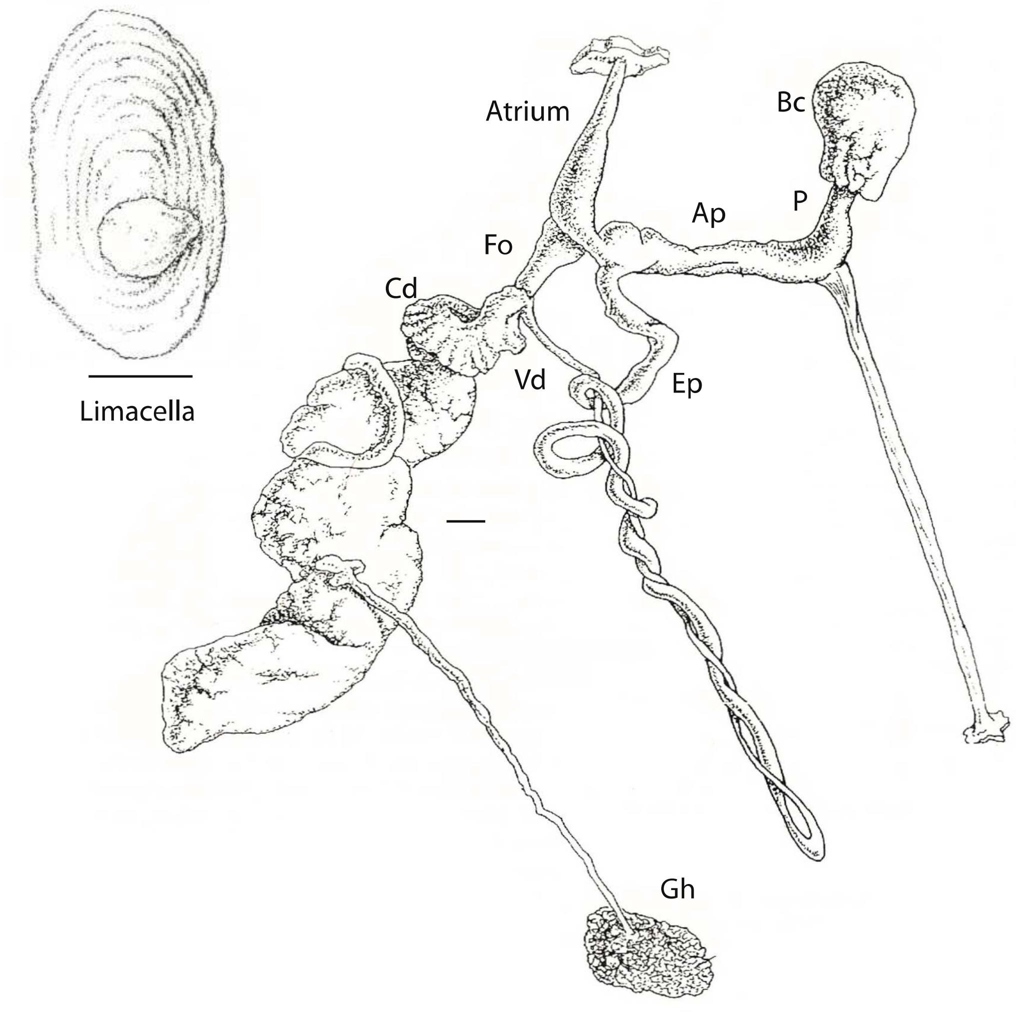

Figure 1.5. *Geomalacus anguiformis* reproductive system: Ep – epiphallus; Bc – bursa copulatrix; P – pedunculus of the bursa copulatrix; Vd – vas deferens; Fo – free oviduct; Cd – Common duct; Gh – glandula hermaphrodita; Ap – atriopenis. Scale bar represents 1 mm. Illustration adapted from (Rodriguez *et al.*, 1993).

Although *G. anguiformis* is mostly active at night, it is also possible to find it at daytime, during or immediately after raining (Rodriguez, 1989). It can be found in barks of oak (*Quercus* sp.), olive (*Olea europaea*) or pine (*Pinus sp*) trees, or in areas with the presence of *Rhododendron ponticum* and *Arbutus unedo*. (Rodriguez *et al.*, 1993).

Geomalacus anguiformis is common in the Southwest part of the Iberian Peninsula: in Serra de Monchique and Serra do Caldeirão in Algarve, Portugal and in Sierra de Aracena, Andaluzia, Spain (Castillejo, 1998), being restricted to the most humid zones of this dry area. *Geomalacus anguiformis* is listed as “vulnerable” in the Invertebrate Red List of Spain (Castillejo & Iglesias-Pineiro, 2011a) (Figure 1.6).

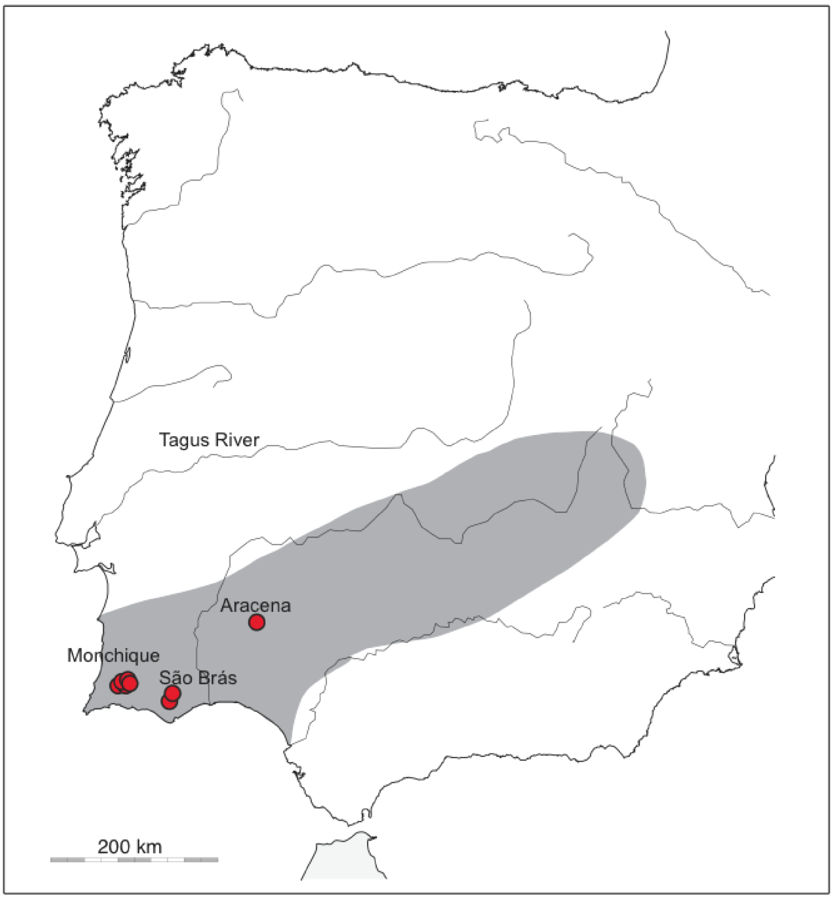

Figure 1.6. Known distribution of *G. anguiformis* in the Iberian Peninsula, inferred from Castillejo (1998). Red circles indicate samples from the present study.

Geomalacus oliveirae Simroth, 1891

Type locality: Guarda, Portugal

Geomalacus oliveirae was described by Simroth (1891) based on two specimens from Guarda, Serra da Estrela, Portugal, who considered this as an intermediate form between *G. maculosus* and *G. anguiformis*. Externally this species is very similar to *G. anguiformis*, and it has been neglected by science. Since its original description, it was mentioned by Hesse (1926) and Nobre (1941), only to mention that he was unaware of the diagnostic characteristics. Half century latter Rodriguez (1989) re-captured and re-described the species in Guarda, the type locality.

Geomalacus oliveirae is the smallest species of its genus (45 mm), brownish with four lateral dark lines (the two internal lines are not totally continuous) and body margins light colored, with yellowish mucus. The limacella is very similar to that of *G. anguiformis* but the growth lines are even more lightly colored. Internally, the species has a cylindrical genital atrium (covered externally by a glandular mass) with 7-9 internal grooves, a short and smooth diverticulum, a spherical bursa copulatrix with a short and thick pedunculus (with internal transverse grooves), joining the epiphallus by a pedunculus with an annular dilation (with short and thick internal grooves). The oviduct is cylindrical and forms the continuation of the atrium. The atrium is slightly shorter and lined with fine grooves. The epiphallus is cylindrical, slightly longer than the vas deferens (lined with 7-9 longitudinal grooves). The retractor muscle inserts close to the bursa copulatrix (Rodriguez *et al.*, 1993) (Figure 1.7).

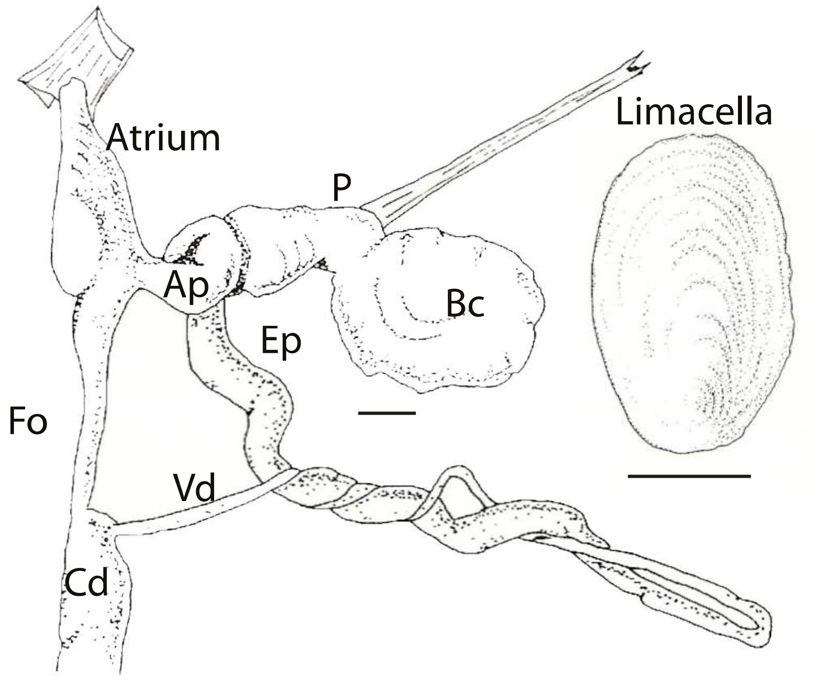

Figure 1.7. *Geomalacus oliveirae* reproductive system: Ep – epiphallus; Bc – bursa copulatrix; P – pedunculus of the bursa copulatrix; Vd – vas deferens; Fo – free oviduct; Cd – Common duct; Gh – glandula hermaphrodita; Ap – atriopenis. Scale bar represents 1 mm. Illustration adapted from (Rodriguez *et al.*, 1993).

This is a nocturnal species that is almost impossible to find during daytime (Rodriguez, 1989). It is associated to granitic areas, in Mediterranean-type vegetation regions below 1300 m. Above this altitude it can be associated with pine (*Pinus silvestris*), downy birch (*Betula pubescens*) and juniper (*Juniperus communis*) (Rodriguez *et al.*, 1993).

This species is appears to the rarest of all *Geomalacus*, since it is always found in small numbers (no more than 5 specimens where collected at any given location), and is present in Serra da Estrela (Portugal), Béjar and Sierra de la Peña de Francia, Sierra de Gredos, Sierra de Guadalupe y Montes de Toledo (Spain), being restricted to the mountain ranges of central Iberia (Castillejo, 1998). *Geomalacus oliveirae* is listed as "vulnerable" in the Invertebrate Red List of Spain (Castillejo & Iglesias-Pineiro, 2011b) (Figure 1.8).

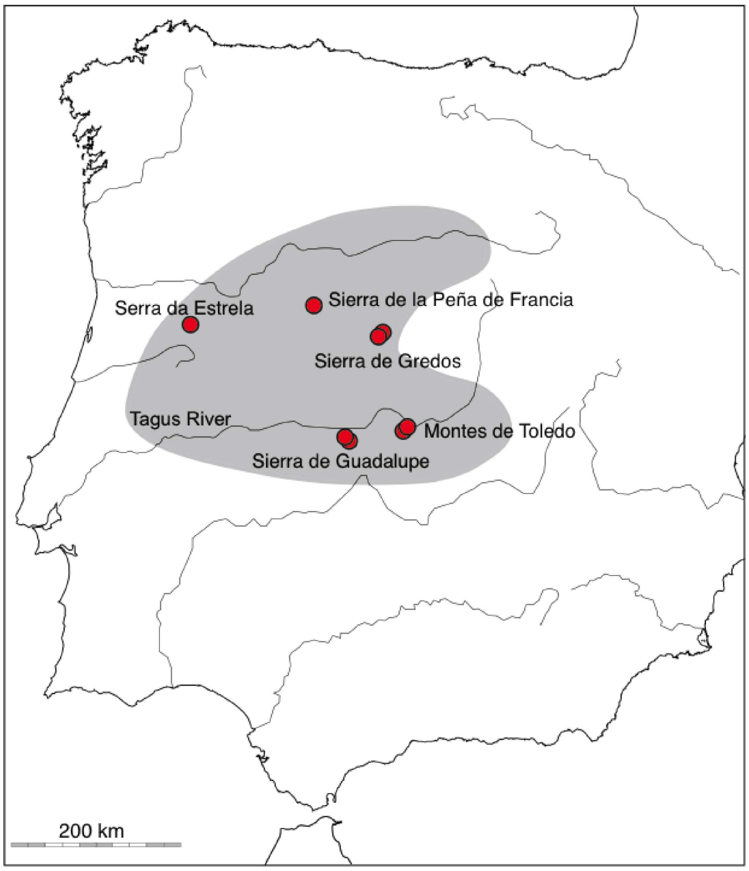

Figure 1.8. Known distribution of *G. oliveirae* in the Iberian Peninsula, inferred from Castillejo (1998). Red circles indicate samples from the present study.

Geomalacus malagensis Wiktor & Norris, 1991

Arion (Letourneuxia) moreleti sensu Norris 1977
Arion moreleti sensu Castillejo, 1996
Holotype: Colmenar, Malaga, Spain (deposited in the Museum of Natural History, Wroclaw University, Wroclaw, Poland)

A big debate regarding the taxonomic interpretation of *Geomalacus malagensis* started in 1991 with the description of this species, by Wiktor & Norris (1991) based on specimens from Malaga, Cadiz (Spain) and Gibraltar. Specimens from Gibraltar, had already been studied by Norris (1977), who synonymized this species with *Arion (Ariunculus) moreleti* Hesse (Hesse, 1884) from Tanger (Morocco). After carefully observing the individuals from Spain, Wiktor & Norris (1991) noted the genital difference between the original description Hesse's *A. moreleti* and the 'redescription' of *A. moreleti* by Norris (1977) from Gibraltar. Therefore they decided that the material studied by Norris (1977) was not Hesse's (1884) *A. moreleti*, but a new species that they described as *Geomalacus malagensis*. Castillejo (1996) challenged Wiktor & Norris (1991) by stating that they were mistaken when correcting the original note of Norris (1977) and that the species Norris (1977) observed is a junior synonym of *A. moreleti*.

The original drawing provided by Hesse (1884) shows a weakly developed epiphallus and a genital atrium onto which three canals indepently insert (epiphallus, spermatheca and oviduct), however Norris (1977) drawings reveal a big, swollen, well-developed epiphallus and a genital atrium onto which only two canals insert independently because the spermatheca is implanted on the basis of the epiphallus. It is based on this observation that Wiktor & Norris (1991) decided that the slugs from southern Spain and Gibraltar were a *Geomalacus* and not the species described by Hesse (1884). The drawings of Castillejo (1996) show a slug with a big, swollen well-developed epiphallus and a spermatheca that inserts at the base of the epiphallus and that does not open independently into the atrium. Hence Castillejo's specimens nicely correspond to the *A. moreleti* of Norris (1977) and the *G. malagensis* of Wiktor & Norris (1991), but not to the moreleti of Hesse (1884). Norris (1977), Wiktor & Norris (1991) and Castillejo (1996) deal with the same species, which is not Hesse's

Arion (Ariunculus) moreleti (1884). Currently, the issue has not been settled definitively and both names, viz. *Geomalacus malagensis* Wiktor & Norris (1991) and *Geomalacus moreleti sensu* Castillejo (1996), are applied. Here we will use *Geomalacus malagensis* Wiktor & Norris (1991) when referring to this species.

Geomalacus malagensis is a medium sized species, that can grow up to 80 mm, featuring a dorso-ventrally flattened and strongly elongated body, with a very short head and relatively long tentacles. The skin is very thin and it’s tubercles extremely delicate. It’s color ranges from beige-greenish to yellowish-orange, with two dark lateral, almost parallel bands. The limacella is strongly flattened, asymmetrical and near oval, with visible growth lines on the upper surface. The reproductive apparatus of. *G. malagensis,* comprises a thin vas deferens, visibly separated from a long and thick epiphallus. The bursa copulatrix is oval and has a short pedunculus, which together with the epiphallus opens in a short atriopenis. The free oviduct is shorter than the epiphallus, opening into a very long, tubular or flattened atrium (Wiktor & Norris, 1991)(Figure 19).

This species prefers living in masses on calcareous soil, occurring in synanthropic environments, such as gardens, in scrub and trees (Wiktor & Norris, 1991). It is endemic from the South Iberian Peninsula from Sierra Nevada to Cape Tarifa (Castillejo 1998). This distribution range is based only on five populations recorded from locations in Gibraltar, Málaga, Cádiz and Granada (Castillejo et al. 1994; Castillejo 1996; Norris 1977; Wiktor and Norris 1991), therefore *G. malagensis* is classified as “vulnerable” in the Red List of the invertebrates of Andalusia (Castillejo and Iglesias-Pineiro 2008) (Figure 1.9).

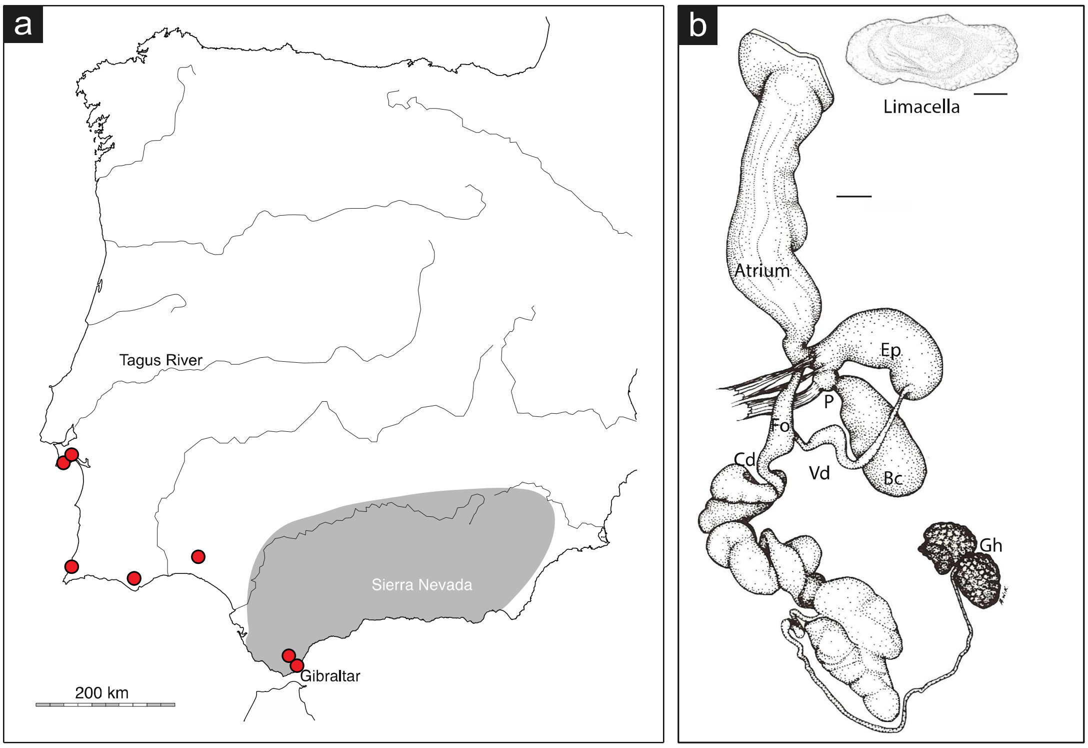

Figure 1.9. *Geomalacus malagensis.* a) known distribution of *G. malagensis* in the Iberian Peninsula, inferred from Castillejo (1998). Red circles indicate samples from the present study and b) Reproductive system: Ep – epiphallus; Bc – bursa copulatrix; P – pedunculus of the bursa copulatrix; Vd – vas deferens; Fo – free oviduct; Cd – Common duct; Gh – glandula hermaphrodita. Scale bar represents 1 mm. Illustration adapted from (Wiktor & Norris, 1991).

4.2 The genus *Letourneuxia*

The description of the genus *Letourneuxia* Bourguignat, 1866, was based on specimens from Algeria. This taxon was described as endemic from North Africa and its taxonomic status has changed several times, including being described as a subgenus of *Geomalacus* (Pollonera, 1890), a subgenus of *Arion* (Hesse, 1926), and finally, acquiring generic status within the family Arionidae (Wiktor, 1983). These slugs are externally similar to *Arion (Mesarion)*, though they are somewhat larger (65 mm), with two lateral bands on the back and mantle. The skin is delicate and thin; the genital orifice is situated in the front of the body, laterally towards the right tentacle (Wiktor, 1983).

Letourneuxia numidica Bourguignat, 1866

L. atlantica Bourguignat, 1883
Arion (Ariunculus) moreleti Hesse, 1884
Geomalacus (L.) turneri Pollonera, 1890
Geomalacus (L.) maroccanus Pollonera, 1916

The four nominal species, *L. numdica, L. atlantica, G. (L.) turneri* and *G. (L.) maroccanus*, together with *A. moreleti* have been synonymized with *L. numidica* by Wiktor (1983), based on external and internal morphology. *Letourneuxia numidica* grows up to 65 mm and has a body shape similar do *Geomalacus*. The pneumostome is located at the first third of the mantle length and presents an extremely fine and delicate skin texture. The color pattern is similar to *G. malagensis*, cream colored with dark pigmentation on the back, two almost black lateral bands, and lighter body margins. The limacella is thick and oval with slightly marked growth lines. This reproductive apparatus of the species has a long hermaphroditic duct. It lacks a penis or epiphallus, but the atrium is slightly swollen at the entrance of the vas deferens. The oviduct is tubular with papilla at its opening in the atrium. The vas deferens is short and the pedunculus is longer than the bursa copulatrix. There is a bifurcated genital rectractor muscle attached to the pedunculus and the atrium. This latter is pear-shaped with a wide upper section that encloses a large, folded, tongue-shaped ligula (Wiktor, 1983) (Figure 1.10).

Letourneuxia numidica is usually found associated with fig (*Ficus carica*), pine (*Pinus sp.*), oak (*Quercus sp.*) or eucalyptus trees, in shrubs or debris (Wiktor, 1983). It is hard to find during broad daylight, but in the evening or dawn, it can be spotted crawling in the dead leaves. It is present in northwest Algeria (Tlemcen and Oran) and Morocco (Tanger, Taza, Tetuan rocky valley, Middle and High Atlas), and, according to Wiktor (1983) probably also in the Iberian Peninsula (Figure 1.10).

Figure 1.10. *Letourneuxia numidica*. Known distribution of *L. numidica* in the Iberian Peninsula (A), inferred from Wiktor(1983). Red circles indicate samples from the present study. Reproductive system (B): Ep – epiphallus; Bc – bursa copulatrix; P – pedunculus of the bursa copulatrix; Vd – vas deferens; Fo – free oviduct; Cd – Common duct; Gh – glandula hermaphrodita. Scale bar represents 1 mm. Illustration adapted from (Wiktor, 1983).

Chapter II

Habitat suitability modelling of four terrestrial slug species in the Iberian Peninsula (Gastropoda, Pulmonata: *Geomalacus sp.*)

Habitat suitability modelling of four terrestrial slug species in the Iberian Peninsula (Gastropoda, Pulmonata: *Geomalacus* sp.)

