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Title	The EU-protected slug geomalacus maculosus: an investigation into its phylogenetics, population densities in conifer plantations and its gut microbial community
Author(s)	Reich, Inga
Publication Date	2016-04-04
Item record	http://hdl.handle.net/10379/5631

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The EU-protected slug *Geomalacus maculosus*: An investigation into its phylogenetics, population densities in conifer plantations and its gut microbial community

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy

April 2016

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Acknowledgements

A huge thank you to my supervisors Mike Gormally and Cindy Smith for helping me in so many different ways with their encouragement, patience and good advice. It was great to have the opportunity to work with ecological as well as molecular methods. You are both so enthusiastic about what you do and were always willing to discuss new ideas for my project - at the beginning of my PhD I never would have thought I'll do a chapter on microbes!

Many thanks also to Rory McDonnell - the pioneer in *Geomalacus maculosus* research at the Applied Ecology Unit who has been very supportive throughout my PhD, and who provided me with lots of Kerry slug knowledge and feedback on papers and conference abstracts despite being all the way across the Atlantic. Chris Williams has been of great assistance with any kind of statistical analysis and Gesche Kindermann has been an immense help with all my GIS related problems. They and Caitriona Carlin were always supportive if I had any problems or queries – thank you so much guys!

I am very grateful to Owen Doherty for assisting with fieldwork and organising all the materials for my second year students, to Angela Trayers-Lynagh for helping with all administrative queries, to Maurice Martin for ordering anything and everything and to Mike Coughlan and Ann Smyth for locating things within the department and helping out in any other possible way.

Many thanks also to Louise Allcock for helping me with my phylogenetic analyses – I don't think she knew how much work was in store for her when I first approached her but with her patience and great teaching skills I went from a complete newbie to a fairly confident BEAST user.

I am very grateful to the members of my Graduate Research Committee, Ger Fleming and Micheline Sheehy-Skeffington for providing me with feedback about my work.

I couldn't have asked for a nicer working atmosphere and throughout my four years in the Applied Ecology Unit my colleagues have been very supportive and great to work with. Thanks to Brendan Canning, John Staunton, Dave Clarke, Tracy Hynes, Margaret Hayes, Mesa Perry, John Carey, Irene Giordani, Erin Johnston, Collette Mulkeen, Aidan O'Hanlon, Lorenzo Sardo, Manuela Carnaghi and Allison Bistline-East for being such fantastic labmates. I'll miss our 'Cake-Tuesdays', lunchtime sessions and general fun times in the lab! A special thanks to Kim O'Meara for being my field assistant in my first year of the PhD and for making fieldwork and writing so much more enjoyable. A pity you didn't stick around longer. Thanks also to the many visiting students as well as fourth year students, in particular Maxime Poissonneau, Cian Blaix, Eugene Cush, Fiachra Tierney, Shane Callanan and Cathal McInerney for helping me with my fieldwork and laboratory experiments.

Thanks to Aoife Duff, Agata Lisik, Cathy Abberton, Limei Zhang and Enrico Tatti for making my start in the world of genetics and microbiology so much easier with their friendly reception in the 'microlab' and by helping me with my gels, extractions and PCRs.

My PhD would have been only half as much fun without my fieldwork in Spain and I am very grateful to José Castillejo and Javier Iglesias from the University of Santiago de Compostela for accompanying me on my sampling trip and showing me the best *G. maculosus* spots. They were such nice and helpful hosts and I'll never forget the extended night sampling trips in the amazing countryside of Northern Iberia. Thanks also to Javier Quinteiro for letting me use his lab for sample preparation and for helping me with my population genetics

analyses, to Jorge Rodríguez-Castro for collecting additional *G. maculosus* specimens and to María Córdoba Otero for help with sending tissue samples from Spain to Ireland.

Many thanks to the National Parks and Wildlife Service for funding parts of my research and providing me with several *G. maculosus* sampling and killing licenses. A special thank you to Brian Nelson who was of great help during the preparation of the Wildlife Manual as well as of two other, sadly not yet published papers.

Thanks to Barry Rintoul from Coillte for letting me conduct my work in Cloosh Forest and for providing me with management plans and information about the area. I am also very grateful to Paul Whelan for assisting me with lichen identification for the Wildlife Manual.

I want to thank all my friends for being so supportive and providing plenty of distraction from my study when needed - even if it was just through a Skype call!

Thanks to my family for their emotional and financial support, interest in my work and belief in me. It's so good to know that you're always there for me.

A huge thanks goes to my partner Peter Kelly for being the most loving and caring person I know. You always had my back and helped me through the stressful and frustrating stretches of my PhD by reminding me that there's always something else apart from work to look forward to. I am so lucky to have you. A special thanks to my dog Talin who always ensured that I made enough time in my day for a walk in the fresh air and some ball games!

Finally thanks to all the Kerry slugs which have been subject to my investigations – you guys have really grown on me!

Abstract

The EU-protected Lusitanian slug species *Geomalacus maculosus* Allman (Gastropoda: Arionidae) occurs only in Northern Iberia and the West of Ireland. It inhabits largely undisturbed habitats such as blanket bogs and forests where it feeds on lower plants growing on tree trunks or rocks. Chapter 1 provides an overview of the current knowledge of the species' distribution, ecology and associated legislation and identifies areas in need of further research.

Chapter 2 investigates the range-wide genetic variability and population structure of *G. maculosus* with the aim of shedding light onto the origin of the Irish population. Tissue samples from 78 specimens were collected from 13 locations within Ireland and ten locations within Iberia and partial sequences of the mitochondrial 16S rRNA and cytochrome oxidase subunit 1 (COI) and from the nuclear internal transcribed spacer 1 region (ITS-1) were compared. The Irish population was found to have a greatly reduced genetic diversity compared to the Iberian populations, with only one (16S rRNA) and two (COI) mitochondrial haplotypes identified respectively in addition to which no private Irish ITS-1 allele was found. Based on the COI sequences, the Irish specimens clustered monophyletically with Spanish specimens from Northern Asturias and Cantabria, suggesting that the Irish population may have originated close to this area. Iberian *G. maculosus* populations were found to be highly structured with some populations being assumed to be separated for millions of years, even if the 16S rRNA gene evolved with a fast divergence rate of 10 % per million years.

Chapter 3 deals with the estimation of population sizes of *G. maculosus* and of the sympatric slug species *Lehmannia marginata* Müller in a commercial conifer plantation in the context of variations in habitat structure and weather conditions. The study follows the

2010 discovery of *G. maculosus* in Cloosh Forest, a conifer plantation in county Galway, more than 200 km north of it's previously known Irish distribution area. Since then its presence has been confirmed in several other commercial plantations located in the south-west of Ireland. This was surprising, considering the species is generally associated with undisturbed habitats such as deciduous woodlands and blanket bog. However, it is likely that conifer plantations may become important refuges for this protected species in the future and research, taking into account commercial forestry practices, is needed to determine the requirements and population densities of *G. maculosus* in these habitats. The mark-recapture method was used to determine population density estimates in six differently managed sites in Cloosh Forest. *Lehmannia marginata* was included in this study as it occupies a very similar niche to *G. maculosus* and we wanted to determine how their abundances relate to each other. *Geomalacus maculosus* was found in higher numbers than *L. marginata* in each surveyed site and the population densities of both species were found to be highest in plantation sites which comprised of mature trees covered with a thick epiphyte layer while they were lowest in a clear-fell