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Submitted to Journal of Zoology

Abstract

Ecological niche modelling (ENM) determines habitat suitability of species by relating presence records to environmental variables and maps the habitat suitability of a landscape for the species in question. Here, we investigated the probability of occurrence of four terrestrial slugs of the genus *Geomalacus* (Mollusca, Pulmonata) from the Iberian Peninsula using ENM. The potential distribution of these species was estimated using the program MaxEnt. For this we used presence records from the literature and from own fieldwork, and 21 layers of environmental variables. The predicted distribution models showed a high accuracy for each of the species. Precipitation, and to a lesser extent temperature, were the most important variables to predict the distribution of *G. maculosus*, *G. anguiformis* and *G. malagensis*. In contrast, the distribution of *G. oliveirae* was best predicted by the occurrence of granite and shale/argilite in an anthrosol-andosol soil type. We then compared the predicted distributions of the four species with their currently known distribution. For *G. anguiformis* and *G. maculosus* the predicted distribution corresponded strongly to the currently known distribution. For *G. malagensis* and *G. oliveirae* the model predicted a much wider distribution than previously known. Based on additional fieldwork we could not confirm the presence of *G. oliveirae* in the newly predicted area. Yet, *G. malagensis* was found at five new localities in the newly predicted area and we report the first records of this species from Portugal.

Introduction

Most of the information on the geographic distribution of species stems from fieldwork data accumulated throughout centuries. Recently, however, the advent of habitat suitability modelling or ecological niche modelling (ENM) has allowed inferring hypothetical geographic species' distributions by relating the presence/absence of a species with environmental variables (Franklin, 2010). Here, we use the term ENM *sensu lato* (for a discussion on the use of the terminology ENM see Warren (2012) and references therein). ENM attempts to predict the environmental conditions that are suitable for a species (Guisan & Thuiller, 2005). Based on these predictions, new putative areas can then be surveyed for the presence of that species. ENM relies on a grid cell map with known species presence/absence records and environmental spatial data layers. ENM then classifies grid cells according to the degree in which they are (un)suitable for a species, resulting in a predictive model describing the suitability of any site for that species. Common applications of ENM include exploring the response of geographic species distributions to climate change (Fouquet *et al.*, 2010; Martinez-Meyer, Townsend Peterson & Hargrove, 2004), predicting range expansions of invasive species (Benedict *et al.*, 2007), supporting conservation planning (Wilson, Roberts & Reid, 2011), identifying areas of endemism (Raxworthy *et al.*, 2007) and facilitating field surveys of species with poorly known geographic distributions (Guisan & Thuiller, 2005; Raxworthy *et al.*, 2003; Rebelo & Jones, 2010).

The terrestrial slugs of the genus *Geomalacus* Allman, 1843 (family Arionidae) comprise four species: *G. anguiformis* (Morelet, 1845), *G. oliveirae* Simroth, 1891 and *G. malagensis* Wiktor and Norris, 1991, all endemic in the Iberian Peninsula, and *G. maculosus,* Allman, 1843 which is also present in SW Ireland. The geographic ranges of these species vary from being restricted to Gibraltar and the adjacent areas in southern Spain, as *G. malagensis* that occurs from Sierra Nevada to Cape Tarifa (Castillejo, 1998) to a larger part of the Iberian Peninsula as *G. maculosus* with more than 90 reported locations over a wider area comprising Central and Northern Portugal and Northern Spain) (Figure 1.1). *Geomalaus maculosus* is legally protected under the Habitats Directive 92/43/CEE and Bern Convention Appendix II, while all *Geomalacus* spp. are listed as "vulnerable" in Red Lists of Spain (Castillejo & Iglesias-Pineiro, 2008; Verdú, Numa & Galante, 2011).

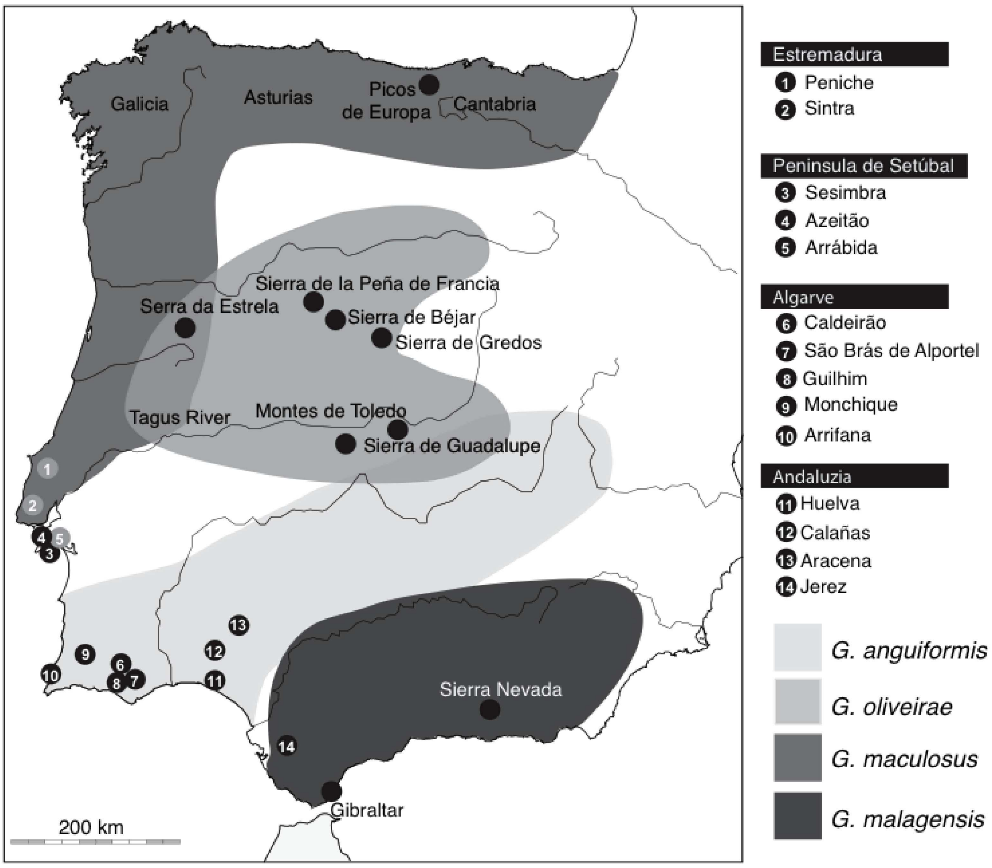

Figure 2.1. Locations mentioned in the manuscript and locations screened for the presence of *G. malagensis* (marked with numbered bullets). Black circles represent locations where the presence of specimens was confirmed and grey circles represent locations where no specimens were found. The known geographic distribution of all *Geomalacus* species, based on Castillejo *et al.* (1994) is represented by shadowed areas.

In the last few decades, the habitat of *Geomalacus* spp. suffered severe fragmentation and deterioration due to an expansion of the tourist industry and urban development (Castillejo & Iglesias-Pineiro, 2008; Verdú *et al.*, 2011). Habitat loss has negative impacts on organisms by narrowing their geographic distribution and decreasing their abundance. Yet, it is presently unknown if the geographical distribution of *Geomalacus* spp. has shrunk as a result of such putatively damaging pressures.

Here, we apply ENM to explore the distributions of the four *Geomalacus* species in the Iberian Peninsula using presence-only data with MaxEnt (Phillips, Anderson & Schapire, 2006; Phillips, Dudík & Schapire, 2004). In this way, we aimed at predicting the potential distribution of these species for future monitoring programs. As such, our aims were (1) to produce models of the potential geographic distribution of the four species and (2) to assess how well the predicted ENM distributions fit to the current distribution records, particularly in rare species such as *G. malagensis.*

Material and methods

Occurrence records

Occurrence records of *Geomalacus* from the Iberian Peninsula were obtained from the literature and field surveys conducted across the known species' geographic ranges during 2007–2010 (Supporting Information Appendix S1). Surveys were done by car and when suitable habitats were found, the sites were recorded by GPS positioning (GARMIN MAP) and subsequently visited at different periods (dawn, morning, evening and night). Specimens were searched in undisturbed areas (*G. maculosus*, *G. oliveirae* and *G. malagensis*), in abandoned rural areas (*G. anguiformis*) or synanthropic areas (*G. maculosus*, *G. malagensis*) following the known habitats of the species. Since specimens were sometimes found outside their known environments, other habitats were also searched. Although *Geomalacus* is supposed to mainly inhabit mountain ranges, we did our field surveys at a range of altitudes from sea level up to Picos de Europa. Slugs were identified with the key of Castillejo, Garrido & Iglesias (1994).

Unprecise locations reported in the literature were replaced, whenever possible, by precise geo-referenced location data from our field trips. Multiple references to the same location were entered only once in the models. We applied a cautionary approach for the selection of literature records. Therefore we did not consider three single occurrence records (*G. anguiformis* from Lisboa and *G. oliveirae* from Alvega and Monchique) in Hidalgo (1916) that were made a century ago and that were never confirmed subsequently. Furthermore, *G. oliveirae* and *G. anguiformis* were reported to co-occur in Aracena (Spain) (Castillejo & Iglesias-Pineiro, 2011a; Castillejo & Iglesias-Pineiro, 2011b). However, the authors made no mention of having found both species in the same habitat and location, which would be expected because the species do not co-occur elsewhere. We have therefore also discarded this record.

Ecological niche modelling

ENM was implemented using the maximum entropy method (MaxEnt 3.3.3) (Phillips *et al.*, 2006; Phillips *et al.*, 2004). This approach models the potential distribution of a species by finding the probability distribution of maximum entropy. The result is a probability distribution that is consistent with known environmental variable constraints, but as unbiased as possible, since it only depends on environmental variables and presence records (Penfield, 2010). MaxEnt performs well in comparison with other approaches (Elith *et al.*, 2006), particularly if only few occurrence records are available (Wisz *et al.*, 2008). We used MaxEnt to produce a logistic output of environmental suitability values since this provides an estimate of the probability that conditions are suitable for a given species, ranging from 0 (unsuitable environment) to 1 (optimal environment) (Phillips & Dudik, 2008). The results were validated with a threshold independent measure, the area under the Receiver Operating Curve (ROC) and 10,000 random background points as training data (Phillips *et al.*, 2006). The Area Under the ROC (AUC) describes the success of the model: AUC = 1 implies a perfect fit and AUC = 0.5 implies that the model accuracy is the same as a random prediction (Baldwin, 2009b). Obviously, dubious or inaccurate locality data from the literature can influence ENM results. However, MaxEnt is somehow insensitive to imprecise locality records of up to 5 km (Baldwin, 2009a). Historical locations are usually recorded in UTM 10X10 km coordinates, therefore we selected random cells and ran MaxEnt repeatedly, each time with one of 12 random points along the raster. All runs were insensitive to the change of the locations, yielding the same ENM output (data not shown). Therefore, in the absence of specific information on the location of the records, we selected the lower left corner of each raster in the model predictions.

The geographic distribution models of *G. maculosus*, *G. anguiformis* and *G. oliveirae*, were based on literature records and fieldwork in areas proposed by the literature. Two distinct geographic distribution models were considered for *G. malagensis* (the rarest of the four species): the first was based on literature records with added locations as a result of fortuitous, preliminary fieldwork by the authors. The second included the same data plus new records resulting from directed fieldwork in areas that were rated as highly suitable by MaxEnt.

Environmental data

Current climate and ecological data were obtained from the European Soil Database (ESDB; (Center, 2006)), WorldClim (WCDB; (Hijmans *et al.*, 2005)) and the European Environment Agency (EEA, 2004) using a 1 km spatial resolution. Only one presence record per grid cell was considered. Twenty-one layers of data (variables) were selected and downloaded, representing putatively relevant physiological and ecological requirements of *Geomalacus*, inferred from the literature (Table 2.1): we selected the ones related with topsoil characteristics, vegetation coverage and climate. We assumed that this limited number of variables would increase model robustness and would reduce the risk of over-fitting (Nogués-Bravo, 2009). The values of each variable for each presence record were analyzed for collinearity (R Development Core Team, 2013), excluding those with a Spearman correlation coefficient $\boldsymbol{\rho} \geq 85\%$ (Walker *et al.*, 2009). Although collinearity between variables is not expected to affect the performance of MaxEnt, it can hinder model interpretation (Evangelista *et al.*, 2009; Kuemmerle *et al.*, 2010). For each correlated pair, the excluded variable was the one with the lower relative contribution in a preliminary MaxEnt model with all 21 variables.

A run was performed with all remaining variables and presence records to rank those variables that contributed most to the model. The importance of each variable was measured by a jackknife validation test (Pearson, 2007) and variables with no, or negative impact on the model, were removed sequentially (Sumarga, 2011). When two models presented the same AUC, the model with the lowest number of variables was preferred as final model. Irrespective of the number of variables that contributed to the model, only the three that contributed most to the model were explained in detail. Excluded variables and criteria for exclusion can be found in Table 2.1.

Table 2.1. Environmental, Climatic and Soil Variables tested for the distribution models of *Geomalacus*, excluded variables, and importance of the included ones for the geographic distribution scenario for each species.

Code	**Description**	**Type**	**Source**	***G. maculosus***	***G. anguiformis***	***G. oliveirae***	***G. malagensis*[1]**	***G. malagensis*[2]**
Parmado	Code for dominant parent material of the STU	CAT	ESDB	N	N	42.2%	0%	N
AWC	Topsoil available water capacity	CAT	ESDB	CO	N	CO	2.9%	N
OC_top	Topsoil organic carbon content	CAT	ESDB	N	0%	10.8%	N	N
WRBFU	Full soil code of the STU from the WRB	CAT	ESDB	9.2%	5.2%	23.3%	N	12.9%
Alt	Elevation	CAT	ESDB	N	CO	N	3.0%	CO
Dimp	Depth to an impermeable layer	CAT	ESDB	0%	0%	0%	0%	0%
Min	Topsoil mineralogy	CAT	ESDB	0%	0%	0%	N	N
physchim	Physi-chemical factor of soil crusting & erodibility	CAT	ESDB	0%	0%	CO	0%	0%
Str	Topsoil structure	CAT	ESDB	CO	N	0%	0%	0%
Text	Dominant surface textural class	CAT	ESDB	N	N	CO	0%	N
Txsrfdo	Dominant surface textural class of the STU	CAT	ESDB	CO	0%	N	0%	0%
Zmax	Maximum elevation above sea level of the STU	CAT	ESDB	N	N	N	20.2%	8.4%
Usedo	Code for dominant land use of the STU	CAT	ESDB	N	N	CO	5.2%	9.8%
vqi_2008	Vegetation quality index	CAT	EEA	N	5.6%	0%	0%	0%
Dem	Altitude	CON	WCDB	N	CO	N	CO	CO
Bio5	Maximum temperature of the warmest month	CON	WCDB	10.3%	13.8%	CO	2.6%	2.5%
Bio6	Minimum Temperature of Coldest Month	CON	WCDB	CO	4.3%	CO	CO	17.6%
Bio12	Annual Precipitation	CON	WCDB	CO	CO	CO	N	7.7%
Bio13	Precipitation of Wettest Month	CON	WCDB	58.8%	12%	12.4%	14.6%	CO
Bio14	Precipitation of Driest Month	CON	WCDB	21.6%	59.2%	CO	CO	CO
Bio17	Precipitation of Driest Quarter	CON	WCDB	CO	CO	11.3%	51.5%	41%

CAT – Categorical, CON – Continuous, ESBD – European Soil Database, EEA – European Environment Agency, WCDB – WorldClim, STU – Soil typological unit , WRB – World reference base for soil resources, [1]prediction 1D for *G. malagens*is, [2]prediction 1E for *G. malagensis*, CO – removed from the model for correlation ($\rho \geq 0.85$) with other variables, as tested in R, 0% - Removed for contributing 0% for the model fitness, N - Removed for hindering model performance (AUC and Jackknife) and not improving the AUC.

Results

Models for the four species performed better than random, with average test AUC values ranging from 0.94 to 0.99. The current distributions of *G. maculosus*, *G. anguiformis* and *G. malagensis* were largely affected by bioclimatic variables, whereas the distribution of *G. oliveirae* was strongly predicted by soil variables (Table 2.1). Values of all environmental variables retained in the models are detailed in the Supporting Information Appendix S1.

Geomalacus maculosus

The predicted geographic distribution of *G. maculosus* was based on 92 locations: 79 from the literature and 17 from our fieldwork (Supporting Information Appendix S1, Figure 2.2a). The model had a high accuracy with an AUC = 0.94, and included four environmental variables (Table 2.1). The three most important variables were “precipitation of wettest month” (Bio13, 58.8%; range: 91 - 269 mm, average: 146 mm); “precipitation of driest month” (Bio14, 21.6%; range: 10 - 54 mm; average: 28 mm) and “maximum temperature of the warmest month” (Bio5, 10.3%; range: 20.5 ºC – 28.6 ºC; average: 24.4 °C). *Geomalacus maculosus* was effectively found in all the regions predicted by the model.

Geomalacus maculosus is a crepuscular and nocturnal slug that was found near houses and gardens, mostly on stony walls or rocks. It is extremely hard to find during early evening unless there is light shower rain. We always found *G. maculosus* in anthropogenic environments and, only in Galicia, Asturias and Cantabria, we found slugs, at early dawn, crawling on oak (*Quercus* sp.) and chestnut (*Castanea* sp.) trees, whereas at all other locations, the species was found crawling on rocks or stony walls.

Geomalacus anguiformis

The predicted geographic distribution of *G. anguiformis* was based on 21 locations: 12 from the literature and nine from our fieldwork (Supporting Information Appendix S1, Figure 2.2b). The accuracy of the model was very high with an AUC =

0.99. The preferred model included six environmental variables (Table 2.1). The three most important variables were “precipitation of driest month” (Bio14, 59.2%; range: 1 – 4 mm; average: 1.9 mm), “maximum temperature of the warmest month” (Bio5, 13.8%; range: 26.4 °C - 33.8 ºC; average: 28.7 ºC), and “precipitation of wettest month” (Bio13, 12%; range: 81 – 104 mm; average: 91.7 mm). *Geomalacus anguiformis* was found in all the regions predicted by the model.

In all locations the specimens were found at abandoned farms, except in Monchique (Figure 2.1), where it was found in pristine habitats, mainly under the bark of oak logs. Although the species is mostly active at night, we also found it active under tree logs during daytime, as long as the air was humid.

Geomalacus oliveirae

The predicted geographic distribution of *G. oliveirae* was based on 23 locations: 14 from the literature and nine from our fieldwork (Supporting Information Appendix S1, Figure 2.2c). The accuracy of the model was high with an AUC = 0.97. The preferred model included five environmental variables (Table 2.1). The three variables that contributed most to the model were: “dominant parent material of the soil typological unit, STU” (Parmado, 42%; being either granite or shale/argilite), “full soil code of the STU from the world reference base for soil resources” (WRBFU, 23%; with anthrosol and andosol as the preferred soil types), and “precipitation of wettest month” (Bio13, 12.4%; range: 81 – 104 mm; average: 91.7 mm). *Geomalacus oliveirae* was found in almost all regions predicted by the model, except for in Sierra Nevada in Southern Spain (Figure. 2.1), which had an occurrence probability of 0.80. *Geomalacus oliveirae* was found at night, crawling on stones near water streams or under rocks in pinewoods in areas with little or no human disturbance.

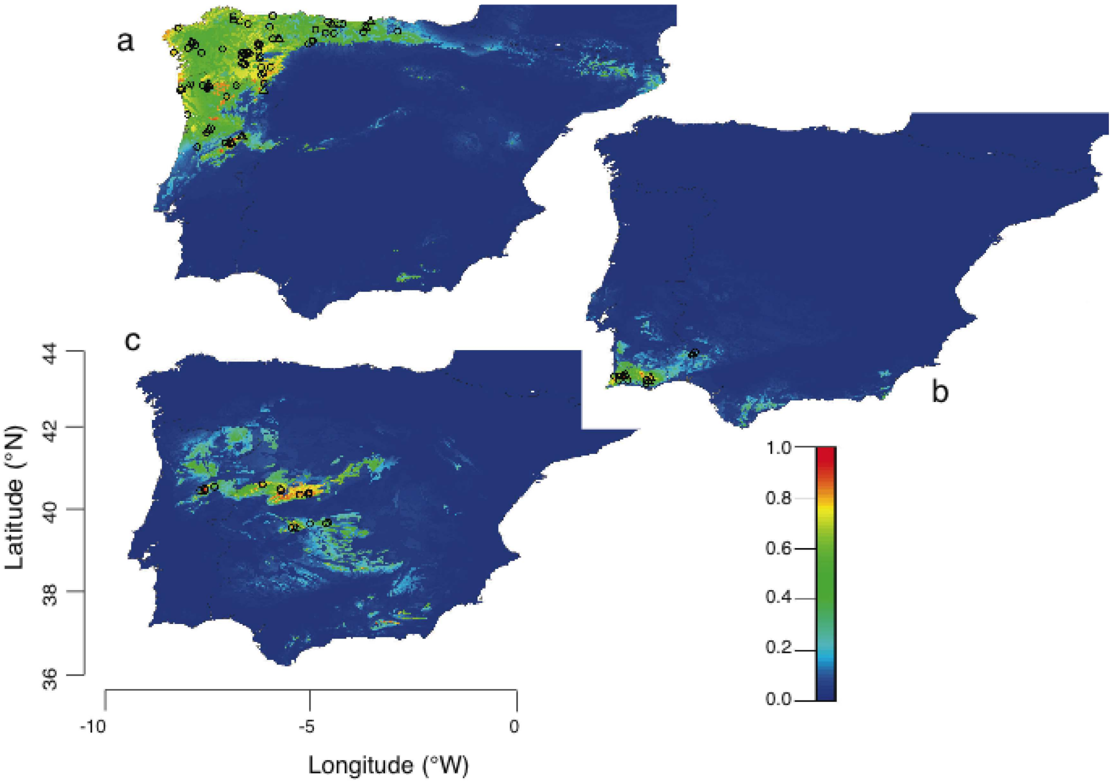

Figure 2.2. Habitat suitability map across the study area predicted from literature records and fieldwork for *Geomalacus maculosus* (a); *G. anguiformis* (b) and *G. oliveirae* (c). Color scale represent high levels of predicted habitat suitability in red and low levels in blue.

Geomalacus malagensis

The first model for *G. malagensis* was based on 11 records from the literature and three records from preliminary fieldwork prior to the ENM analyses, *viz.* Arrifana, Jerez, and Gibraltar (Figure 2.1), with Arrifana being the first record for this species in Portugal (Supporting Information Appendix S1, Figure 2.3a). The preferred model included seven environmental variables (Table 2.1) and had a high accuracy (AUC = 0.99). The three environmental variables that contributed most to this model were: “precipitation in the driest quarter” (Bio17, 51.5%; range: 13 – 42 mm; average: 23.5 mm), “maximum elevation above sea level of the STU” (zmax, 20.2%; range: 400 – 3000 m; average: 2145 m), and “precipitation in the wettest month” (Bio13, 14.6% range: 73 – 144 mm; average: 115.3 mm).

Jackson & Robertson (2011) proposed to look for additional records of species in areas with a presence probability > 40%. We identified four such areas for *G.*

malagensis (for details on locations see Figure 2.1 and Supporting Information Appendix S1): (1) Huelva in Andalusia, (2) Monchique, São Brás de Alportel, Estoi, and Caldeirão, in Algarve, (3) areas near Sesimbra, Arrábida, and Azeitão, and (4) Sintra and Peniche in Estremadura. We surveyed ten localities and recorded the species in five of these, *viz.* Calañas in Spain and Guilhim, Brejos de Azeitão, Fonte de Sesimbra and Cabedal in Portugal (Supporting Information Appendix S1).

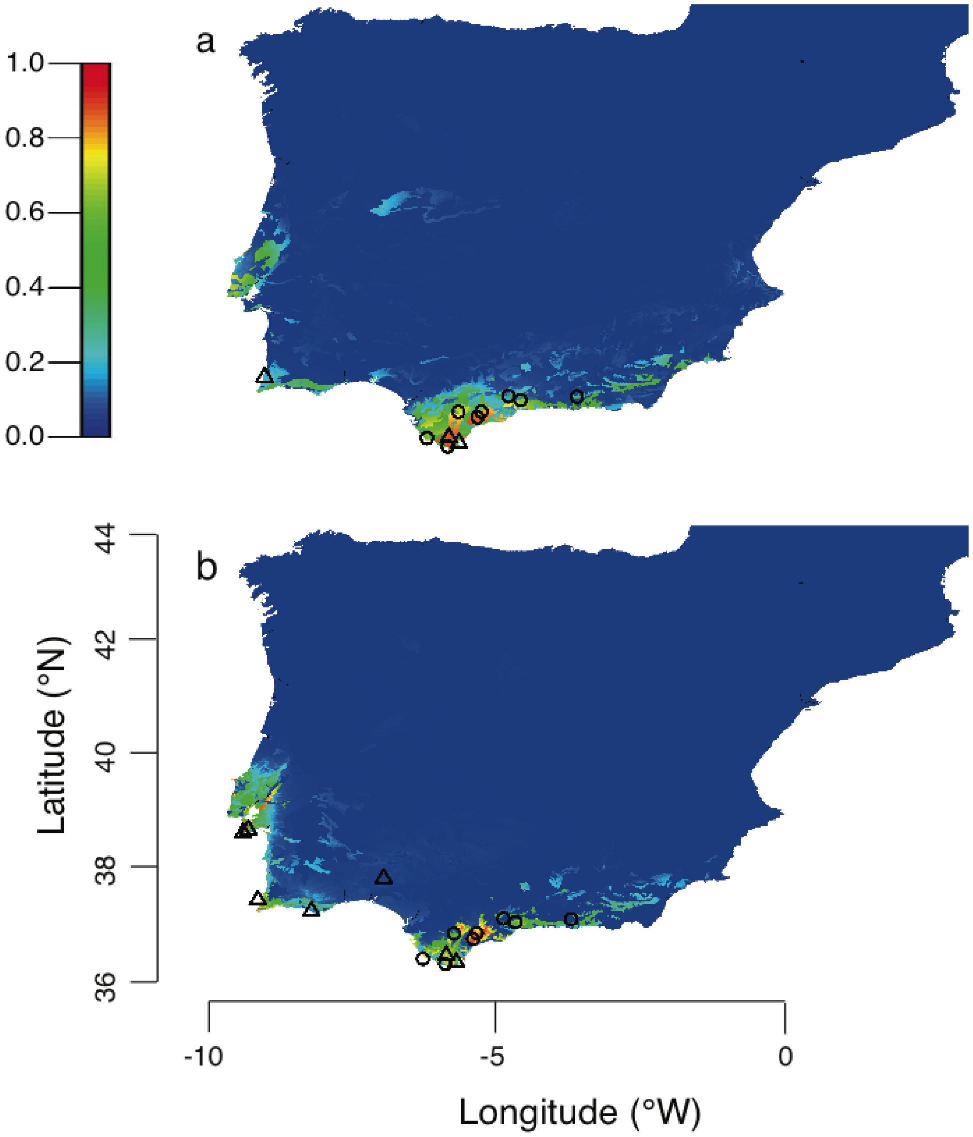

Figure 2.3. Habitat suitability map across the study area for *Geomalacus malagensis* based on sampling locations obtained from the literature and initial fieldwork (a) and based on all locations (literature and new locations) (b). Color scales represent high levels of predicted habitat suitability in red, and low levels in blue. Circles represent data points obtained from the literature, and triangles represent specimens sampled during the present work.

To improve the first distribution model, we added the five new records to the 11 localities of the first model and reran MaxEnt. The new, enhanced model (Figure 2.3b) suggested a greatly expanded geographic distribution: northwards up to 39 ºN and westwards to 9 ºW. The accuracy of this second model was very high with an AUC = 0.99. It included seven variables (Table 2.1), the most important of which were: “precipitation in the driest quarter” (Bio17, 41%; range: 13 - 42 mm; average 24.3 mm), “minimum temperature in the coldest month” (Bio 6, 17.6%; range: -1.5 ºC – 8.9 ºC; average 5.1 ºC) and the “full soil code of the STU from the world reference base for soil resources” (WRBFU, 12.9%; with specimens recorded in arenosol, cambisol, luvisol, histosol, gleysol, leptosol or marsh soils). Although both models predicted a habitat suitability of > 50% for *G. malagensis* in Estremadura, north of the river Tagus (Portugal), we were unable to confirm the presence of the species in this area. *Geomalacus malagensis* was always found near houses and gardens (feeding on dog food), on walls or hiding under piles of firewood.

Discussion

Distribution models obtained from few presence records should be interpreted as regions with environmental conditions (habitat similarity) similar to those where a species presently occurs (Pearson, 2007), but do not necessarily indicate the presence of a species. Hence, distribution scenarios do not outline the actual distribution range of a species but its putative distribution, meaning that they identify areas of high suitability for a species. Based on predictive species distribution models generated by MaxEnt we were able to significantly extend the known distribution range of *G. malagensis,* uncovering five new populations. Our results emphasize the usefulness of MaxEnt to predict geographic ranges of rare species. However, at least three caveats must be taken into account when ENM methods are employed (Anderson, 2012; Yackulic *et al.*, 2013). Firstly, with only few records available, ENM methods may overestimate the predicted range. Secondly, ENM methods assume that spatial sampling is such that the relationship between environmental variables and species occurrence accurately represents the suitability of habitats. Finally, estimation biases may be introduced by disparities in sampling effort between locations, spatial bias in sampling, and differences resulting from habitat availability in areas where a species is present. Below, we discuss the implication of these caveats for the distribution models that we obtained for the four *Geomalacus* species.

All distribution models had a high accuracy, with AUC values above 0.94. Bioclimatic variables, especially precipitation and to a lesser extent temperature, rather than soil characteristics, seem the most important variables to explain the distribution of three *Geomalacus* species. Only for *G. oliveirae* soil characteristics, especially the occurrence of granite or shale/argilite in an anthrosol-andosol soil type, seem important to explain its distribution. This fundamental difference in model composition might explain the absence of *G. oliveirae* in areas predicted by the model (discussed further below).

The predictive distribution models for *G. maculosus* and *G. anguiformis* agree with the known geographic ranges of these species (Castillejo *et al*., 1994). For *G. maculosus* the model encompasses the northwestern coastal area of the Iberian Peninsula, a region with constant humidity and high rainfall, even during the driest months (Altonaga *et al*., 1994). The third environmental variable that contributed to explain the distribution of *G. maculosus* was the maximum temperature of the warmest month, indicating that in humid conditions the species may endure temperatures of up to 28 ºC. Based on the model predictions and the microhabitat where this species is present, the common slug *G. maculosus* can be found in areas with a high precipitation, even during the dry season, on a calcareous soil and with an annual mean temperature of 25-35 °C. Within these areas the species can often be observed on rocky walls in oak or chestnut orchards, in ruins, near houses, churches and cemeteries. For *G. anguiformis* the model includes the southwestern mountains of Monchique, Caldeirão and Aracena. Yet, the populations are restricted to the most humid parts of these mountains since rainfall and humidity appear to be limiting factors for the distribution of this species (Castillejo & Iglesias-Pineiro, 2005). Nevertheless, *G. anguiformis* can withstand dry (precipitation ≤ 1 mm) and warm (up to 34 °C) periods.

In contrast, the distribution models for *G. oliveirae* and *G. malagensis* suggested much wider ranges than previously reported. Hitherto, *G. oliveirae* was known to occur in the central mountains of the Iberian Peninsula, namely in Serra da Estrela (Hidalgo, 1916; Nobre, 1941; Pollonera, 1890b; Rodriguez *et al*., 1993; Simroth, 1888), Béjar and Sierra de La Peña de Francia (Hermida, 1992), Sierra de Gredos (Castillejo *et al.*, 1994), Sierra de Guadalupe (Bech Taberner *et al.*, 2005; Castillejo *et al.*, 1994) and Montes de Toledo (Wiktor & Parejo, 1989) (for details on locations see Supporting Information Appendix S1, Figure 2.1). Yet, the MaxEnt model expanded this area by including the southern mountains of Sierra Nevada. This areas has both granite and argilite soils (either anthrosols or andosols), which together with heavy rainfall during the wettest month were the most important variables of the predicted model for this species. However, the species was never confirmed to occur in the Sierra Nevada. The central mountain ranges where this species is present (Serra da Estrela, Sierras de Gualdalupe, Gredos, Béjar and Peña de Francia) and Sierra Nevada share similar soil characteristics (granitic and shale/argillite soils and andosol), and that may have biased the prediction. Also, it is possible that with only few records, predicted range overestimation may have occurred. Because MaxEnt assumes that spatial sampling is such that the relationship between environmental variables and species occurrence accurately represents the suitability of habitats, it is possible that the habitat is suitable but the species is absent (Hirzel *et al.*, 2002; Raxworthy *et al.*, 2003; Pearson *et al.*, 2007).

The predicted range of *G. malagensis* includes Andalusia and expands to the adjacent areas around Huelva, as well as to the more distant areas in Portugal, where this species was never previously reported, *viz.* Algarve, Setúbal Peninsula and Estremadura. These regions are characterized by more than 13 mm of rain in the driest quarter, a minimum temperature of -1.5 °C, and moist, calcareous soils with high levels of decomposing organic matter.

Contrary to expectations from the literature (Castillejo *et al.*, 1994) where *G. malagensis* from outside Gibraltar was described as a non-anthropophilous organism, this species was only found in highly anthropogenic and disturbed habitats, suggesting that the species may survive well when subjected to human impact. We confirmed the occurrence of the species in some of the predicted areas such as Huelva, Algarve and Setúbal Peninsula. However, we were unable to find it in two areas with high probability of suitability of the habitat, namely Arrábida Natural Park and north of the Tagus River in Estremadura (Sintra and Peniche – Portugal). The absence North of the Tagus River might best be explained by an overestimation of the predictive range, due to the few records, resulting in an artificially larger predicted area. However alternative, non-mutually exclusive, explanations are possible, such as competition or dispersal limitations (Guisan & Thuiller, 2005). Obviously, species with poor dispersal abilities, as slugs allegedly are, comply with this latter possibility and this may also explain why ranges were over-predicted. The putative absence in Arrábida Natural Park may be explained similarly, but *G. malagensis* is not easily found in undisturbed forests. Because it was not possible to implement a grid-sampling throughout the entire predicted range, the absence of *G. malagensis* in the region is subject to further confirmation.

To conclude, we present maps of high suitability areas for the four Iberian *Geomalacus* species based on ENM. Guided by these suitability maps, we conducted directed fieldwork, which allowed us to report the first record of *G. malagensis* in Portugal, resulting in a substantial extension of the distribution area of this species. We further identified new and highly suitable regions where *G. malagensis* and *G. oliveirae* may occur.

Acknowledgments

The authors wish to thank K. Erzini, M.C. Ribeiro, P. Cesário, A. Patrão and D. Reis for collecting specimens in the field and T. Rodriguez for assistance with the identification of the specimens. This work was partially funded by Fundação para a Ciência e Tecnologia (Portugal) through C. Patrão PhD. grant SFRH/BD/30024/2006, and the project “Pillars of Hercules - Gene flow across the pillars of Hercules: the role of North Africa in the postglacial recolonization of Europe” PTDC/BIA-BDE/74349/2006. T. Backeljau was supported by BELSPO projects MO/36/017 and SPEEDY.

Appendix S1 - Sampling sites and literature referenced sites for all the *Geomalacus* species and value of the variables included in the geographic distribution models. In the Source column, "Fieldwork" refers to locations where samples were collected prior to ENM analysis, while "Present work" refers to locations that were subsequently added as a result of post-ENM fieldwork conducted in areas of high suitability proposed by the models.

Appendix S1.1 - Sampling sites and literature referenced sites for *Geomalacus anguiformis* and value of the variables included in the geographic distribution models. In the Source column, "Fieldwork" refers to locations where samples were collected prior to ENM analysis.

Species	Site	Country	Province	Coordinates		Source	Variables					
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 6 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU	Vqi
G. anguiformis	Cerro del Hierro (Sevilla)	Spain	Andalusia	37.954	-5.614	Castillejo & Iglesias-Pineiro (2011)	33.8	1.8	95	4	Anthrosol	moderate
G. anguiformis	El Repilado (Aracena)	Spain	Andalusia	37.920	-6.766	Castillejo, Garrido & Iglesias (1994)	31.8	3.8	87	3	Ferralic Anthrosol	moderate
G. anguiformis	El Quejigo (Aracena)	Spain	Andalusia	37.838	-6.841	Castillejo, Garrido & Iglesias (1994)	31.7	4.6	82	3	Ferralic Anthrosol	moderate
G. anguiformis	Serra do Caldeirão	Portugal	Algarve	37.225	-8.436	Hidalgo (1916)	29.0	7.8	82	1	Ferralic Anthrosol	moderate
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.316	-8.661	Mabille (1867); Simroth (1891); Hidalgo (1916); Rodriguez *et al.* (1993)	26.8	6.1	96	2	Eutric Arenosol	moderate
G. anguiformis	Serra do Caldeirão	Portugal	Algarve	37.316	-8.549	Morelet (1845); Hidalgo (1916)	27.1	5.8	98	2	Terric Anthrosol	moderate
G. anguiformis	Caldas de Monchique	Portugal	Algarve	37.285	-8.553	Rodriguez *et al*. (1993)	27.8	6.7	90	1	Ferralic Anthrosol	moderate
G. anguiformis	Alferce (Monchique)	Portugal	Algarve	37.337	-8.489	Rodriguez *et al*. (1993)	28.2	6.7	90	1	Eutric Arenosol	moderate
G. anguiformis	Road Monchique-Foia	Portugal	Algarve	37.226	-8.662	Rodriguez *et al*. (1993)	27.9	7.6	83	1	Siltic Chernozem	moderate
G. anguiformis	Barrance do Velho (Caldeirão)	Portugal	Algarve	37.222	-7.986	Rodriguez *et al*. (1993)	29.0	6.4	91	1	Ferralic Anthrosol	moderate
G. anguiformis	Alportel (Caldeirão)	Portugal	Algarve	37.132	-7.987	Rodriguez *et al*. (1993)	28.7	6.7	91	1	Hypoduric Arenosol	moderate
G. anguiformis	Marmelete (Monchique)	Portugal	Algarve	37.316	-8.774	Rodriguez *et al*. (1993)	27.7	8.2	81	1	Siltic Chernozem	moderate
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.307	-8.570	Fieldwork	27.1	5.8	98	2	Terric Anthrosol	moderate

Appendix S1.1 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables					
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 6 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU	Vqi
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.305	-8.588	Fieldwork	26.4	5.2	104	3	Terric Anthrosol	moderate
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.320	-8.536	Fieldwork	27.5	6.0	96	2	Terric Anthrosol	moderate
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.342	-8.488	Fieldwork	28.5	7.0	88	1	Eutric Arenosol	moderate
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.313	-8.549	Fieldwork	27.1	5.8	98	2	Terric Anthrosol	moderate
G. anguiformis	Serra de Monchique	Portugal	Algarve	37.306	-8.499	Fieldwork	27.9	6.4	92	1	Ferralic Anthrosol	moderate
G. anguiformis	São Brás de Alportel	Portugal	Algarve	37.194	-7.919	Fieldwork	28.5	5.7	95	2	Ferralic Anthrosol	moderate
G. anguiformis	São Brás de Alportel	Portugal	Algarve	37.273	-7.875	Fieldwork	28.3	4.9	100	2	Ferralic Anthrosol	moderate
G. anguiformis	Aracena	Spain	Andalusia	37.878	-6.817	Fieldwork	31.4	3.7	88	4	Melanic Andosol	moderate

Appendix S1.2 - Sampling sites and literature referenced sites for all *Geomalacus maculosus* and value of the variables included in the geographic distribution models. In the Source column, "Fieldwork" refers to locations where samples were collected prior to ENM analysis.

Species	**Site**	**Country**	**Province**	**Coordinates**		**Source**	**Variables**			
				Lat °N	**Long °W**		**Bio 5 (ºC)**	**Bio 13 (mm)**	**Bio14 (mm)**	**WRBFU**
G. maculosus	Monte Ganekogorta	Spain	Pais Vasco	43.199	-2.987	Altonga (1994)	22.0	131	54	Skeletic Acrisol
G. maculosus	Parada del Caurel	Spain	Galicia	42.629	-7.145	Castillejo (1981)	24.3	105	40	Hydric Andosol
G. maculosus	Seoane del Caurel	Spain	Galicia	42.641	-7.155	Castillejo (1981)	25.3	186	22	Terric Anthrosol
G. maculosus	Pendella (EI Caurel)	Spain	Galicia	42.583	-7.217	Castillejo (1981)	24.4	158	14	Terric Anthrosol
G. maculosus	Casela (El Caurel)	Spain	Galicia	42.633	-7.267	Castillejo (1981)	20.7	265	20	Hydric Andosol
G. maculosus	La Campa (El Caurel)	Spain	Galicia	42.567	-7.200	Castillejo (1981)	26.7	176	19	Terric Anthrosol
G. maculosus	Acebeiro (Forcarey)	Spain	Galicia	42.622	-8.301	Castillejo (1981)	NA	NA	NA	NA
G. maculosus	Argomoso (Mondonedo)	Spain	Galicia	43.400	-7.350	Castillejo (1981)	28.5	121	15	Terric Anthrosol
G. maculosus	Pico Sacro	Spain	Galicia	42.807	-8.447	Castillejo (1981)	24.6	144	27	Hydric Andosol
G. maculosus	Aramio (Santiago)	Spain	Galicia	42.864	-8.508	Castillejo (1981)	25.7	130	23	Hydric Andosol
G. maculosus	Pico de los Tres Obispos (Los Ancares)	Spain	Galicia	42.807	-6.868	Castillejo (1981)	25.5	123	25	Hydric Andosol
G. maculosus	VilareIlo (Los Ancares)	Spain	Galicia	42.846	-6.901	Castillejo (1981)	23.6	130	33	Terric Anthrosol
G. maculosus	Puente Ceso	Spain	Galicia	43.231	-8.877	Castillejo (1981)	23.6	130	33	Terric Anthrosol

Appendix S1.2 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables			
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU
G. maculosus	La Curota (Lesón)	Spain	Galicia	42.632	-8.986	Castillejo (1981)	23.6	130	33	Terric Anthrosol
G. maculosus	Herbón (Padrón)	Spain	Galicia	42.739	-8.639	Castillejo (1981)	NA	NA	NA	NA
G. maculosus	Los Cabaniños (Los Ancares)	Spain	Galicia	42.842	-6.904	Castillejo (1981)	24.5	161	31	Terric Anthrosol
G. maculosus	Camporredondo (Piomedo)	Spain	Galicia	42.838	-6.862	Castillejo (1981)	20.8	159	40	Hydric Andosol
G. maculosus	Viveiró (Serra do Xistral)	Spain	Galicia	43.520	-7.606	Castillejo (1998)	26.3	121	23	Terric Anthrosol
G. maculosus	O'Grove (Campos)	Spain	Galicia	42.488	-8.868	Castillejo (1998)	25.5	123	25	Hydric Andosol
G. maculosus	Gedrez (Ranhadoiro)	Spain	Asturias	42.969	-6.536	Castillejo, Garrido & Iglesias (1994)	25.5	123	25	Hydric Andosol
G. maculosus	Reserva del Saja (Cantabria)	Spain	Cantabria	43.130	-4.298	Castillejo, Garrido & Iglesias (1994)	23.1	155	34	Terric Anthrosol
G. maculosus	Carrejo (Cantabria)	Spain	Cantabria	43.294	-4.239	Castillejo, Garrido & Iglesias (1994)	22.5	141	39	Hydric Andosol
G. maculosus	Puerto de los Tornos (Cantabria)	Spain	Cantabria	43.152	-3.439	Castillejo, Garrido & Iglesias (1994)	22.3	149	40	Hydric Andosol
G. maculosus	Valporqueros (Sierra del Gato)	Spain	Castilla y Léon	42.907	-5.560	Castillejo, Garrido & Iglesias (1994)	23.9	125	34	Terric Anthrosol
G. maculosus	Puerto del Pontón (León)	Spain	Castilla y Léon	43.099	-5.019	Castillejo, Garrido & Iglesias (1994)	21.1	156	37	Hydric Andosol
G. maculosus	Biobra (Ourense)	Spain	Galicia	42.481	-6.863	Castillejo, Garrido & Iglesias (1994)	23.2	105	40	Terric Anthrosol
G. maculosus	Santa Albas	Spain	Asturias	43.197	-5.474	Castillejo & Rodriguez (1991)	23.4	114	39	Haplic Acrisol

Appendix S1.2 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables			
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU
G. maculosus	Serra da Estrela	Portugal	Beira Alta	40.372	-7.587	Castillejo & Rodriguez (1991)	22.3	121	46	Terric Anthrosol
G. maculosus	Embalse Puente Porto Sierra Segundera (Zamora)	Spain	Castilla Y Léon	42.071	-6.824	Castillejo & Rodriguez (1991)	26.6	103	20	Terric Anthrosol
G. maculosus	Oporto	Portugal	Douro Litoral	41.101	-8.643	Castillejo & Rodriguez (1991)	23.1	108	43	Hydric Andosol
G. maculosus	Santiago de Compostela*	Spain	Galicia	NA	NA	Castillejo & Rodriguez (1991)	22.3	110	47	Terric Anthrosol
G. maculosus	Feces da Cima	Spain	Galicia	41.855	-7.381	Castillejo & Rodriguez (1991)	23.4	122	37	Terric Anthrosol
G. maculosus	Montefurado	Spain	Galicia	42.350	-7.300	Castillejo & Rodriguez (1991)	25.4	123	25	Hydric Andosol
G. maculosus	Puebla de Trives	Spain	Galicia	42.339	-7.253	Castillejo & Rodriguez (1991)	25.2	125	26	Hydric Andosol
G. maculosus	La Rogueira (El Caurel)	Spain	Galicia	42.618	-7.171	Castillejo & Rodriguez (1991)	26.3	116	23	Terric Anthrosol
G. maculosus	Bosque de los Cabaniños	Spain	Galicia	42.842	-6.904	Castillejo & Rodriguez (1991)	24.9	133	27	Hydric Andosol
G. maculosus	Camporredondo	Spain	Galicia	42.963	-7.218	Castillejo & Rodriguez (1991)	25.0	131	27	Terric Anthrosol
G. maculosus	Las Morteiras	Spain	Galicia	42.794	-6.921	Castillejo & Rodriguez (1991)	23.1	220	38	Hydric Andosol
G. maculosus	Puente Bibey	Spain	Galicia	42.334	7.215	Castillejo & Rodriguez (1991)	23.6	129	35	Hydric Andosol
G. maculosus	Xiân (Taboada)	Spain	Galicia	42.716	-7.779	Castillejo & Rodriguez (1991)	24.4	210	39	Terric Anthrosol
G. maculosus	Lairoso (Peña Trevinca)	Spain	Galicia	42.251	-6.818	Castillejo & Rodriguez (1991)	24.5	188	35	Terric Anthrosol

Appendix S1.2 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables			
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU
G. maculosus	Folgoso del Caurel	Spain	Galicia	42.530	-7.295	Castillejo & Rodriguez (1991)	20.5	161	47	Hydric Andosol
G. maculosus	Ferreiria del Caurel	Spain	Galicia	42.618	-7.171	Castillejo & Rodriguez (1991)	24.2	123	31	Hydric Andosol
G. maculosus	Mercurin	Spain	Galicia	42.618	-7.171	Castillejo & Rodriguez (1991)	22.9	140	28	Terric Anthrosol
G. maculosus	Piedrafrita del Caurel	Spain	Galicia	42.620	-7.293	Castillejo & Rodriguez (1991)	22.5	184	31	Hydric Andosol
G. maculosus	Ferreira (Valle del Oro)	Spain	Galicia	43.523	-7.515	Castillejo & Rodriguez (1991)	24.0	179	31	Hydric Andosol
G. maculosus	Puente debajo del Cuadramón	Spain	Galicia	43.433	-7.517	Castillejo & Rodriguez (1991)	24.2	123	31	Hydric Andosol
G. maculosus	Taramundi (Asturias)	Spain	Galicia	43.338	-7.150	Castillejo & Rodriguez (1991)	22.1	143	40	Hydric Andosol
G. maculosus	Caldas de Gerês	Portugal	Minho	41.729	-8.158	Castillejo & Rodriguez (1991)	22.6	142	38	Terric Anthrosol
G. maculosus	Viana do Castelo	Portugal	Minho	41.820	-8.278	Castillejo & Rodriguez (1991)	23.2	174	27	Terric Anthrosol
G. maculosus	Viana do Castelo	Portugal	Minho	41.841	-8.571	Castro (1873)	24.0	217	24	Terric Anthrosol
G. maculosus	Arenas de Cabrales (Arangas)	Spain	Asturias	43.341	-4.798	Hermida (1992)	22.6	114	45	Hyperochric Acrisol
G. maculosus	Arenas de Cabrales	Spain	Asturias	43.323	-4.872	Hermida (1992)	23.5	108	40	Lamellic Arenosol
G. maculosus	Cangas de Narcea (Pola de Allande)	Spain	Asturias	43.270	-6.608	Hermida (1992)	24.3	117	35	Hydric Andosol
G. maculosus	Cangas de Onis (La Morca – Cruce a Cosiella)	Spain	Asturias	43.385	-5.171	Hermida (1992)	23.8	107	38	Hyperochric Acrisol

Appendix S1.2 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables			
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU
G. maculosus	Covadonga*	Spain	Asturias	NA	NA	Hermida (1992)	NA	NA	NA	NA
G. maculosus	Luarca	Spain	Asturias	43.535	-6.536	Hermida (1992)	24.6	111	33	Hydric Andosol
G. maculosus	Tarna	Spain	Asturias	43.108	-5.224	Hermida (1992)	23.2	107	44	Hyperochric Acrisol
G. maculosus	La Bañeza (Encinedo)	Spain	Castilla Y Léon	42.263	-6.588	Hermida (1992)	25.8	103	23	Terric Anthrosol
G. maculosus	La Robla (Felmin)	Spain	Castilla Y Léon	42.919	-5.541	Hermida (1992)	24.3	100	40	Terric Anthrosol
G. maculosus	Villafranca del Bierzo (Cruce a Trabadelo)	Spain	Castilla Y Léon	42.648	-6.876	Hermida (1992)	26.4	101	22	Hydric Andosol
G. maculosus	Villafranca del Bierzo (Friera)	Spain	Castilla Y Léon	42.526	-6.837	Hermida (1992)	26.2	105	22	Hydric Andosol
G. maculosus	Puebla de Sanabria (Moncabril)	Spain	Castilla Y Léon	42.122	-6.755	Hermida (1992)	25.6	113	23	Hydric Andosol
G. maculosus	Huergas de Gordón (South 0.5Km)	Spain	Castilla y Léon	42.838	-5.652	Platts & Speight (1988)	25.7	91	34	Hyperochric Acrisol
G. maculosus	Herbon	Spain	Galicia	42.738	-8.627	Platts & Speight (1988)	24.0	181	31	Hydric Andosol
G. maculosus	Covadonga	Spain	Asturias	43.307	-5.053	Fieldwork	25.0	168	16	Terric Anthrosol
G. maculosus	Covadonga	Spain	Asturias	43.341	-5.084	Fieldwork	23.8	179	14	Terric Anthrosol
G. maculosus	Caldas de Manteigas	Portugal	Beira Alta	40.383	-7.544	Fieldwork	27.4	104	14	Terric Anthrosol
G. maculosus	Guarda	Portugal	Beira Alta	40.535	-7.273	Fieldwork	25.2	207	12	Terric Anthrosol

Appendix S1.2 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables			
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU
G. maculosus	Serra da Estrela	Portugal	Beira Alta	40.414	-7.588	Fieldwork	24.6	189	12	Terric Anthrosol
G. maculosus	Chãos	Portugal	Beira Alta	40.539	-7.313	Fieldwork	21.5	269	17	Hydric Andosol
G. maculosus	Vouzela	Portugal	Beira Alta	40.721	-8.109	Fieldwork	26.0	178	11	Terric Anthrosol
G. maculosus	Santillana del Mar	Spain	Cantabria	43.388	-4.110	Fieldwork	27.7	160	12	Terric Anthrosol
G. maculosus	Saja Besaya	Spain	Cantabria	43.238	-4.227	Fieldwork	24.9	191	23	Hydric Andosol
G. maculosus	Caboalles Arriba	Spain	Castilla Y Léon	42.949	-6.395	Fieldwork	26.0	181	21	Hydric Andosol
G. maculosus	Caboalles Abajo	Spain	Castilla Y Léon	42.955	-6.372	Fieldwork	23.0	122	41	Skeletic Acrisol
G. maculosus	Santiago de Compostela	Spain	Galicia	42.878	-8.555	Fieldwork	23.2	112	40	Terric Anthrosol
G. maculosus	Sé	Portugal	Minho	41.774	-8.619	Fieldwork	23.6	108	41	Lamellic Arenosol
G. maculosus	Portuzelo	Portugal	Minho	41.705	-8.792	Fieldwork	24.0	105	37	Hyperochric Acrisol
G. maculosus	Gerês Albergaria	Portugal	Minho	41.793	-8.138	Fieldwork	23.9	116	36	Hydric Andosol
G. maculosus	Portela do Homem	Portugal	Minho	41.834	-8.119	Fieldwork	23.7	117	37	Hydric Andosol
G. maculosus	Bragança	Portugal	Trás os Montes e Alto Douro	41.6878	-6.7508	Fieldwork	24.2	188	35	Terric Anthrosol
G. maculosus	Sabugueiro (Serra da Estrela)	Portugal	Beira Alta	40.402	-7.641	Rodriguez *et al*. (1993)	23.9	232	14	Hydric Andosol

Appendix S1.2 – (Continuation)

Species	Site	Country	Province	Coordinates		Source	Variables			
				Lat ºN	Long ºW		Bio 5 (ºC)	Bio 13 (mm)	Bio14 (mm)	WRBFU
G. maculosus	São Romao (Serra da Estrela)	Portugal	Beira Alta	40.397	-7.717	Rodriguez *et al*. (1993)	27.5	181	12	Terric Anthrosol
G. maculosus	Ermita Nossa Sra. do Desterro (Serra da Estrela)	Portugal	Beira Alta	40.395	-7.695	Rodriguez *et al*. (1993)	26.0	203	13	Terric Anthrosol
G. maculosus	Chãos (Serra da Estrela)*	Portugal	Beira Alta	NA	NA	Rodriguez *et al*. (1993)	NA	NA	NA	NA
G. maculosus	São Pedro do Sul	Portugal	Beira Alta	40.757	-8.065	Rodriguez *et al*. (1993)	NA	NA	NA	NA
G. maculosus	Vouzela*	Portugal	Beira Alta	NA	NA	Rodriguez *et al*. (1993)	23.6	201	26	Hydric Andosol
G. maculosus	Paços (São Pedro do Sul)	Portugal	Beira Alta	40.648	-8.172	Rodriguez *et al*. (1993)	24.1	194	22	Hydric Andosol
G. maculosus	Luso	Portugal	Beira Litoral	40.289	-8.412	Rodriguez *et al*. (1993)	NA	NA	NA	NA
G. maculosus	Portela do Homem*	Portugal	Minho	NA	NA	Rodriguez *et al*. (1993)	28.0	96	14	Terric Anthrosol
G. maculosus	Albergaria (Serra de Gerês)*	Portugal	Minho	NA	NA	Rodriguez *et al*. (1993)	28.4	150	11	Terric Anthrosol
G. maculosus	Curral de Leonte (Serra de Gerês)	Portugal	Minho	41.768	-8.147	Rodriguez *et al*. (1993)	NA	NA	NA	NA
G. maculosus	Quintás (Chaves)	Portugal	Trás os Montes e Alto Douro	41.5438	-7.6806	Rodriguez *et al*. (1993)	24.9	211	16	Terric Anthrosol
G. maculosus	Rabal (Bragança)	Portugal	Trás os Montes e Alto Douro	41.8691	-6.7507	Rodriguez *et al*. (1993)	28.6	134	10	Arenic Anthrosol
G. maculosus	Viana do Castelo	Portugal	Minho	41.696	-8.826	Rodriguez *et al*. (1993); Castillejo & Rodriguez (1991)	23.6	179	14	Glossic Chernozem

Appendix S1.3 - Sampling sites and literature referenced sites for all *Geomalacus malagensis* and value of the variables included in the geographic distribution models. In the Source column, "Fieldwork" refers to locations where samples were collected prior to ENM analysis, while "Present work" refers to locations that were subsequently added as a result of post-ENM fieldwork conducted in areas of high suitability proposed by the models.

Species	**Site**	**Country**	**Province**	**Coordinates**		**Source**	**Variables**				
				Lat °N	**Long °W**		**Bio 6 (°C)**	**Bio 13 (mm)**	**Bio17 (mm)**	**WRBFU**	**Zmax** (m)
G. malagensis	Torcal de Antequera	Spain	Andalusia	36.972	4.547	Castillejo *et al*. (1996)	0.9	100	31	Rendzic Leptosol	2000
G. malagensis	Capileira	Spain	Andalusia	36.961	3.359	Castillejo *et al*. (1996)	-0.5	73	42	Eutric Cambisol	2500
G. malagensis	Sierra de las Nieves	Spain	Andalusia	36.700	5.017	Castillejo *et al*. (1996)	-1.5	133	41	Eutric Cambisol	2500
G. malagensis	Puerto del Madroño	Spain	Andalusia	36.601	5.081	Castillejo *et al*. (1996)	3.1	117	23	Eutric Cambisol	2500
G. malagensis	Brejos de Azeitão	Portugal	Sesimbra, Setúbal Peninsula	38.4761	9.1143	Present work	8.1	103	28	Haplic arenosol	100
G. malagensis	Fonte de Sesimbra	Portugal	Sesimbra, Setúbal Peninsula	38.5409	9.03	Present work	8	107	31	Calcaric cambisol	400
G. malagensis	Cabedal	Portugal	Sesimbra, Setúbal Peninsula	38.4864	9.1206	Present work	8.1	106	31	Haplic arenosol	100
G. malagensis	Arrifana	Portugal	Algarve	37.296	8.865	Fieldwork	8.3	82	18	Vertic Luvisol	400
G. malagensis	Guilhim	Portugal	Algarve	37.098	7.922	Present work	7.3	88	16	Salic Histosol	300
G. malagensis	Calañas	Spain	Andalusia	37.679	6.653	Present work	4.5	85	23	Haplic Gleysol	N/A
G. malagensis	Jerez	Spain	Andalusia	36.255	5.582	Fieldwork	7.8	144	15	Marsh	3000
G. malagensis	Gibraltar	Spain	Gibraltar	36.160	5.350	Fieldwork	8.9	137	13	Calcaric Gleysol	2800
G. malagensis	Benaocaz	Spain	Andalusia	36.700	5.421	Wiktor & Norris (1991)	3.0	126	23	Rendzic Leptosol	2000
G. malagensis	Colmenar	Spain	Andalusia	36.902	4.339	Wiktor & Norris (1991)	3.4	87	23	Eutric Cambisol	2500
G. malagensis	Sierra del Niño	Spain	Andalusia	36.183	5.600	Wiktor & Norris (1991)	4.5	141	16	Marsh	3000
G. malagensis	Vejer de la Fronteira	Spain	Andalusia	36.255	5.962	Wiktor & Norris (1991)	8.3	128	14	Ferric Luvisol	400

Appendix S1.3 - Sampling sites and literature referenced sites for all *Geomalacus oliveirae* and value of the variables included in the geographic distribution models. In the Source column, "Fieldwork" refers to locations where samples were collected prior to ENM analysis.

Species	Site	Country	Province	Coordinates		Source					
				Lat °N	Long °W		Bio 13 (mm)	Bio17 (mm)	WRBFU	oc_top	Parmado
G. oliveirae	Garganta Salóbriga (Navalvillar de Ibor)	Spain	Estremadura	39.594	-5.412	Bech Tabernet *et al.* (2005)	57	40	Terric Anthrosol	very low	granite
G. oliveirae	Arroyo de la Barquera (Hoyos)	Spain	Estremadura	40.177	-6.769	Bech Tabernet *et al.* (2005)	112	57	Luvic Andosol	low	shale/argilite
G. oliveirae	Hoyos del Collado (Sierra de Gredos)	Spain	Castilla Y Léon	40.269	-5.235	Castillejo, Garrido & Iglesias (1994)	86	94	Terric Anthrosol	very low	granite
G. oliveirae	Guadalupe	Spain	Estremadura	39.454	-5.441	Castillejo, Garrido & Iglesias (1994)	68	49	Hydric Andosol	very low	granite
G. oliveirae	Bejar (Alto Los Quemados)	Spain	Castilla Y Léon	40.368	-5.700	Hermida (1992)	100	94	Terric Anthrosol	low	shale/argilite
G. oliveirae	Bejar (Navacarros y Vallejera de Riofrio)	Spain	Castilla Y Léon	40.404	-5.716	Hermida (1992)	68	62	Terric Anthrosol	low	shale/argilite
G. oliveirae	Peña de Francia (Las Batuecas)	Spain	Castilla Y Léon	40.520	-6.162	Hermida (1992)	95	71	Placic Andoso	low	shale/argilite
G. oliveirae	Guadalupe	Spain	Estremadura	39.440	-5.315	Fieldwork	57	35	Placic Andoso	medium	shale/argilite
G. oliveirae	Guadalupe	Spain	Estremadura	39.474	-5.368	Fieldwork	70	53	Ferralic Anthrosol	very low	granite
G. oliveirae	Montes Toledo	Spain	Castilla La Mancha	39.563	-4.585	Fieldwork	56	50	Ferralic Anthrosol	medium	shale/argilite
G. oliveirae	Montes Toledo	Spain	Castilla La Mancha	39.585	-4.527	Fieldwork	58	51	Terric Anthrosol	medium	shale/argilite
G. oliveirae	Peña de Francia	Spain	Castilla Y Léon	40.514	-6.157	Fieldwork	92	70	Placic Andoso	low	shale/argilite
G. oliveirae	Gredos	Spain	Castilla Y Léon	40.322	-5.014	Fieldwork	68	71	Placic Andoso	very low	granite
G. oliveirae	Gredos	Spain	Castilla Y Léon	40.308	-5.000	Fieldwork	60	64	Hydric Andosol	very low	granite
G. oliveirae	Serra da Estrela	Portugal	Beira Alta	40.414	-7.588	Fieldwork	269	103	Terric Anthrosol	low	granite
G. oliveirae	Gredos	Spain	Castilla Y Léon	40.276	-5.018	Fieldwork	51	51	Terric Anthrosol	low	shale/argilite
G. oliveirae	Caldas de Manteigas	Portugal	Beira Alta	40.395	-7.550	Rodriguez *et al.* (1993)	249	94	Terric Anthrosol	low	granite
G. oliveirae	Chãos	Portugal	Beira Alta	40.543	-7.322	Rodriguez *et al.* (1993)	176	73	Terric Anthrosol	low	granite

Appendix S1.3 – (Continuation)

Species	Site	Country	Province	Coordinates		Source					
				Lat °N	Long °W		Bio 13 (mm)	Bio17 (mm)	WRBFU	oc_top	Parmado
G. oliveirae	Chãos	Portugal	Beira Alta	40.543	-7.322	Rodriguez *et al.* (1993)	176	73	Terric Anthrosol	low	granite
G. oliveirae	Sameiro	Portugal	Beira Alta	40.424	-7.471	Rodriguez *et al.* (1993)	188	76	Terric Anthrosol	low	granite
G. oliveirae	Crossroad Guarda-Manteigas-Gouveia	Portugal	Beira Alta	40.373	-7.704	Rodriguez *et al.* (1993)	217	84	Placic Andoso	low	granite
G. oliveirae	Serra da Estrela	Portugal	Beira Alta	40.372	-7.587	Simroth (1888); Nobre (1941); Hidalgo (1916); Pollonera (1890)	265	111	Terric Anthrosol	low	granite
G. oliveirae	Guarda	Portugal	Beira Alta	40.459	-7.349	Simroth (1891); Rodriguez *et al.* (1993)	199	80	Ferralic Anthrosol	low	granite
G. oliveirae	Robledo del Mazo	Spain	Castilla La Mancha	39.553	-4.979	Wiktor & Parejo (1989)	53	42	Placic Andosol	medium	shale/argilite

*We collected species at these locations, and therefore the precise geo-referenced location data from out field trips were used instead of the less precise literature reference

Bio 5 - Maximum temperature of the warmest month; Bio 6 - Minimum temperature of coldest month; Bio 13 - Precipitation of Wettest Month; Bio 14 - Precipitation of driest month; Bio 17 - Precipitation of Driest Quarter; OC_top - Topsoil organic carbon content; Parmado - Code for dominant parent material of the STU; Vqi - Vegetation quality index; WRBFU - Full soil code of the STU from the World Reference Base for Soil Resources.

Chapter III

Geomalacus and *Letourneuxia* (Mollusca, Pulmonata): A cytogenetic assessment

Geomalacus and *Letourneuxia* (Mollusca, Pulmonata): A cytogenetic assessment

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Accepted for publication in Malacologia

Introduction

The terrestrial malacofauna of the Iberian Peninsula is extremely rich and shows the highest diversity of arionid slug species in Europe (and probably worldwide), with 30 to 50 species, including several endemic ones (Castillejo, 1998). However, the taxonomic status of several of these species remains unclear due to the extreme variability in body size and color (Backeljau & De Bruyn, 1990). Moreover, these slugs often lack reliable diagnostic morphological traits (Backeljau & De Bruyn, 1990). The taxonomy of terrestrial slugs is based almost entirely on the morphology of their variable reproductive apparatus, which varies according to developmental stage and sexual maturation, often preventing the correct identification of juveniles at the species level (Backeljau & De Bruyn, 1990; and references therein, Backeljau *et al.*, 1996). As a consequence, the taxonomic status of supraspecific arionid groupings, such as (sub) genera, is still controversial. This applies to the genera *Geomalacus* Allman, 1843, and *Letourneuxia* Bourguignat, 1866, which have undergone several taxonomic changes since their original descriptions.

Geomalacus presently comprises four species grouped into two subgenera: *G.* (*Arrudia*) *anguiformis* (Morelet, 1845), *G.* (*A.*) *oliveirae* Simroth, 1891, *G.* (*A.*) *malagensis* Wiktor & Norris, 1991 and *G.* (*Geomalacus*) *maculosus* Allman, 1843. The first three species are endemic to the Iberian Peninsula, whereas *G. maculosus* is also found in southwestern Ireland. Although *G. maculosus* is easily identified by its unique color pattern, *G. anguiformis* and *G. oliveirae* are very similar in their external morphology, showing only subtle differences in their reproductive organs (Rodriguez *et al.,* 1993). Often, these two species have been identified solely from the geographical origin of specimens (Castillejo & Rodriguez, 1991). But this is not the only problem in *Geomalacus* taxonomy. When Wiktor & Norris (1991) originally described *G. malagensis*, the set of existing features for the classification of Arionidae provided by Hesse (1926) was “considered unsatisfactory” (Wiktor & Norris, 1991), since this species could have been easily classified within any of the three genera (*Arion*, *Geomalacus* or *Letourneuxia*) of this family. Therefore, Wiktor & Norris (1991) proposed additional diagnostic characters, and the new species was included in the genus *Geomalacus*.

The description of the genus *Letourneuxia* Bourguignat, 1866, was based on specimens from Algeria. This taxon is described as endemic from North Africa, and it has suffered successive changes in its taxonomic status, including being described as a subgenus of *Geomalacus* (Pollonera, 1890), a subgenus of *Arion* (Hesse, 1926), and, finally, acquiring generic status within the family Arionidae (Wiktor, 1983). The four nominal species, viz. G. (*L.) numidica* Bourguignat, 1866, *L. atlantica* Bourguignat, 1883, *L. maroccanus* Pollonera, 1916, and G. (*L.) turneri* Pollonera, 1890, together with *Arion moreleti* Hesse, 1884, have been synonymized with *L. numidica* by Wiktor (1983).

The debate as to whether *Letourneuxia* and *Geomalacus* should be kept in separate genera was fueled by the description of *G. malagensis*. However, even if *L. numidica* and *G. malagensis* are very similar in external morphology and color, they present two major differences in their reproductive organs: (1) *G. malagensis* has a large, thick epiphallus that is lacking in *L. numidica*, and (2) *L. numidica* has a voluminous atrium with a ligula inside, whereas the atrium of *G. malagensis* is slender and lacks a ligula (Wiktor & Norris, 1991). Because of the variability of these diagnostic features, and the fact that they mostly rely on fully mature individuals, additional evidence is needed to evaluate the degree of differentiation between *Letourneuxia* and *Geomalacus*.

Cytogenetic studies in slugs and snails have yielded important taxonomic insights (e.g.Colomba *et al.*, 2009; Kongim *et al.*, 2009; Kongim *et al.*; Vitturi *et al.*, 2005). However, for slugs, these reports are exclusively based on the use of gonadal tissue for chromosome preparations. This constitutes a serious drawback as it excludes juvenile individuals in which the ovotestis is not yet fully developed. Juvenile terrestrial slugs are particularly difficult to identify considering that the taxonomy is based on the morphology of their highly variable reproductive apparatus. So, if other organs provide reliable cytogenetic results, then this traditional disadvantage would be overcome.

In the past, chromosome studies of slugs (Beeson, 1960) and of euthyneuran gastropods in general (Burch, 1965) suggested that karyological data might be useful to distinguish (sub)genus level taxa in limacid and arionid slugs. There seems to be a suggestive correspondence between haploid chromosome numbers and subgeneric groupings in the genus *Arion*: n = 25 in *Mesarion* Hesse, 1926, n = 26 in *Arion* Férussac, 1819, n = 28 in *Kobeltia* Seibert, 1873 and *Microarion* Hesse, 1926, and n = 29 in *Carinarion* Hesse, 1926 (Beeson, 1960). This observation was, amongst others, used to include *Microarion* in the subgenus *Kobeltia* (Backeljau & De Bruyn, 1990). However, no karyotypes are available for any *Arion*, and no cytogenetic study (chromosome number and karyotypic formula) has been conducted in *Letouneuxia* or *Geomalacus.*

Here we present karyotypes and a comparative karyological study of the four *Geomalacus* species and *L. numidica* after testing different somatic tissues (mouth and both optical and sensory tentacles) to evaluate their suitability for karyological studies. We also assess the contribution of cytogenetics to provide additional evidence to resolve the taxonomy of these slugs.

Materials and methods

Biological Material

Specimens of all five nominal species were collected in the Iberian Peninsula and Morocco as detailed in Table 2.1 and identified following Castillejo *et al.* (1994) (1994). Animals were kept alive at 4 ºC and fed with lettuce. Prior to the experiments (48 h), the slugs were kept at room temperature.

Chromosome Preparation

Whole individuals were submerged for 75 min in a 0.01% solution of colchicine at room temperature. Then ovotestis, mouth, and optical and sensory tentacles were dissected. Somatic tissues were chosen as representative structures with high mitotic rates: the mouth for the constant renewal of the radula by odontoblastic and membranoblastic cells and the tentacles for their ability to regenerate (Barker, 2001).

All structures were subjected to a hypotonic treatment for 45 min in 0.9% sodium citrate and fixed in a freshly prepared mixture of absolute ethanol and glacial acetic acid (3:1). Fixed pieces of ovotestis, mouth and tentacles were dissociated in 50% glacial acetic acid and distilled water. Slides were prepared following an air-drying technique (Thiriot-Quiévreux & Ayraud, 1982). Slides were stained with Giemsa (4%, pH 6.8) for 10 min.

Table 3.1. Localities, number of specimens (n), diploid chromosome number and karyotypic formulae for the four *Geomalacus* and the *Letourneuxia* species used in this study. Sp – Spain; Pt – Portugal.

Genus/Species	Locality	n	Geographical coordinates	Chromosome number	Karyotypic formula
G. oliveirae	Gredos, Sp	1	40.3217°N/ 5.0135°W	2n = 62	15m + 13sm + 3st
G. oliveirae	Gredos, Sp	3	40.3151°N/ 5.0090°W	2n = 62	15m + 13sm + 3st
G. oliveirae	Pena de Francia, Sp	3	40.5144°N/ 6.1567°W	2n = 62	15m + 13sm + 3st
G. maculosus	Chãos, Serra Estrela, Pt	1	40.5386°N/ 7.3125°W	2n = 62	14m + 12sm + 5st
G. maculosus	Caldas Manteigas, Serra Estrela, Pt	8	40.3825°N/ 7.5442°W	2n = 62	14m + 12sm + 5st
G. maculosus	Viana do Castelo, Minho, Pt	5	41. 7739ºN/ 8.6186ºW	2n = 62	14m + 12sm + 5st
G. anguiformis	São Brás de Alportel, Algarve, Pt	4	37.2728°N/ 7.8753°W	2n = 62	14m + 10sm + 7st
G. malagensis	Fonte Sesimbra, Setúbal, Pt	2	38.4761°N/ 9.1143°W	2n = 62	10m + 12sm + 9st
G. malagensis	Guilhim, Algarve, Pt	15	37.1016°N/ 7.9279°W	2n = 62	10m + 12sm + 9st
L. numidica	Tanger, Morocco	7	35.7844°N/ 5.9011°W	2n = 62	10m + 12sm + 9st
L. numidica	Tanger, Morocco	2	35.7827°N/ 5.8506°W	2n = 62	10m + 12sm + 9st

Karyotyping

Images of Giemsa-stained metaphases were acquired with a digital camera (Nikon DSFi 1) coupled to a light microscope (Nikon Eclipse 80i). Digital images were processed with Adobe Photoshop (edition CS3) using functions only affecting the whole image. Ten karyotypes per species were performed in which chromosomes were organized based on relative length and centromeric position; terminology followed Levan et *al.* (Levan, Fredga & Sandberg, 1964).

Results

To test the suitability of different organs to provide usable chromosome images, we performed a number of trials with different individuals from all five species. Chromosomes were not obtained from preparations of ovotestis from juvenile individuals, regardless the analyzed species, but only from specimens where the ovotestis was well differentiated. However, using mouth and both optical and sensory tentacles, it was possible to obtain diploid chromosome preparations independently of the individual stage of development (Figure 3.1 b, c and d).

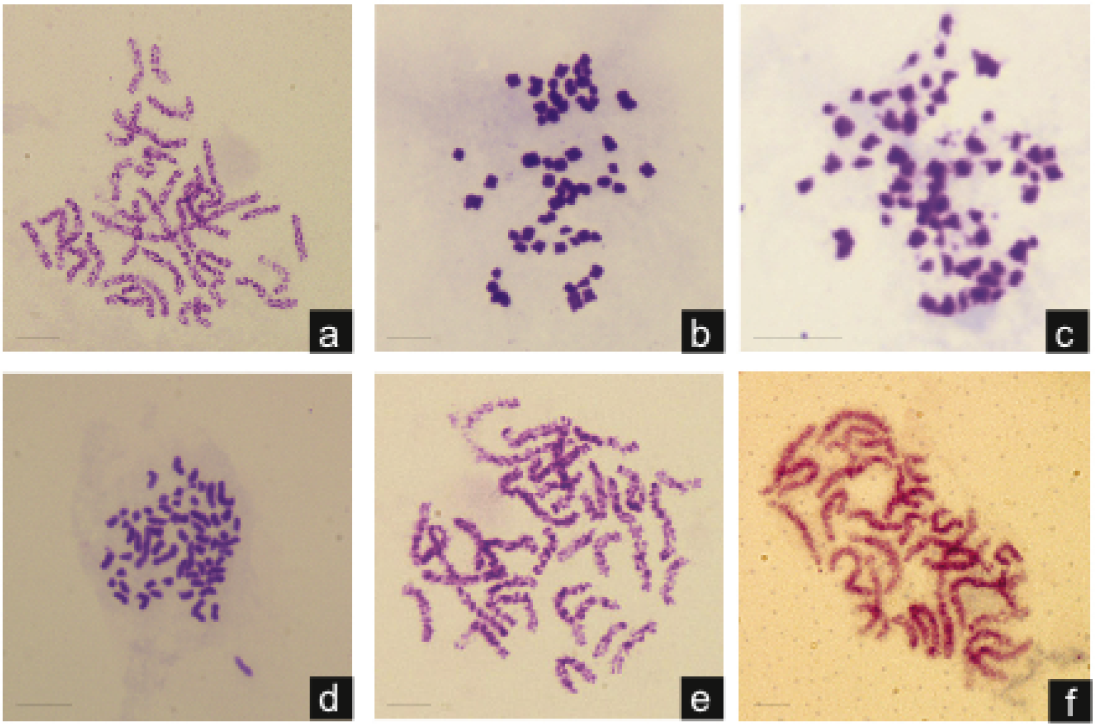

Figure 3.1. Giemsa stained metaphases of *Geomalacus* and *Letourneuxia*: a) *G. oliveirae* meiotic metaphase II (n = 31); b) *G. oliveirae* mitotic metaphase (2n = 62); c) *G. maculosus* mitotic metaphase (2n = 62); d) *G. anguiformis* mitotic metaphase (2n = 62); e) *G. malagensis* meiotic metaphase II (n = 31); f) *L. numidica* meiotic metaphase II (n = 31). Scale bar represents 4 μm.

Concerning the number of chromosomes, the five species presented the same diploid chromosome number of 2n = 62 (n = 31). However, karyotypic formulae are different and unique to each *Geomalacus* species, with karyotypes mainly consisting of metacentric (m) and submetacentric (sm) chromosomes; with few subtelocentric (st) and lacking telocentric chromosomes (*G. oliveirae* 15m + 13sm + 3st; *G. maculosus* 14m + 12sm + 5st, *G. anguiformis* 14m + 10sm + 7st, *G. malagensis* 10m + 12sm + 9st) (Figures 3.2,and 3.3 and Table 3.1). *Geomalacus malagensis* and *L. numidica* share the same karyotypic formula, yet, in *L. numidica* 6 of the 12 submetacentric chromosome pairs presented a submetacentric/subtelocentric tendency, while in *G. malagensis* only four chromosome pairs show this trend (chromosomes marked with an * in Figure 3.3).

Discussion

The use of somatic organs for cytogenetic studies instead of ovotestis proved to be effective. Mouth and both optical and sensory tentacles yielded several mitotic metaphases and hence were successfully used to determine diploid chromosome numbers. In previous cytogenetic studies of terrestrial slugs (Beeson, 1960; Burch, 1965; Colomba *et al.*, 2009; Patterson, 1969), only well-developed ovotestis were used for karyotyping. The new approach presented here using somatic tissues allows karyological studies to be performed regardless of the sexual developmental stage of the specimens.

The five species studied displayed an invariant chromosome number (n = 31) so that the observed interspecific karyotypic differences only involved structural chromosomal rearrangements without affecting chromosome number. Such patterns of chromosomal change have been previously observed in the neogastropod family Muricidae (Leitão *et al.*, 2009). Unlike the genus *Arion*, in which chromosome numbers were useful to distinguish between subgenera (Beeson, 1960), it is clear that it is not possible to distinguish between these five species based on the chromosome number alone. The haploid chromosome number in *Geomalacus* and *Letourneuxia* is the highest observed within the Arionidae (with n = 25-29) (Beeson, 1960). Also, this chromosome number is among the highest of all terrestrial pulmonate gastropod mollusks (order Stylommatophora). Haploid chromosome numbers within this group vary between n = 18 and n = 34 (Colomba *et al.*, 2009; Park & Kim, 1997; Thiriot-Quiévreux, 2003), with Athoracophoridae being an outlier with n = 44 (Burch & Patterson, 1971; Patterson, 1969).

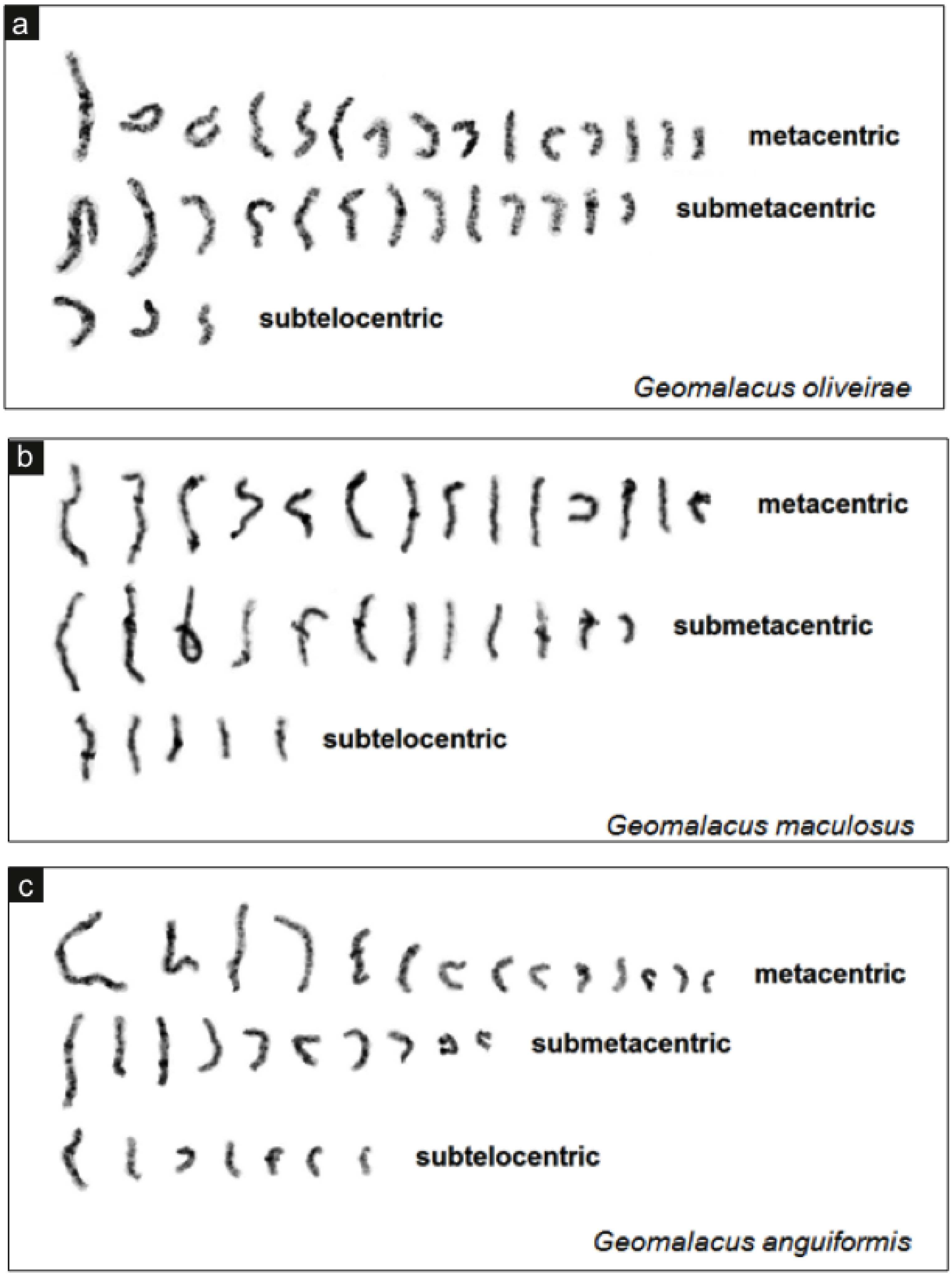

Figure 3.2. Giemsa stained karyotypes of *Geomalacus*. a) *G. oliveirae* (15m + 13sm + 3st), b) *G. maculosus* (14m + 12sm + 5st), c) *G. anguiformis* (14m + 10sm + 7st) (m - metacentric chromosomes, sm - submetacentric chromosomes and st – subtelocentric chromosomes).

Ancestral character state reconstruction tracing chromosome numbers in *Geomalacus* and *Letourneuxia* on a molecular phylogenetic tree, would possibly allow the inference of a chromosome number evolutionary trend for the family Arionidae. However, currently too few karyotypic data are available to conduct such analysis.

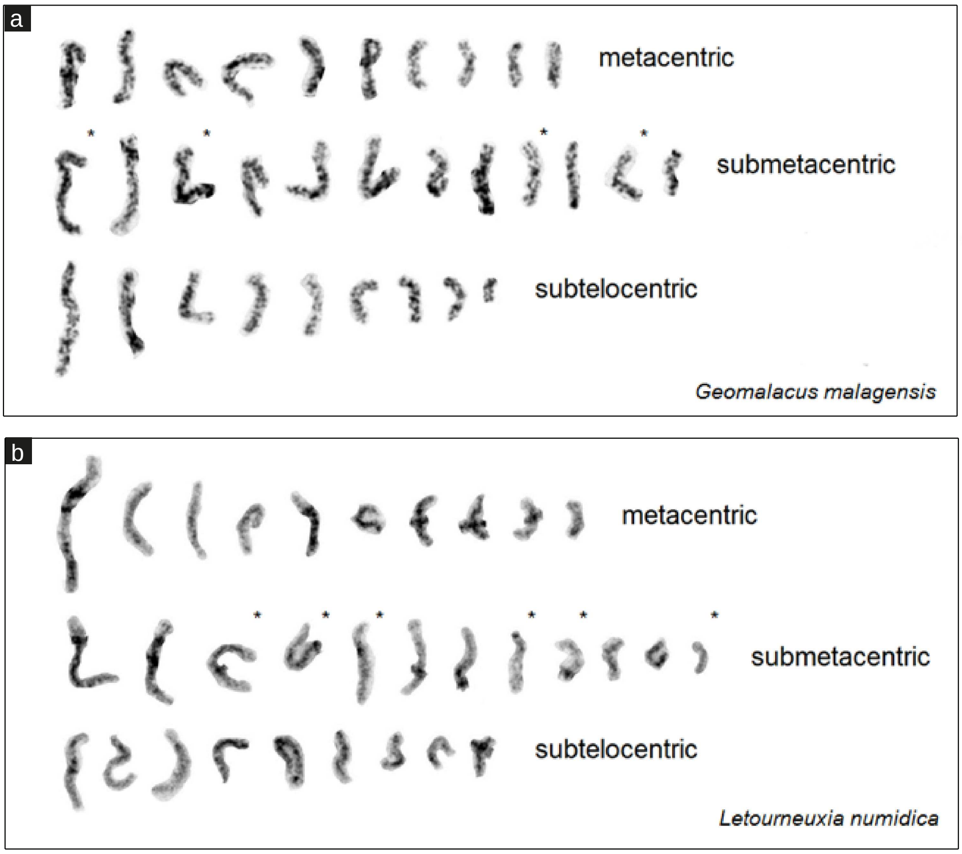

Figure 3.3. Giemsa stained karyotypes of *Geomalacus* and *Letourneuxia*. a) *G. malagensis* (10m + 12sm + 9st); b) *L. numidica* (10m + 12sm + 9st). Asterisks (*) indicate submetacentric chromosomes that present a submetacentric /subtelocentric tendency.

The karyotypes of the species in this study showed a prevalence of metacentric and submetacentric chromosomes, which follows the general trend in gastropod karyotypes (Thiriot-Quiévreux, 2003). Despite presenting the same chromosome number, each *Geomalacus* species displays different and diagnostic karyotypes (Figure 3.1 a-e and Table 3.1). *Geomalacus oliveirae* has the most symmetric karyotype of the studied species, with the highest number of metacentric and submetacentric chromosomes, while *G. malagensis* and *L. numidica* present the more asymmetrical ones. Symmetrical karyotypes are often considered plesiomorphic, since a higher proportion of metacentric pairs may point to relative chromosomal evolutionary stability (White, 1978).

It is taxonomically relevant that *G. malagensis* is karyotypically different from its congeners and shares the same karyotypic formula and similar chromosome morphology with *L. numidica.* The difference between *G. malagensis* and *L. numidica* resides solely in the numbers of chromosome pairs showing a submetacentric/subtelocentric tendency, i.e respectively, 4 and 6. Even if the present chromosomal data alone is not conclusive to establish the taxonomic status of *Geomalacus* and *Letourneuxia,* this finding suggests that both genera may be closely related and perhaps should be merged in a single genus-level taxon. Similarly, Backeljau & De Bruyn (1990) used chromosome numbers, together with morphology and allozyme data, to merge the arionide subgenera *Microarion* and *Kobeltia*. Yet, whether such conclusion is also warranted for *Geomalacus* and *Letourneuxia* requires further corroboration.

In conclusion, our work showed that somatic tissues are perfectly suitable for cytogenetic studies and that the chromosome number of the genera *Geomalacus* and *Letourneuxia* is n = 31, which is among the highest of all Stylommatophora. The five described karyotypes constitute the first record for Arionidae, with *G. malagensis* and *L. numidica* presenting similar chromosome morphologies and karyotypic formulae. Cytogenetic studies may significantly contribute to clarify the taxonomy of these, and other, pulmonate gastropods.

Acknowledgements

The authors wish to thank K. Erzini, A. Patrão, T. Pereira, D. Reis and G. Silva for collecting specimens in the field and T. Rodriguez for assistance with the identification of the specimens. The authors are also grateful to S. Natale, for revising the English. This work was partially funded by “Fundação para a Ciência e Tecnologia” (Portugal): doctoral grant SFRH/BD/30024/2006 (CP), project “Pillars of Hercules – Gene flow across the Pillars of Hercules: the role of North Africa in the postglacial recolonization of Europe” PTDC/BIA-BDE/74349/2006 (RC) and “Programa Ciência 2007” (AL). T. Backeljau was supported by BELSPO project MO/36/017 and FWO research network W0.009.11N.

Chapter IV

Cryptic diversity of *Geomalacus* and *Letourneuxia* in the Iberian-Moroccan region

Cryptic diversity of *Geomalacus* and *Letourneuxia* in the Iberian-Moroccan region

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Introduction

Cryptic species represent a significant component of total biodiversity that is often underestimated when detection relies exclusively on diagnostic morphological characters (Carter, 2012). The use of molecular data in species delimitation removed most of the challenges underlying the quantification of such hidden diversity (Welton *et al.*, 2013). While the recognition of cryptic speciation represented by reciprocally monophyletic lineages is elementary (De Queiroz, 2007), its perception in recently diverged lineages, or in lineages undergoing differentiation, poses greater difficulties. Lack of congruence between gene trees and the species tree, due to incomplete lineage sorting (Knowles & Carstens, 2007) or paraphyletic species, are some of the problems that frequently occur in species delimitation. Species with widespread distributions, conserved morphologies and long demographic histories are more expected to exhibit cryptic diversity (Omland *et al.*, 2000). Further, after genetic surveys, many cryptic lineages in morphologically undistinguishable species emerge as evolutionary distinct units (Webb, Marzluff & Omland, 2011). However, restricted dispersal abilities and habitat fragmentation are also critical factors in generating cryptic diversity (Jörger *et al.*, 2012).

Southern Europe has a particular geography with three relevant peninsular areas: the Balkans, Iberia and Italy. These peninsulas have played a major role as Pleistocene refugia because they have preserved a remarkable amount of diversity lost in higher latitudes due to the setting of extensive glaciated areas during the Last Glacial Maxima (LGM) (for a review see (Weiss & Ferrand, 2007; Feliner, 2011)). The conjunction of particular physiography and micro-climates (desert, alpine, mediterranean and atlantic) was responsible for the existence multiple refugia (the so-called "refugia within refugia" (Gómez & Lunt, 2007)) associated to mountain ranges instead of a large single refugial area during the LGM. Several cryptic species were described for Iberia (e.g. rotifer in Gómez *et al.*, 2002; wall lizards in Pinho, Harris & Ferrand, 2007; Kaliontzopoulou, Carretero & Llorente, 2012; and lamprey Mateus *et al.*, 2013) and some of this cryptic diversity can be found associated within these refugial areas (e.g. land snails in Elejalde *et al.*, 2008; fire salamander in Reis *et al.*, 2011; for a review Gómez & Lunt, 2007).

There is little information on the genetic diversity and population structure of Iberian terrestrial gastropods, particularly in arionid slug species. The Iberian Peninsula holds the highest diversity of arionids (genus *Arion)* in Europe (Castillejo, 1998) and the existence of cryptic speciation within the genus *Arion* was recently identified (PCR-RFLP, allozymes and 16SrRNA (Jordaens *et al.*, 2006), and morphological data (Pinceel *et al.*, 2004; Jordaens *et al.*, 2010)). In the present study we focused on the endemic slugs of the genus *Geomalacus,* which geographic distribution is mostly restricted to the Iberian Peninsula, with three endemic Iberian species (*Geomalacus oliveirae*, *G. anguiformis*, *G. malagensis*) and one species *G. maculosus* occurs both in the northern Iberia and Ireland (see map from Figure 4.1 for further details on the geographic distribution of each species) and on the genus *Letourneuxia* from the Moroccan region that is presumed to be its sister group. The taxonomic classification of arionid species is based on extremely labile characters such as body size and color that depend both on diet and environment, as well as age (Jordaens *et al.*, 1999; Jordaens *et al.*, 2001). Other characters used for taxonomic purposes include the morphology of reproductive apparatus, which varies according to developmental stage and sexual maturation, often preventing the correct identification of juveniles at the species level (Backeljau & De Bruyn, 1990 and references therein; Backeljau *et al.*, 1996).

Arion and *Geomalacus* found in sympatry in some locations, suggesting that they share biological life traits and ecological requirements, but show a striking difference in the number of species. While the Iberian number of *Arion* species ranges between 30 and 50 species (Castillejo, 1998), only four species are currently recognized within *Geomalacus* and one in *Letourneuxia*. The disparity between the number of species between genera might result from the following alternative hypotheses: the genus *Arion* is considerably older than *Geomalacus* and had more time to diversify, or genera have similar ages and *Geomalacus* may exhibit cryptic diversity. Here, we focused on the Iberian land slugs *Geomalacus* because they show some intrinsic features (low dispersal abilities, lack of reliable diagnostic morphological characters and a reduced number of species compared to other Iberian slugs) that suggest the existence of cryptic speciation within the genus.

We present the molecular phylogeny of *Geomalacus* across its Iberian distribution using the mitochondrial cytochrome oxidase subunit I (COI), and the nuclear ribosomal small subunit (18S rRNA) genes. The specific aims of the present study are to: (1) analyze phylogenetic patterns within *Geomalacus*; (2) estimate the age of *Arion* and *Geomalacus* and date major lineage splitting events within Arionidae, and (3) analyze phylogeographic patterns within *Geomalacus* and propose a biogeographic scenario in the Iberian Peninsula for this group of land slugs.

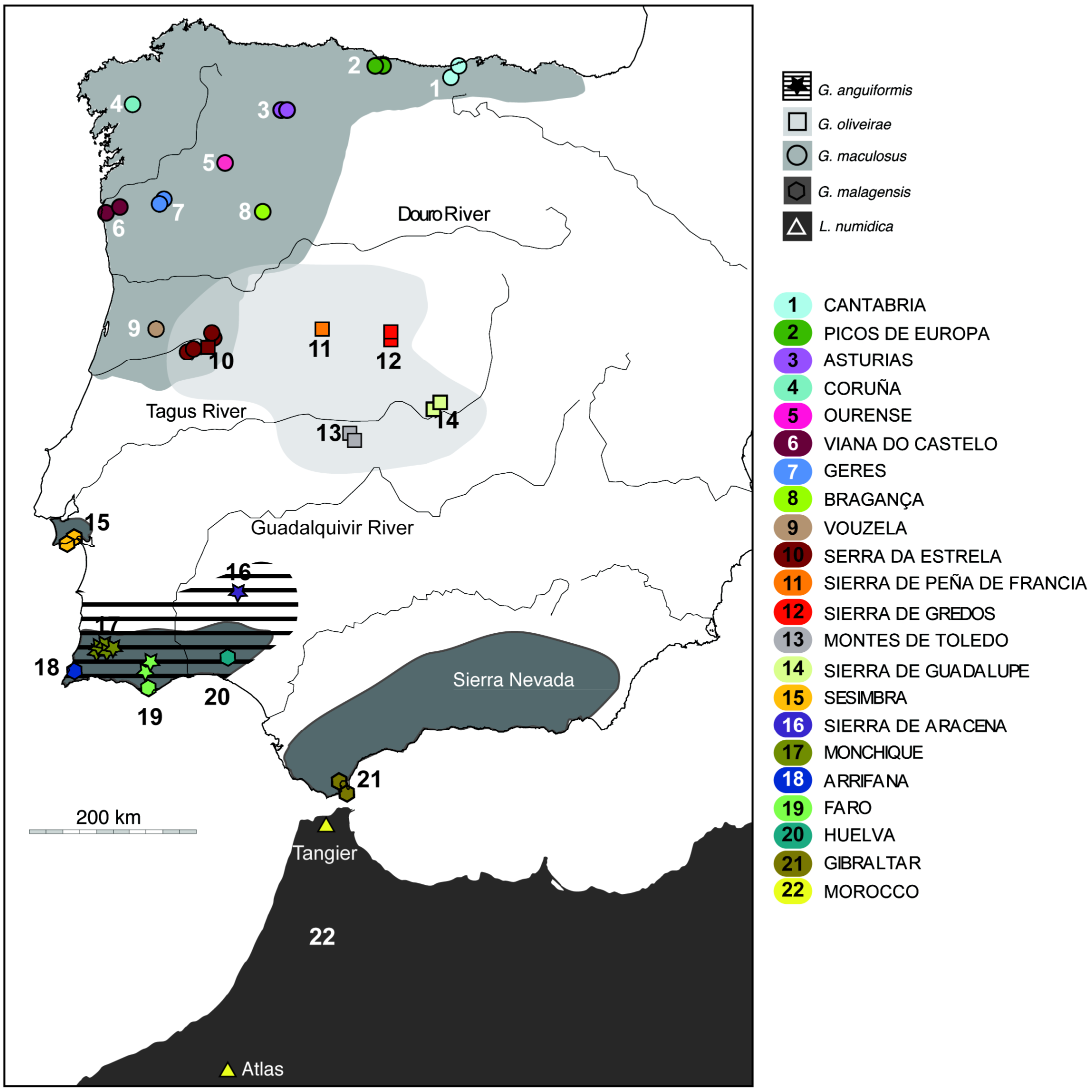


Figure 4.1. Map showing the current approximate distribution of *Geomalacus* and *Letourneuxia* (based on Castillejo (1998)) and on Figures 2.2 and 2.3) and sampled localities (details of sampling locations are as in Annexe 4.1).

Material and Methods

Sampling

Sampling was done by field surveys conducted across the species geographic ranges between 2007–2010 (Figure 4.1 and Annexe 4.1) in 49 different sites, grouped into 22 main regions. These included all the four nominal *Geomalacus* species as well as *L. numidica* from North Africa. Slugs were morphologically identified with the key of Castillejo, Garrido & Iglesias (1994). Foot tissue was extracted and stored in 96% ethanol at −20ºC.

DNA extraction, amplification and sequencing

Total genomic DNA for 497 individuals (Table 4.1) was extracted with a chelex buffer, using a modified protocol of Walsh et al. (1991): foot tissue was added to 250 µL of 5% (w/v) solution of chelex with 5µL of Proteinase K, and incubated with thorough mixing at 55°C for 60 min, followed by a 20 min incubation at 95ºC. The samples were then placed on ice for 2 min and centrifuged at 11000 rpm for 3 min. The supernatant containing genomic DNA was removed and used directly as a template in downstream polymerase chain Reaction (PCR) analysis. Amplifications of a 750 bp portion of mtDNA COI, were obtained by PCR using primers LCO1490 5’ - GGTCAACAAATCATAAAGATATTGG - 3’ and HCO2198 5’ - TAAACTTCAGGGTGACCAAAAAATCA - 3’ (Folmer *et al.*, 1994). For selected individuals representing the major evolutionary lineages inferred in the previous analysis, a 700 bp fragment of the small subunit nuclear ribosomal gene (18S) was additionally amplified and sequenced with primers 4F18S 5’ - CTGGTTGATYCTGCCAGT - 3’ and 1R18S 5’ - ATTACCGCGGCTGCTGGC - 3’ (Winnepenninckx, Backeljau & Dewachter, 1994). PCR profiles were 1 cycle of 5min at 95°C, 40 cycles of 40 sec at 95ºC, 40 sec at 40ºC and 1min at 72ºC and a last elongation step of 5min at 72ºC. PCR amplifications were performed in a total 25µl reaction volume of 1X buffer, 2mM $MgCl_2$, 0.2mM DNTP’s, 0.2µM of each primer and 1U Taq DNA polymerase Promega (Madison, USA). Negative controls were included in each set of reactions. Amplifications were checked by electrophoresis in a

1% agarose gel and purified by ethanol precipitation. Sequencing was performed on an ABI 3130xl (Applied Biosystems) automated sequencer using the described primers.

Phylogenetic analyses and lineage identification

The Akaike Information Criterion (AIC) (Akaike, 1973) implemented in Modeltest v.3.7 (Posada & Crandall, 1998) was used to determine the evolutionary models that best fitted the data sets and used in maximum likelihood (ML) and Bayesian Inference (BI) analyses.

BI analyses based on combined data set (82 taxa, 1119 bp) were conducted with MrBayes v3.2.1 (Ronquist *et al.*, 2012). Model parameters were estimated independently for each of the data partitions in MrBayes using the unlink command. Analyses accommodated among-partition rate variation through use of the “prset applyto=(all) ratepr=variable;” command. Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for two million generations, and sampled every 100 generations. The burn-in was set to 1000,000 generations and robustness of the inferred trees was evaluated using Bayesian posterior probabilities (BPPs). Two independent runs were performed for each data set.

ML analyses were performed with RAxML ver. 7.0.0 (Stamatakis, 2006). Initially, the best-scoring ML tree was determined from 100 randomized maximum-parsimony starting trees using the rapid hill-climbing algorithm (Stamatakis *et al.*, 2007) as implemented in RaxML. ML model parameters were estimated separately for each partition (COI: GTR+I+Γ; ITS1: H80+Γ). Individual per-partition branch length optimization and 1,000 thousand bootstrap replicates were drawn on the best-scored ML tree using the exhaustive bootstrap algorithm. The resulting tree with the highest bootstrap support values was visualized using FigTree ver. 1.4 (Rambaut, 2009).

We used a Bayesian relaxed molecular-clock approach and the new Species Tree Ancestral reconstruction (*Beast) as implemented in Beast v1.7.4 (Drummond *et al.*, 2012) to date lineage-splitting events within Arionidae using the mitochondrial COI data set (146 taxa; 594 bp) and the GTR+I+Γ evolutionary model. The *Beast method estimates the species tree and gene trees, allows the use of multiple individuals per species without causing overestimation of the ages (Heled & Drummond, 2010). We used a Yule tree prior that assumes a constant speciation rate among lineages. This method allows the incorporation of fossil uncertainties because it uses probabilistic calibration priors instead of point calibrations (Drummond *et al.*, 2006).

Estimates of divergence are generally based upon known historical events, geologic or fossil, which can be used as calibration points for a taxon-specific mutation rate estimate (McCormack *et al.*, 2011). For terrestrial slugs there are no fossils that can with certainty be attributed to species of interest, so we used an historical biogeographic event, the opening of the Gibraltar strait 5.45 ± 0.15 million years (MYA) ago (Duggen *et al.*, 2003), to calibrate the divergence between *G. malagensis* and *L. numidica*, by placing a normal distribution in the divergence of these two taxa (mean=5.645 stdev=0.045).

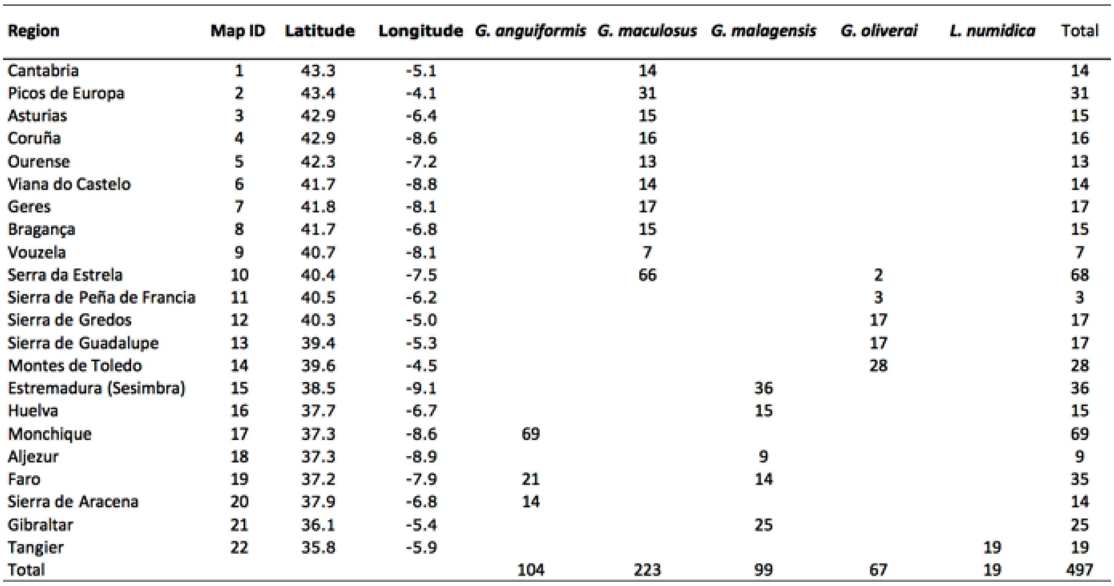
MCMC analyses of data sets (a) and (b) were run for 300,000,000 generations with a sample frequency of 10,000, following a discarded burn-in of 3,000,000 steps. The convergence to the stationary distributions was confirmed by inspection of the MCMC samples using Tracer v1.5 (Rambaut & Drummond, 2007). All dating analyses were performed on the CCmar Computational Cluster Facility (http://gyra.ualg.pt) at the University of Algarve.

We applied the Indicator Vectors Method to assign sequences to groups of organisms (Sirovich, Stoeckle & Zhang, 2009; Sirovich, Stoeckle & Zhang, 2010). The indicator vector representation is not as dependent on the variation of evolutionary rates in the different species as the GMYC and ABGD methods (Puillandre *et al.*, 2012). We generated a Klee diagram based on indicator vector correlations for analysing and displaying affinities of COI haplotypes (Sirovich *et al.*, 2009; Sirovich *et al.*, 2010). Using this method, nucleotide symbol sequences were transformed into numerical vectors, called indicator vectors. The vectors for each taxa is obtained under the condition that it is maximally correlated with the taxa and simultaneously that it is minimally correlated with all other taxa. The matrix of intertaxa correlations is depicted as a Klee diagram, given its similarity with the artwork of Paul Klee. Two advantages the method has are that although the diagrams are intrinsic to the data they are also independent of evolutionary models and the diagrams are intuitively interpreted. Moreover, unlike phylogenetic trees, which lose distance accuracy with size, the Klee diagram faithfully retains its accuracy at all scales. In the diagram, areas of congruence are shown in gradations of red (>0.8) and yellow (0.7< >0.8).

Results

A total of 497 slugs were sampled from 49 sites across the Iberian distribution of *Geomalacus* spp. while *Letourneuxia numidica* was sampled in two sites in Morocco. Sites were grouped into 22 regions shown in Figure 4.1, Table 4.1 and details are in Annexe 4.1.

Table 4.1. Table of sampling sites and presumed taxon. Geographical coordinates are an approximation, true values for all the 49 different sites can be found in Annexe 4.1.



Region	Map ID	Latitude	Longitude	*G. anguiformis*	*G. maculosus*	*G. malagensis*	*G. oliverai*	*L. numidica*	Total
Cantabria	1	43.3	-5.1		14				14
Picos de Europa	2	43.4	-4.1		31				31
Asturias	3	42.9	-6.4		15				15
Coruña	4	42.9	-8.6		16				16
Ourense	5	42.3	-7.2		13				13
Viana do Castelo	6	41.7	-8.8		14				14
Geres	7	41.8	-8.1		17				17
Bragança	8	41.7	-6.8		15				15
Vouzela	9	40.7	-8.1		7				7
Serra da Estrela	10	40.4	-7.5		66		2		68
Sierra de Peña de Francia	11	40.5	-6.2				3		3
Sierra de Gredos	12	40.3	-5.0				17		17
Sierra de Guadalupe	13	39.4	-5.3				17		17
Montes de Toledo	14	39.6	-4.5				28		28
Estremadura (Sesimbra)	15	38.5	-9.1			36			36
Huelva	16	37.7	-6.7			15			15
Monchique	17	37.3	-8.6	69					69
Aljezur	18	37.3	-8.9			9			9
Faro	19	37.2	-7.9	21		14			35
Sierra de Aracena	20	37.9	-6.8	14					14
Gibraltar	21	36.1	-5.4			25			25
Tangier	22	35.8	-5.9					19	19
Total				104	223	99	67	19	497

All COI sequences represented uninterrupted open reading frames, with no gaps or premature stop codons, suggesting they are functional mitochondrial DNA copies, yielding 109 unique haplotypes. Of a total of 594 sites sequenced, 226 were variable, from which 215 were parsimony informative. Considering the five nominal species, the less diverse taxa is *G. malagensis* with intra-specific p-distance of 0.009 (SD ± 0.002) and the most heterogeneous group is *G. oliveirae*, with 0.119 (SD ± 0.009) (Table 3.2). When considering the five *G. oliveirae* lineages, the inter-specific p-distances varied between 0.114 (SD ± 0.012) (*G. oliveirae* Sierra de Gredos / *G. oliveirae* Sierra Peña de Francia) and 0.206 (SD ± 0.014) (*G. maculosus / G. oliveirae*

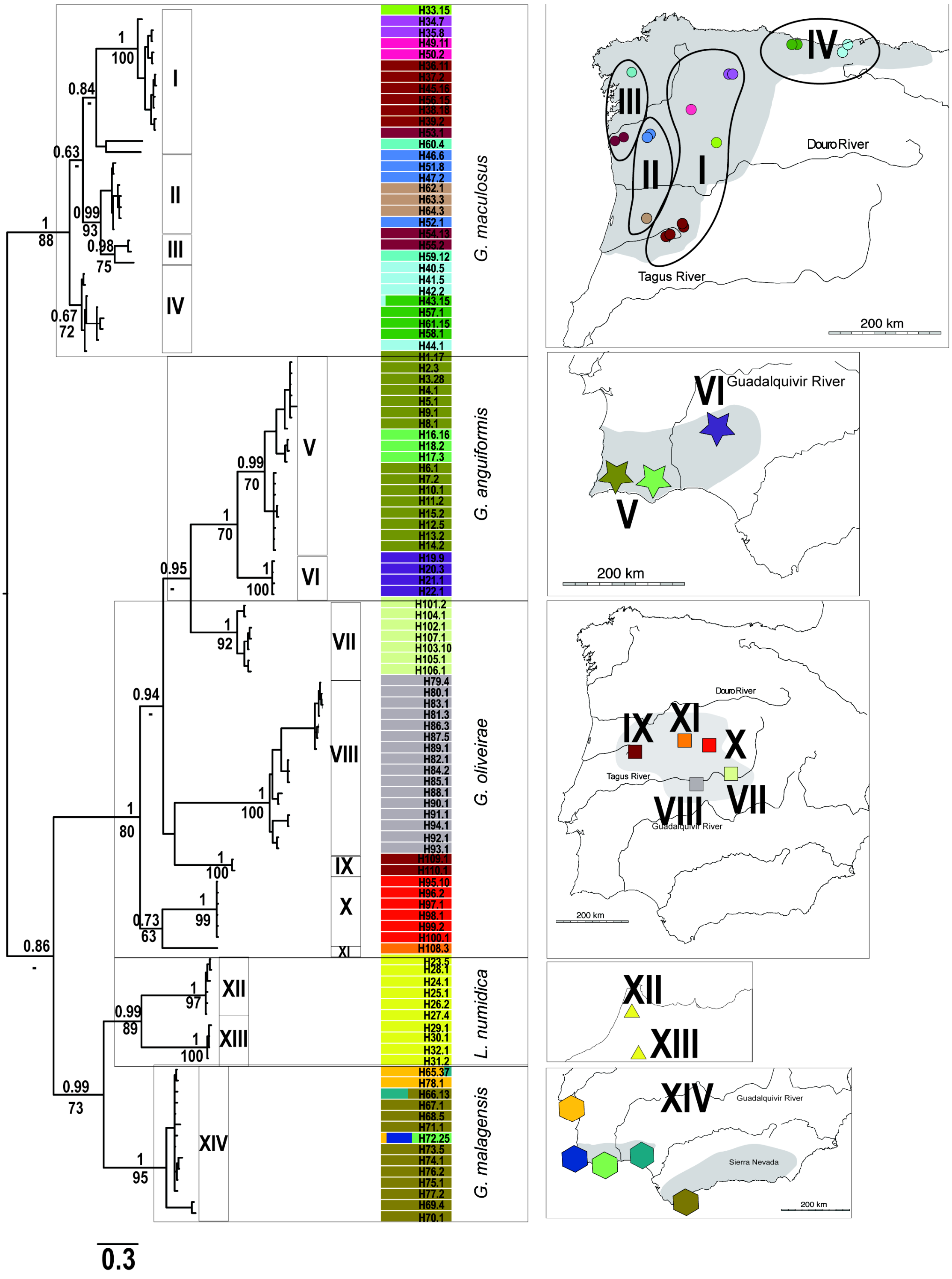
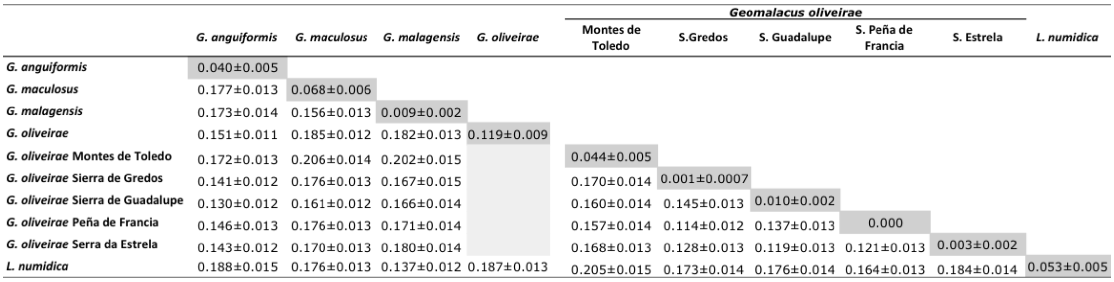


Figure 4.2. Maximum-likelihood topology inferred from a concatenated set of COI for *Geomalacus* spp. and *L. malagensis* from 22 sampling sites throughout the distributional range of these taxa. Tip labels are sampling haplotype codes and number of sequences in each haplotype. The maximum-likelihood phylogeny used unique haplotypes only (n = 109) and is shown with outgroups removed (*Geomalacus* spp. and *L. numidica* formed a monophyletic group). Maximum-likelihood bootstrap values larger than 70% and Bayesian posterior probabilities (BPP) greater than 0.90

for major supported clades are shown above and below branches, respectively. Proportional geographic origin of shared haplotypes is indicated in colour at the branch tips. Haplotype codes are listed in Annexe 4.1

Montes de Toledo) (Table 4.2). Nuclear 18S sequences were 527 bp long, with 3 variable sites all parsimony informative, and the combination of mutations is diagnostic for *G. oliveirae* (C/C/C), *G. anguiformis* (C/T/T), *G. malagensis* and *L. numidica* (T/T/C) and *G. maculosus* (T/C/C).

Table 4.2. Pairwise uncorrected COI sequence divergence among species and *G. oliveirae* lineages (mean ± s.e).



					Geomalacus oliveirae					
	G. anguiformis	***G. maculosus***	***G. malagensis***	***G. oliveirae***	**Montes de Toledo**	**S.Gredos**	**S. Guadalupe**	**S. Peña de Francia**	**S. Estrela**	***L. numidica***
G. anguiformis	0.040±0.005									
G. maculosus	0.177±0.013	0.068±0.006								
G. malagensis	0.173±0.014	0.156±0.013	0.009±0.002							
G. oliveirae	0.151±0.011	0.185±0.012	0.182±0.013	0.119±0.009						
***G. oliveirae* Montes de Toledo**	0.172±0.013	0.206±0.014	0.202±0.015		0.044±0.005					
***G. oliveirae* Sierra de Gredos**	0.141±0.012	0.176±0.013	0.167±0.015		0.170±0.014	0.001±0.0007				
***G. oliveirae* Sierra de Guadalupe**	0.130±0.012	0.161±0.012	0.166±0.014		0.160±0.014	0.145±0.013	0.010±0.002			
***G. oliveirae* Peña de Francia**	0.146±0.013	0.176±0.013	0.171±0.014		0.157±0.014	0.114±0.012	0.137±0.013	0.000		
***G. oliveirae* Serra da Estrela**	0.143±0.012	0.170±0.013	0.180±0.014		0.168±0.013	0.128±0.013	0.119±0.013	0.121±0.013	0.003±0.002	
L. numidica	0.188±0.015	0.176±0.013	0.137±0.012	0.187±0.013	0.205±0.015	0.173±0.014	0.176±0.014	0.164±0.013	0.184±0.014	0.053±0.005

Phylogenetic analyses and lineage identification

Maximum likelihood analyses based on the COI data set yielded the topology depicted in Figure 4.2. All nominal species were reciprocally monophyletic (Figure 4.2) with the exception of *G. oliveirae* that was retrieved as paraphyletic. ML and BI analyses recovered four well-supported clades but phylogenetic relationships within the genus were mostly unresolved (Figure 4.2). *L. numidica* and *G. malagensis* were recovered as sister species.

The dating analysis obtained with Beast (Figure 4.3) estimated the origin of the most recent common ancestor (MRCA) of *Geomalacus* during the Miocene at 11.8 [95% HPD: 7.2-22.5] MYA. The splitting of *G. oliveirae* (lineage VII) and *G. anguiformis* was estimated at 4.8 [95% HPD: 2.2-9.5] MYA. The MRCA of *G. maculosus* originated at 3.6 [95% HPD: 1.8-6.9] MYA. The two main lineages of *G. anguiformis* diverged at 2.6 [95% HPD: 1.1-5.2] MYA. *G. malagensis* and *L. numidica* diverged at 6.5 [5.6-12] MYA.

The Klee diagram (Figure 4.4) revealed 14 MOTU pairs with correlation values > 0.75. Results yielded *L. numidica* and *G. malagensis* as independent ESUs, *G. anguiformis* containing two independent ESUs and *G. oliveirae* and *G. maculosus* with five independent ESUs each (Figure 4.4). The indicator vector analysis results provide support for the phylogenetic results.

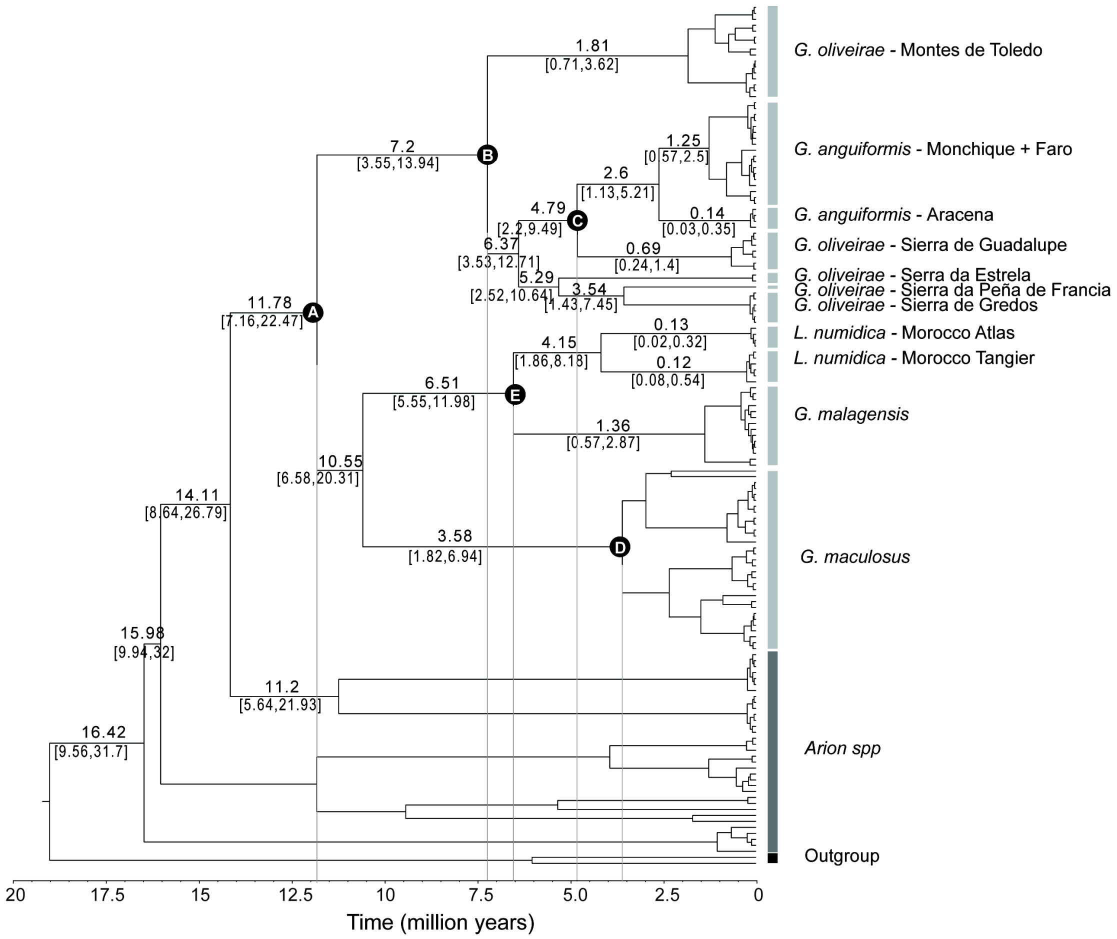


Figure 4.3. Bayesian tree showing the phylogenetic relationships among the studied samples of the *Geomalacus* and *Letourneuxia*. Numbers above branches are node posterior probabilities. The ages of the nodes discussed in the text are indicated by a black circle on the tree.

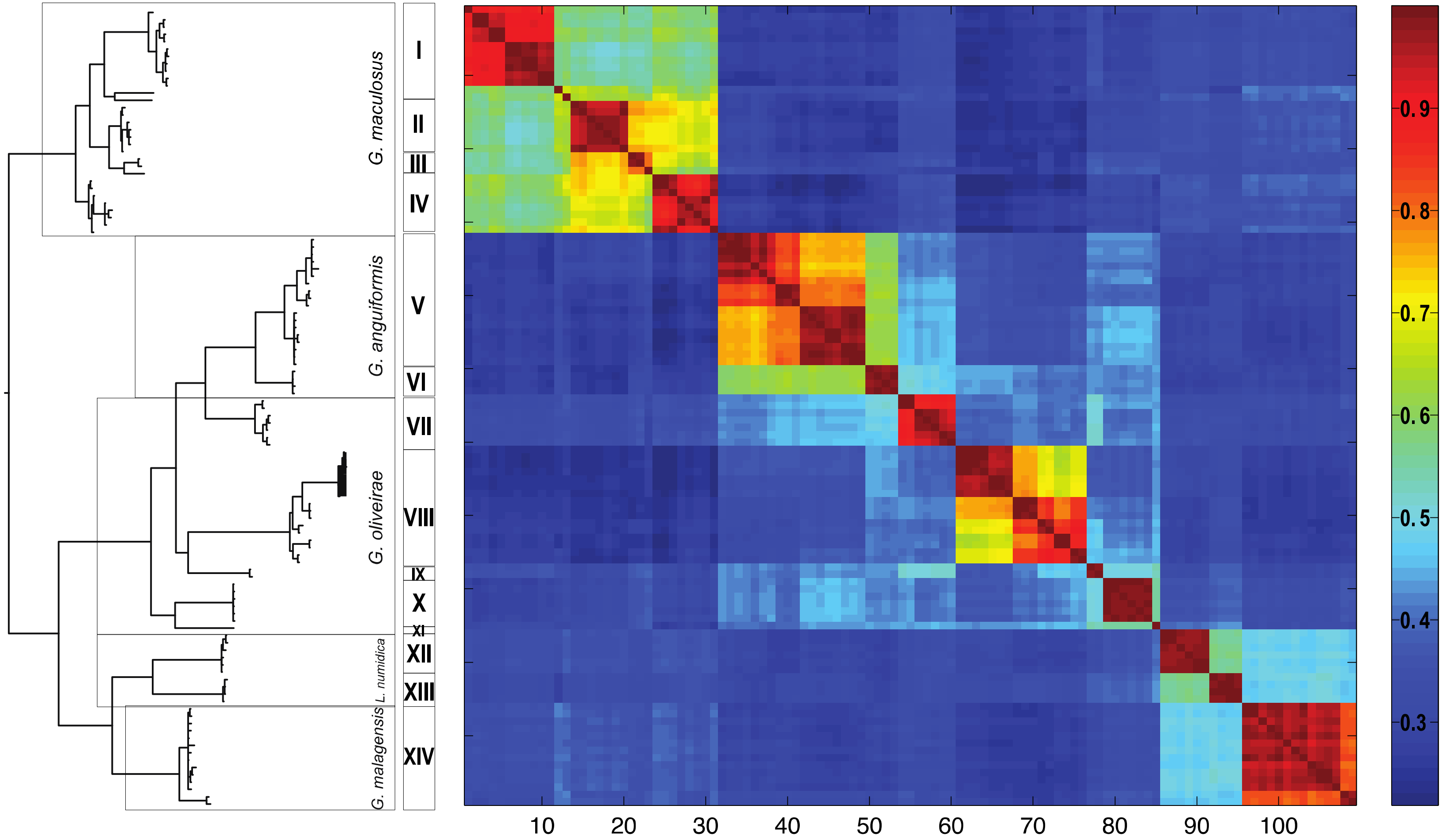


Figure 4.4. Indicator vector analysis of 109 haplotypes of the COI gene fragment of *Geomalacus* and *Letourneuxia* species using the methods of Sirovich et al. (2009, 2010). On the left the maximum likelihood tree represented in Figure 4.2 for comparison.

Discussion

In the present study we assessed the differentiation of the *Geomalacus* and *Letourneuxia* genera to explore genetic diversity of arionid slug populations in the Iberia-Moroccan region. We sampled 497 specimens representing almost the entire range distribution of the four currently recognized species within *Geomalacus* (*Geomalacus maculosus*, *G. malagensis*, *G. oliveirae* and *G. anguiformis*) and two Moroccan locations for *Letourneuxia numidica*, the presumed the sister group of *Geomalacus* (Backeljau, T. personal communication). Considering the reduced number of reliable diagnostic morphological characters to distinguish between species, we used sequence-based methods to investigate the putative existence of cryptic diversity. Dating estimates allowed us to determine major lineage splitting events within three arionid genera (*Arion*, *Geomalacus* and *Letourneuxia*) and propose a biogeographic scenario for the Iberian Peninsula.

Cryptic diversity and phylogeographic patterns within Geomalacus and Letourneuxia land slugs

Genetic analyses often reveal the existence of cryptic lineages in morphologically uniform species (Webb *et al.*, 2011). Cryptic diversification may result from intrinsic morphological features of the species (e.g. low vagility or the developing of ecological specializations) and habitat fragmentation and/or historical climatic events (Pearson & Dawson, 2005). There is a clear correspondence between the retrieved molecular clades and their geographical location (with the exception of *G. malagensis,* Figure 4.2). The low active dispersal ability of slugs and their high sensitivity to biotic parameters such as suitable vegetation, humidity and temperature (Quinteiro *et al.*, 2005) may have promoted the isolation of some lineages that evolved into cryptic intraspecific diversity.

Phylogenetic (Figure 4.2) and indicator vector (Figure 4.4) analyses identified 14 highly divergent lineages within *Geomalacus*: five cryptic lineages within *G. oliveirae*, five within *G. maculosus*, two within *G. anguiformis* and a single *G. malagensis*. Also, the analyses recovered two lineages within *Letourneuxia.* Our results suggest the existence of five cryptic lineages within *G. oliveirae* with narrow distributional ranges, each of one associated to a mountain complex (Serra da Estrela, Sierra de Gredos, Montes de Toledo, Sierra de Guadalupe and Sierra de la Peña de

Francia) (Figure 4.1). There are some biological features that might explain this pattern, as habitat suitability modeling for *G. oliveirae* suggests marked discontinuity between patches of suitable habitat in the central region of the Iberian Peninsula where this species occurs (Patrão et al., submitted). Similarly, the recovered two *G. anguiformis* lineages show a geographic distribution that corresponds to Portuguese (Monchique and Faro) and Spanish (Aracena) mountain areas. The reconstructed phylogenetic patterns also indicate the existence of four cryptic lineages within *G. maculosus* (Figure 4.2). While in the remaining *Geomalacus* lineages are often associated to mountain complexes, clades within *G. maculosus* suggest an east-to-west genetic differentiation (Clade I: Bragança +Ourense +Asturias; Clade II: Gerês +Vouzela; Clade III: Viana Castelo +Compostela, see Figure 4.2 for details). This longitudinal grouping of specimens may have been driven by lineage-specific responses to climatic changes, probably related to precipitation levels but further analyses including other abiotic factors are needed to test this hypothesis. *Geomalacus malagensis* presents no geographical structure regardless the existence of non-suitable habitat areas along its distributional range (Patrão et al., submitted), which should promote isolation, and ultimately, genetic differentiation. This is the only species within *Geomalacus* that shows shared haplotypes between locations that are apart by more than 200 km (Figure 4.1). The irregular distribution of this species may result from human-mediated transport through the removal of garden debris where they are frequently found (pers. obs.), associated to the particularly high activity of *G. malagensis* (Castillejo & Iglesias-Pineiro, 2008).

The existence of cryptic lineages within *Geomalacus*, restricted to particular mountain systems (Figure 4.2), suggests the occurrence of differentiation under allopatric conditions. The levels of genetic divergence found between lineages (Table 4.2) indicate extended periods of isolation, particularly within *G. oliveirae.* Our reconstructed phylogeographic patterns are consistent with the “refugia-within-refugia” theory that proposes the existence of local refugia within main refugial areas, determined by distinct responses of intraspecific lineages to climate change, which restricts their ranges (Gomez & Dantart, 1996).

Evidence presented here contradict the previous idea that *L. numidica* is the sister group to all *Geomalacus*. Our results (Figure 4.2) showed a sister relationship between *L. numidica* and *G. malagensis.* Moreover, the two highly divergent lineages within *L. numidica* are associated to distant mountain systems, the North of the Atlas Mountains (Tangier) and the Atlas Mountains. A similar divergence was observed in the stripe-necked terrapin (*Mauremys leprosa*) where the Atlas Chain represents a significant biogeographic barrier to dispersal in the Moroccan region (Fritz *et al.*, 2005).

Biogeographic scenario for the *Geomalacus/Letourneuxia* Iberian land slugs

The accuracy of temporal inferences is crucial for the establishment of biogeographic scenarios, however, precise estimates of divergence times are difficult to obtain given the associated potential sources of error (e.g. when the calibration of the phylogenies is based on the fossil record) (McCormack *et al.,* 2011). Here, estimates of the major lineage splitting events within the Arionidae of the genera *Arion*, *Geomalacus* and *Letourneuxia* were based on a biogeographical event (the end of the "Messinian Salinity Crisis" with the opening of the Gibraltar strait at 5.45 MYA ± 0.15) that was used to calibrate the divergence between *L. numidia* and *G. malagensi*s.

The family Arionidae may have originated in the Iberian Peninsula, since most of the endemic Arionidae species occur in this region (and in North Africa) and all of the genus-level taxa are represented in that area (Likharev & Wiktor, 1980). It is possible that during the Quaternary the Iberian Peninsula might have been the distribution center from where the arionids radiated to the vast areas of the Palearctic (Wiktor & Norris, 1991).

Our dating analysis estimated the age of the most recent common ancestor (MRCA) of *Geomalacus* during the Miocene at about 12 MYA (Figure 4.3), which diverged into three main lineages (Figure 4.4). One included the ancestor of *G. oliveirae* and *G. anguiformis* that most likely occupied the central and southeast area of the Iberian Peninsula. The second lineage included the ancestor of *G. maculosus* present in the north and northeast of Iberia. The ancestor of *L. numidica* and *G. malagensis* probably occupied the area of the Betic-Rifian massif represents the third lineage. During the Zanclean period (5.3−3.6 MYA) (Fauquette *et al.*, 1999), the Iberian Peninsula was characterized by a dry climate that probably restricted the distribution of terrestrial gastropods of the central and south Iberia to more humid areas, such as Monchique, Faro and Sierra de Aracena in the South (current geographical distribution of *G. anguiformis*) or to the mountains of the central massif (current geographical distribution of *G. oliveirae*) (Figure 4.1). The divergence between and *G. anguiformis* and *G. oliveirae* (lineage VII, Figures 4.1 and 4.2) estimated at 4.8 [2.2-9.5] MYA (Figures 4.3) coincided with this period and was most likely triggered by these unsuitable climatic conditions that restricted their ranges to the areas referred above. The two main lineages of *G. anguiformis* diverged at 2.6 [1.1−5.2] MYA isolating Sierra de Aracena from South Portugal (Serra de Monchique and Faro) populations. This time frame is consistent with the emergence of the principal Iberian hydrogeographic basins (1.8–2.5 MYA) (Calvo *et al.*, 1992), in particularly the Guadiana basin, that might have caused the isolation of these two lineages. *Geomalacus maculosus* MRCA split into two major clades at 3.6 [1.8–6.9] MYA during the Pliocene, also fits the Zanclean period, suggesting that the dry climatic period might have restricted some lineages with a northern distribution to two different and restricted areas in north and northeastern. *Geomalacus maculosus* presents a geographically structured mitochondrial genetic variability (Figure 4.2), and it is possible to identify five different lineages within this species (Figure 4.4), however intraspecific diversity is much lower, probably due to the continuum of suitable habitat in the north and northeast of Iberia (Patrão et al., submitted).

Concluding remarks

This study revealed the existence of high levels of cryptic diversity in arionid land slugs throughout their Iberian/Moroccan distribution. Phylogenetic and species delimitation analyses identified 14 lineages within *Geomalacus* and *Letourneuxia* with geographic ranges mostly restricted to mountain systems. We found a clear correspondence between the recovered clades and their geographical location. The allopatric distribution of *Geomalacus* species is consistent with a “refugia-within-refugia” scenario (Gómez & Lunt, 2006). The longitudinal grouping of *G. maculosus* specimens suggests an east-to-west genetic differentiation most likely promoted by lineage-specific responses to climate changes. A more detailed fine-scale study on the identified cryptic lineages would provide valuable information regarding the demographic events that independently occurred on each lineage. In conclusion, the land slugs *Geomalacus* and *Letouneuxia* show a pattern of deep divergences and cryptic diversity, that appears to be a response to climatic and geological events during the Miocene and Pliocene as well as a regional population structure associated to more recent Pleistocene events.

Annexe 4.1. Detailed information on 512 samples used in this study. Collection ID, COI haplotype membership and number of sequences in each COI haplotype, latitude and longitude.

Collection ID	Species	Haplotype number	Number of sequences	Location Code	Region	Latitude	Longitude
ANGMOa0147	*G. anguiformis*	1	17	MOa	Monchique	37.307	-8.570
ANGMOa0319	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0321	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0329	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0316	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0326	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0327	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0330	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0322	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0323	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0324	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0328	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0318	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0320	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOa0317	*G. anguiformis*			MOa	Monchique	37.307	-8.570
ANGMOb0260	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0254	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0261	*G. anguiformis*	2	3	MOb	Monchique	37.305	-8.588
ANGMOb0266	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0262	*G. anguiformis*			MOb	Monchique	37.305	-8.588

ANGMOb0264	*G. anguiformis*	3	28	MOb	Monchique	37.305	-8.588
ANGMOb0257	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0263	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0268	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0265	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOb0267	*G. anguiformis*			MOb	Monchique	37.305	-8.588
ANGMOc0331	*G. anguiformis*			MOc	Monchique	37.320	-8.536
ANGMOc0333	*G. anguiformis*			MOc	Monchique	37.320	-8.536
ANGMOc0332	*G. anguiformis*			MOc	Monchique	37.320	-8.536
ANGMOc0334	*G. anguiformis*			MOc	Monchique	37.320	-8.536
ANGMOd0337	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOd0338	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOd1718	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOd0341	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOd0336	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOd0417	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOd0339	*G. anguiformis*			MOd	Monchique	37.342	-8.488
ANGMOe0532	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0531	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0533	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe1147	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe1149	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0607	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0530	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0526	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0608	*G. anguiformis*			MOe	Monchique	37.313	-8.549

ANGMOe0528	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOe0534	*G. anguiformis*			MOe	Monchique	37.313	-8.549
ANGMOb0256	*G. anguiformis*	4	1	MOb	Monchique	37.305	-8.588
ANGMOc0335	*G. anguiformis*	5	1	MOc	Monchique	37.320	-8.536
ANGMOd0340	*G. anguiformis*	6	1	MOd	Monchique	37.342	-8.488
ANGMOd0418	*G. anguiformis*	7	2	MOd	Monchique	37.342	-8.488
ANGMOf0556	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOe0529	*G. anguiformis*	8	1	MOe	Monchique	37.313	-8.549
ANGMOe0527	*G. anguiformis*	9	1	MOe	Monchique	37.313	-8.549
ANGMOe0505	*G. anguiformis*	10	1	MOe	Monchique	37.313	-8.549
ANGMOf0560	*G. anguiformis*	11	2	MOf	Monchique	37.306	-8.499
ANGMOf0561	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0552	*G. anguiformis*	12	5	MOf	Monchique	37.306	-8.499
ANGMOf0551	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0550	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0558	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0553	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0548	*G. anguiformis*	13	2	MOf	Monchique	37.306	-8.499
ANGMOf0559	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0554	*G. anguiformis*	14	2	MOf	Monchique	37.306	-8.499
ANGMOf0555	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGMOf0549	*G. anguiformis*	15	2	MOf	Monchique	37.306	-8.499
ANGMOf0557	*G. anguiformis*			MOf	Monchique	37.306	-8.499
ANGSBb1162	*G. anguiformis*	16	16	SBb	Faro	37.273	-7.875
ANGSBb1863	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1174	*G. anguiformis*			SBb	Faro	37.273	-7.875

ANGSBb1864	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1171	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1170	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1158	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1867	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1175	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1154	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1160	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1155	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1163	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1159	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1176	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1865	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1153	*G. anguiformis*	17	3	SBb	Faro	37.273	-7.875
ANGSBb1169	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1866	*G. anguiformis*			SBb	Faro	37.273	-7.875
ANGSBb1161	*G. anguiformis*	18	2	SBb	Faro	37.273	-7.875
ANGSBb1172	*G. anguiformis*			SBb	Faro	37.273	-7.875
ARAARA0525	*G. anguiformis*	19	9	ARA	Aracena	37.878	-6.817
ARAARA0536	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0538	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0540	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0542	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0543	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0544	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0545	*G. anguiformis*			ARA	Aracena	37.878	-6.817

ARAARA0546	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0535	*G. anguiformis*	20	3	ARA	Aracena	37.878	-6.817
ARAARA0537	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0541	*G. anguiformis*			ARA	Aracena	37.878	-6.817
ARAARA0539	*G. anguiformis*	21	1	ARA	Aracena	37.878	-6.817
ARAARA0547	*G. anguiformis*	22	1	ARA	Aracena	37.878	-6.817
LETTAa1851	*L. numidica*	23	5	TAa	Morocco	35.784	-5.901
LETTAa1860	*L. numidica*			TAa	Morocco	35.784	-5.901
LETTAa1857	*L. numidica*			TAa	Morocco	35.784	-5.901
LETTAb1889	*L. numidica*			TAb	Morocco	35.783	-5.851
LETTAb1890	*L. numidica*			TAb	Morocco	35.783	-5.851
LETTAa1855	*L. numidica*	24	1	TAa	Morocco	35.784	-5.901
LETTAa1850	*L. numidica*	25	1	TAa	Morocco	35.784	-5.901
LETTAa1849	*L. numidica*	26	2	TAa	Morocco	35.784	-5.901
LETTAa1848	*L. numidica*			TAa	Morocco	35.784	-5.901
LETTAa1856	*L. numidica*	27	4	TAa	Morocco	35.784	-5.901
LETTAa1854	*L. numidica*			TAa	Morocco	35.784	-5.901
LETTAa1853	*L. numidica*			TAa	Morocco	35.784	-5.901
LETTAa1847	*L. numidica*			TAa	Morocco	35.784	-5.901
LETTAa1852	*L. numidica*	28	1	TAa	Morocco	35.784	-5.901
LETLET0605	*L. numidica*	29	1	LET	Morocco	31.130	-7.900
LETLET0622	*L. numidica*	30	1	LET	Morocco	31.130	-7.900
LETLET0630	*L. numidica*	31	2	LET	Morocco	31.130	-7.900
LETLET0636	*L. numidica*			LET	Morocco	31.130	-7.900
LETLET0635	*L. numidica*	32	1	LET	Morocco	31.130	-7.900

MACBRA0203	*G. maculosus*	33	15	BRA	Bragança	41.688	-6.751
MACBRA0204	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0205	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0206	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0207	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0208	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0209	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0210	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0211	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0212	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0213	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0214	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0215	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0216	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACBRA0492	*G. maculosus*			BRA	Bragança	41.688	-6.751
MACCAa0922	*G. maculosus*	34	7	CAa	Asturias	42.949	-6.395
MACCAa0923	*G. maculosus*			CAa	Asturias	42.949	-6.395
MACCAa0924	*G. maculosus*			CAa	Asturias	42.949	-6.395
MACCAa0925	*G. maculosus*			CAa	Asturias	42.949	-6.395
MACCAa0926	*G. maculosus*			CAa	Asturias	42.949	-6.395
MACCAa0927	*G. maculosus*			CAa	Asturias	42.949	-6.395
MACCAa0928	*G. maculosus*			CAa	Asturias	42.949	-6.395
MACCAb0806	*G. maculosus*	35	8	CAb	Asturias	42.955	-6.372
MACCAb0807	*G. maculosus*			CAb	Asturias	42.955	-6.372
MACCAb0808	*G. maculosus*			CAb	Asturias	42.955	-6.372
MACCAb0809	*G. maculosus*			CAb	Asturias	42.955	-6.372

MACCAb0810	*G. maculosus*			CAb	Asturias	42.955	-6.372
MACCAb0811	*G. maculosus*			CAb	Asturias	42.955	-6.372
MACCAb0812	*G. maculosus*			CAb	Asturias	42.955	-6.372
MACCAb0813	*G. maculosus*			CAb	Asturias	42.955	-6.372
MACCHA1708	*G. maculosus*	36	11	CHA	Serra da Estrela	40.539	-7.313
MACCHA1765	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0284	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0285	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0287	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0289	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0290	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0424	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0425	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0426	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0427	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCHA0286	*G. maculosus*	37	2	CHA	Serra da Estrela	40.539	-7.313
MACCHA0288	*G. maculosus*			CHA	Serra da Estrela	40.539	-7.313
MACCAM0146	*G. maculosus*	38	18	CAM	Serra da Estrela	40.383	-7.544
MACCAM1877	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM1878	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM1879	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM1882	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0269	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0270	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0271	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0272	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544

MACCAM0273	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0275	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0276	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0277	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0278	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0279	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0281	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0282	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM0283	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCAM1880	*G. maculosus*	39	2	CAM	Serra da Estrela	40.383	-7.544
MACCAM1881	*G. maculosus*			CAM	Serra da Estrela	40.383	-7.544
MACCOa0868	*G. maculosus*	40	5	COa	Cantabria	43.307	-5.053
MACCOa0869	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0870	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0876	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0877	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0871	*G. maculosus*	41	5	COa	Cantabria	43.307	-5.053
MACCOa0872	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0874	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0875	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0878	*G. maculosus*			COa	Cantabria	43.307	-5.053
MACCOa0873	*G. maculosus*	42	2	COa	Cantabria	43.307	-5.053
MACCOb1116	*G. maculosus*			COb	Cantabria	43.307	-5.053
MACCOa0879	*G. maculosus*	43	15	COa	Cantabria	43.307	-5.053
MACSAA0714	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0715	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227

MACSAA0716	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0717	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0718	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0719	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0720	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0721	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0722	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0724	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0725	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0726	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0728	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACSAA0729	*G. maculosus*			SAA	Picos de Europa	43.238	-4.227
MACCOb1117	*G. maculosus*	44	1	COb	Cantabria	43.341	-5.084
MACGUA0230	*G. maculosus*	45	16	GUA	Serra da Estrela	40.535	-7.273
MACGUA0401	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0402	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0403	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0404	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0405	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0406	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0408	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0409	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0410	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0411	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0412	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0413	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273

MACGUA0414	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0415	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGUA0416	*G. maculosus*			GUA	Serra da Estrela	40.535	-7.273
MACGEf1499	*G. maculosus*	46	6	GEf	Geres	41.793	-8.138
MACGEf0817	*G. maculosus*			GEf	Geres	41.793	-8.138
MACGEf0818	*G. maculosus*			GEf	Geres	41.793	-8.138
MACGEf0819	*G. maculosus*			GEf	Geres	41.793	-8.138
MACGEf0822	*G. maculosus*			GEf	Geres	41.793	-8.138
MACGEf0823	*G. maculosus*			GEf	Geres	41.793	-8.138
MACGEf0820	*G. maculosus*	47	2	GEf	Geres	41.793	-8.138
MACGEf0821	*G. maculosus*			GEf	Geres	41.793	-8.138
MACMAN1231	*G. maculosus*	49	11	MAN	Ourense	42.310	-7.237
MACMAN1232	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1233	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1234	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1235	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1236	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1237	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1238	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1239	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1241	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1243	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACMAN1240	*G. maculosus*	50	2	MAN	Ourense	42.310	-7.237
MACMAN1242	*G. maculosus*			MAN	Ourense	42.310	-7.237
MACHOb0357	*G. maculosus*	51	8	HOb	Geres	41.834	-8.119
MACHOb0358	*G. maculosus*			HOb	Geres	41.834	-8.119

MACHOb0359	*G. maculosus*			HOb	Geres	41.834	-8.119
MACHOb0929	*G. maculosus*			HOb	Geres	41.834	-8.119
MACHOb0930	*G. maculosus*			HOb	Geres	41.834	-8.119
MACHOb0931	*G. maculosus*			HOb	Geres	41.834	-8.119
MACHOb0933	*G. maculosus*			HOb	Geres	41.834	-8.119
MACHOb0934	*G. maculosus*			HOb	Geres	41.834	-8.119
MACHOb0932	*G. maculosus*	52	1	HOb	Geres	41.834	-8.119
MACPOR0232	*G. maculosus*	53	1	POR	Viana do Castelo	41.705	-8.792
MACSEE0217	*G. maculosus*	54	13	SEE	Viana do Castelo	41.774	-8.619
MACSEE0218	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0219	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0221	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0222	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0223	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0224	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0226	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0234	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0235	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0236	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0237	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0238	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEE0220	*G. maculosus*	55	2	SEE	Viana do Castelo	41.774	-8.619
MACSEE0225	*G. maculosus*			SEE	Viana do Castelo	41.774	-8.619
MACSEb0239	*G. maculosus*	56	15	SEb	Serra da Estrela	40.414	-7.587
MACSEb0240	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0241	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587

MACSEb0242	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0243	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0244	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0245	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0246	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0247	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0248	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0249	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0250	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0251	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0252	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSEb0253	*G. maculosus*			SEb	Serra da Estrela	40.414	-7.587
MACSAA0723	*G. maculosus*	57	1	SAA	Picos de Europa	43.238	-4.227
MACSAA0727	*G. maculosus*	58	1	SAA	Picos de Europa	43.238	-4.227
MACCOM0841	*G. maculosus*	59	12	COM	Coruña	42.878	-8.555
MACCOM0844	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0845	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0846	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0847	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0848	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0849	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0850	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0851	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0852	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0853	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0855	*G. maculosus*			COM	Coruña	42.878	-8.555

MACCOM0842	*G. maculosus*	60	4	COM	Coruña	42.878	-8.555
MACCOM0843	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0854	*G. maculosus*			COM	Coruña	42.878	-8.555
MACCOM0856	*G. maculosus*			COM	Coruña	42.878	-8.555
MACMAR0762	*G. maculosus*	61	15	MAR	Picos de Europa	43.388	-4.110
MACMAR0763	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0764	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0765	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0766	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0767	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0768	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0769	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0770	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0771	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0772	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0773	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0774	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0775	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACMAR0776	*G. maculosus*			MAR	Picos de Europa	43.388	-4.110
MACVOa0967	*G. maculosus*	62	1	VOa	Vouzela	40.721	-8.109
MACVOa0968	*G. maculosus*	63	3	VOa	Vouzela	40.721	-8.109
MACVOa0969	*G. maculosus*			VOa	Vouzela	40.721	-8.109
MACVOa0972	*G. maculosus*			VOa	Vouzela	40.721	-8.109
MACVOa0970	*G. maculosus*	64	3	VOa	Vouzela	40.721	-8.109
MACVOa0971	*G. maculosus*			VOa	Vouzela	40.721	-8.109
MACVOa0973	*G. maculosus*			VOa	Vouzela	40.721	-8.109

MALBRE0184	*G. malagensis*	65	37	BRE	Sesimbra	38.541	-9.030
MALBRE0183	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALBRE1224	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALBRE1225	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALBRE1226	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALBRE1229	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALBRE0181	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALBRE0182	*G. malagensis*			BRE	Sesimbra	38.541	-9.030
MALSSb1831	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1832	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1833	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1834	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1835	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1836	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1837	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1838	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1839	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1840	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1841	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1842	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1843	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALSSb1844	*G. malagensis*			SSb	Sesimbra	38.479	-9.113
MALCLb1535	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALCLb1536	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALCLb1542	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALCLb1543	*G. malagensis*			CLb	Huelva	37.679	-6.650

MALSSa0154	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0155	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0156	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0159	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0160	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0161	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0681	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0682	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0683	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0498	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0153	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALCLa0390	*G. malagensis*	66	13	CLa	Huelva	37.679	-6.653
MALCLb1530	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALCLb1537	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALCLb1538	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALCLb1539	*G. malagensis*			CLb	Huelva	37.679	-6.650
MALGIa0510	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0512	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0517	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0518	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0520	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0523	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0601	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa1603	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALCLb1531	*G. malagensis*	67	1	CLb	Gibraltar	37.679	-6.650
MALCLb1532	*G. malagensis*	68	5	CLb	Gibraltar	37.679	-6.650

MALCLb1533	*G. malagensis*			CLb	Gibraltar	37.679	-6.650
MALCLb1534	*G. malagensis*			CLb	Gibraltar	37.679	-6.650
MALCLb1540	*G. malagensis*			CLb	Gibraltar	37.679	-6.650
MALCLb1541	*G. malagensis*			CLb	Gibraltar	37.679	-6.650
MALJER0563	*G. malagensis*	69	4	JER	Gibraltar	36.255	-5.582
MALJER0564	*G. malagensis*			JER	Gibraltar	36.255	-5.582
MALJER0566	*G. malagensis*			JER	Gibraltar	36.255	-5.582
MALJER0568	*G. malagensis*			JER	Gibraltar	36.255	-5.582
MALJER0565	*G. malagensis*	70	1	JER	Gibraltar	36.255	-5.582
MALJER0567	*G. malagensis*	71	1	JER	Gibraltar	36.255	-5.582
MALGUE1845	*G. malagensis*	72	25	GUE	Faro	37.098	-7.922
MALGUE1846	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1861	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1862	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1876	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1883	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1884	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1885	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1798	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1803	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1804	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1805	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1806	*G. malagensis*			GUE	Faro	37.098	-7.922
MALGUE1807	*G. malagensis*			GUE	Faro	37.098	-7.922
MALSSa0684	*G. malagensis*			SSa	Sesimbra	38.486	-9.121
MALSSa0497	*G. malagensis*			SSa	Sesimbra	38.486	-9.121

MALARR0977	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0978	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0979	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0980	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0981	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0982	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0983	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0984	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALARR0985	*G. malagensis*			ARR	Aljezur	37.296	-8.865
MALGIb0605	*G. malagensis*	73	5	GIb	Gibraltar	36.118	-5.345
MALGIa0511	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0514	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0515	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0524	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIb0606	*G. malagensis*	74	1	GIb	Gibraltar	36.118	-5.345
MALGIa0513	*G. malagensis*	75	1	GIa	Gibraltar	36.131	-5.351
MALGIa0516	*G. malagensis*	76	2	GIa	Gibraltar	36.131	-5.351
MALGIa0519	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALGIa0522	*G. malagensis*	77	2	GIa	Gibraltar	36.131	-5.351
MALGIa0602	*G. malagensis*			GIa	Gibraltar	36.131	-5.351
MALSSa0231	*G. malagensis*	78	1	SSa	Sesimbra	38.486	-9.121
OLITOa1151	*G.oliveirae*	79	4	TOa	Montes de Toledo	39.563	-4.585
OLITOa1177	*G.oliveirae*			TOa	Montes de Toledo	39.563	-4.585
OLITOa1178	*G.oliveirae*			TOa	Montes de Toledo	39.563	-4.585
OLITOa1179	*G.oliveirae*			TOa	Montes de Toledo	39.563	-4.585
OLITOa1164	*G.oliveirae*	80	1	TOa	Montes de Toledo	39.563	-4.585

OLITOa1165	*G.oliveirae*	81	3	TOa	Montes de Toledo	39.563	-4.585
OLITOa1475	*G.oliveirae*			TOa	Montes de Toledo	39.563	-4.585
OLITOa0421	*G.oliveirae*			TOa	Montes de Toledo	39.563	-4.585
OLITOa1180	*G.oliveirae*	82	1	TOa	Montes de Toledo	39.563	-4.585
OLITOa1181	*G.oliveirae*	83	1	TOa	Montes de Toledo	39.563	-4.585
OLITOa1182	*G.oliveirae*	84	2	TOa	Montes de Toledo	39.563	-4.585
OLITOa1808	*G.oliveirae*			TOa	Montes de Toledo	39.563	-4.585
OLITOa1280	*G.oliveirae*	85	1	TOa	Montes de Toledo	39.563	-4.585
OLITOb1118	*G.oliveirae*	86	3	TOb	Montes de Toledo	39.585	-4.527
OLITOb1119	*G.oliveirae*			TOb	Montes de Toledo	39.585	-4.527
OLITOb1121	*G.oliveirae*			TOb	Montes de Toledo	39.585	-4.527
OLITOb1120	*G.oliveirae*	87	5	TOb	Montes de Toledo	39.585	-4.527
OLITOb1124	*G.oliveirae*			TOb	Montes de Toledo	39.585	-4.527
OLITOb1126	*G.oliveirae*			TOb	Montes de Toledo	39.585	-4.527
OLITOb1128	*G.oliveirae*			TOb	Montes de Toledo	39.585	-4.527
OLITOb1129	*G.oliveirae*			TOb	Montes de Toledo	39.585	-4.527
OLITOb1122	*G.oliveirae*	88	1	TOb	Montes de Toledo	39.585	-4.527
OLITOb1123	*G.oliveirae*	89	1	TOb	Montes de Toledo	39.585	-4.527
OLITOb1125	*G.oliveirae*	90	1	TOb	Montes de Toledo	39.585	-4.527
OLITOb1127	*G.oliveirae*	91	1	TOb	Montes de Toledo	39.585	-4.527
OLITOb1130	*G.oliveirae*	92	1	TOb	Montes de Toledo	39.585	-4.527
OLITOb1131	*G.oliveirae*	93	1	TOb	Montes de Toledo	39.585	-4.527
OLITOb0488	*G.oliveirae*	94	1	TOb	Montes de Toledo	39.585	-4.527
OLIGRa1762	*G.oliveirae*	95	10	GRa	Sierra de Gredos	40.322	-5.014
OLIGRa1763	*G.oliveirae*			GRa	Sierra de Gredos	40.322	-5.014
OLIGRa1802	*G.oliveirae*			GRa	Sierra de Gredos	40.322	-5.014

OLIGRb1752	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1755	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1758	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1759	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1760	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1761	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1764	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1145	*G.oliveirae*	96	2	GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1757	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1146	*G.oliveirae*	97	1	GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1753	*G.oliveirae*	98	1	GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1754	*G.oliveirae*	99	2	GRb	Sierra de Gredos	40.308	-5.000
OLIGRb1756	*G.oliveirae*			GRb	Sierra de Gredos	40.308	-5.000
OLIGRc1775	*G.oliveirae*	100	1	GRc	Sierra de Gredos	40.308	-5.000
OLILPa0420	*G.oliveirae*	101	2	LPa	Sierra de Guadalupe	39.440	-5.315
OLILPa0490	*G.oliveirae*			LPa	Sierra de Guadalupe	39.440	-5.315
OLILPb1778	*G.oliveirae*	102	1	LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb1779	*G.oliveirae*	103	10	LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb1781	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb1795	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0370	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0371	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0378	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0379	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0380	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0400	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368

OLILPb0489	*G.oliveirae*			LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb1780	*G.oliveirae*	104	1	LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0372	*G.oliveirae*	105	1	LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0373	*G.oliveirae*	106	1	LPb	Sierra de Guadalupe	39.474	-5.368
OLILPb0375	*G.oliveirae*	107	1	LPb	Sierra de Guadalupe	39.474	-5.368
OLIPEa1766	*G.oliveirae*	108	3	PEa	Sierra de Peña de Francia	40.514	-6.157
OLIPEb1771	*G.oliveirae*			PEb	Sierra de Peña de Francia	40.514	-6.157
OLIPEa0382	*G.oliveirae*			PEa	Sierra de Peña de Francia	40.514	-6.157
OLISEa0422	*G.oliveirae*	109	1	SEa	Serra da Estrela	40.461	-7.587
OLISEb1279	*G.oliveirae*	110	1	SEb	Serra da Estrela	40.414	-7.587

Chapter V

General Conclusions

General Conclusions

The terrestrial slugs of the genera *Geomalacus* and *Letourneuxia* constitute interesting model organisms for biogeographical studies due to their habitat specificity, low dispersal abilities and the high levels of endemism in the Iberian Peninsula. The taxonomy of both two genera is quite challenging due to the existence of a small number of morphological diagnostic characters. The genetic structure is completely unknown for both genera and their geographical distribution remains poorly understood. With the present thesis we aim to perceive the evolutionary history of *Geomalacus* and *Letourneuxia,* understand the impact of past climate changes and physiography in shaping present-day species distribution in the Iberian Peninsula and evaluate the existence of cryptic diversity given the small number of *Geomalacus* species currently recognized within this region. For the present thesis 498 specimens of *Geomalacus spp* and *Letourneuxia numidica* were sampled in 49 different locations of Portugal, Spain, Gibraltar and Morocco. Results revealed deep divergences between and within species, cryptic diversity, and regional population structure. The main conclusions of this thesis are summarized below.

Distribution of *Geomalacus spp*.

New distribution maps are proposed, based on empirical observations and on results from the habitat suitability modeling. The combined distribution map (Figure 4.1) reveals more conservative geographical areas for *G. oliveirae*, *G. malagensis* and *G. anguiformis*, when compared to the maps proposed by Castillejo (1998) and a much wider distribution for *G. malagensis*, with new records in Portugal. The extensive list of locations available in Table ?S1, and the obtained distribution models constitute valuable information for molecular and taxonomic studies within the genus *Geomalacus*, and for conservation purposes, since these new ranges can be used in future editions of the Invertebrate Red Book of Spain.

Novel use of somatic tissue in cytogenetic studies of land slugs

The use of somatic organs for cytogenetic studies instead of the classically used ovotestis (Beeson, 1960; Burch, 1965; Patterson, 1969; Colomba et al., 2009) proved to be effective. Mouth and both optical and sensory tentacles yielded several mitotic metaphases and hence were successfully used to determine diploid chromosome numbers and obtaining diploid karyotypes. When using reproductive tissue, only well-developed ovotestis yields chromosome images usable for this purpose. The new approach using somatic tissues allows karyological studies to be performed regardless of the sexual developmental stage of the specimens. This methodology can be tested in other terrestrial gastropods, since it facilitates cytological studies and allows using terrestrial gastropods of any age and development stage.

Phylogenetic assessment and taxonomic implications

This thesis provides the first phylogenetic analysis of the genus *Geomalacus*, based on mtCOI and 18S rRNA gene fragments. The present results reveal *Letourneuxia numidica* as sister group of *Geomalacus malagensis* and *G. oliveirae* paraphyletic with respect to *G. anguiformis.* Several lines of evidence (cytogenetic, nuclear and mitochondrial sequences, and morphology (Wiktor, 1983; Wiktor & Norris, 1991)) suggest that *Letourneuxia* does not deserve generic status, but could instead be considered a species within the genus *Geomalacus*.

The genus *Geomalacus* is more diverse than previously expected based on morphology. All *Geomalacus* species, with the exception of *G. malagensis*, present high geographically structured mitochondrial genetic variability. Five different cryptic lineages were identified within *G. oliveirae*. At the present, it is not possible to access if the cryptic lineages of *G. oliveirae* are different species, therefore collecting new specimens and performing new morphological analysis may uncover new diagnostic characters that might allow the morphological distinction between lineages. A more detailed fine-scale study focusing on the recovered cryptic lineages would provide valuable information regarding the biogeographic events that shaped species distribution and diversity.

According to our dating estimates, the most recent common ancestor of *Geomalacus* dates back to the Middle Miocene (end of the Serravallian stage). Most of the lineage splitting events within *Geomalacus* occurred during the dry periods of the Zanclean stage (5.3-3.6 million years) that restricted the geographic distribution of some lineages to more humid mountain areas of the Iberian Peninsula. These Pliocene climatic events contributed to the present highly geographically structured mitochondrial genetic diversity of the genus *Geomalacus*.

Conservation implications

The natural autochthonous habitats of *Geomalacus spp.* have been under threat by fragmentation and/or destruction (Castillejo & Iglesias-Pineiro, 2008; Castillejo & Iglesias-Pineiro, 2011c; Castillejo & Iglesias-Pineiro, 2011a; Castillejo & Iglesias-Pineiro, 2011b). Habitat fragmentation may strongly disturb species distribution patterns, with consequences on metapopulation dynamics and the genetic structure of populations. Moreover, synergistic interactions of climate change, species interactions and other enhancers of habitat destruction may contribute to population fragmentation and demographic decline. It is extremely important to establish and maintain ecological corridors between patches of suitable habitats to avoid the loss of species genetic diversity and, at the extreme, extinction (Bennett, 1999).

Geomalacus maculosus is a very common slug that seems to co-habit with humans. In fact, we were able to find this species in synantropic environments, such as houses, gardens, stonewalls, ruins and cemeteries, as well as in chestnut and oak tree orchards. Although *G. maculosus* is considered a pest species for agriculture, we believe that the populations of this species can thrive as long as the considerations in the red book of invertebrates of Spain (Verdú *et al.*, 2011) are met: destruction of chestnut and oak tree woods must be under strict surveillance, maintenance of water lines (streams, rivers and groundwater) and moderation in the use of phytosanitary compounds.

With the exception of Serra de Monchique, *G. anguiformis* is common in areas previously abandoned by humans as already reported by Castillejo, (2011a), being easily found at daylight, during or immediately after raining. *Geomalacus anguiformis* displays a strong geographical structure and the loss of local and isolated populations may present serious consequences for local biodiversity. The destruction of Serra de Monchique, due to logging and fire (2003), where several patches of autochthonous forest were destroyed, together with the 2012 fires in Serra do Caldeirão, were responsible for severe recent habitat destruction, forcing the reduction of ecological corridors and escalated the fragmentation of the existing habitat. Conservation measures ensuring the protection of these areas should allow a rapid recovery of the habitats, therefore reducing the risk of extinction of the local populations and biodiversity. We recommend that the conservation status of *G.*

anguiformis should be given special attention due to the recent events that destroyed this species natural habitat.

Geomalacus oliveirae is a quite small and hard to find slug, mainly because we were unable to locate it during daylight, even when light showers occurred. Also, the number of individuals captured at all locations was small (3-4 specimens); following what was already stated in the Red Book of Invertebrates of Spain (Castillejo & Iglesias-Pineiro, 2011b), *Geomalacus oliveirae* is listed as "vulnerable" in the Invertebrate Red List of Spain (Castillejo & Iglesias-Pineiro, 2011b). The discovery of five cryptic lineages harboring unique diversity makes the protection of each distinct ESU even more pressing, since the geographical range of each lineage is much smaller than previously expected based when considering *G. oliv*eirae one single ESU. Highly geographically structured mitochondrial genetic variability, associated with relative "old age" of the mitochondrial lineages suggested that each ESU should be considered a distinct management unit. The present "hidden" diversity in *G. oliveirae* might also prompt the revision of the protection level of the Sierras of the central Massif of the Iberian Peninsula.

Based on literature (Castillejo & Iglesias-Pineiro, 2008), outside Gibraltar, *G. malagensis* is antrophobic. However, we found the species in highly anthropogenic-disturbed habitats revealing a positive response in traditionally negative drivers to species thriving. There is an apparent adaptation of the species to disturbed habitats and present thesis reports a wider geographic distribution range than previously known. There is still no legal protection to *G. malagensis*, probably since it was a poorly studied species so far. We propose that the present findings will allow a conservation status to be issued and included by proper authorities in a future revision of the Red Book of Invertebrates Species of Spain (Verdú *et al.*, 2011).

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A final, phylosophical remark

I opened this thesis with a quote from Darwin, and as I lay my pencil (computer keys) to rest, I can’t stop thinking about that same sentence: for the sake of “convenience” we classify and aggregate entities, sometimes in such artificial ways that biology no longer is the center of our concern. Biologist spend more and more hours facing a computer screen than in the lab, and when we compare the amount of hours spend in the lab with the ones spend in the field, I wonder to what extent biology still reflects real life. More and more, habitat, ecological and in-situ behavioral descriptions are frowned upon while computer modelling and complicated biostatistics are hiper-valued on any peer-reviewed indexed journal. I have fellow biologist colleagues that never even laid eye on the object of their study, neither in their natural habitat, nor even in the lab… Biology is more and more a science of pipettes and pieces of tissue that lay pickled in a tiny jar.

I can’t seem to forget attending a very respected phylogeny course and in one particular presentation, by a world renown molecular evolutionist, when I asked him: “But, what is the biological meaning of these two clades clustering together?”, he simple answered – “I don’t really care…” Have we really lost this much touch with reality? Do we really care more about the outcome of a computer program that the real reason why it was developed in first place? Do we value more and more the silicon cells than the beauty of real cells and real life? How many of us, biologists, can afford to spend hours in the field understanding and validating the results from this myriad of very respectable software analysis? Time is money; time is published papers… but taking the time to actually SEE what we now know, in the field, in real life… is completely underrated.

As for me, one thing is sure, biology as we know it today, cannot live without biostatistics and complex molecular techniques, but I will forever do my best, to spend some of my very “valuable” time admiring the object of my study where it belongs, in nature, in the middle of the forest or in the middle of the desert, I shall make an effort to never forget the real reason why any study first begins.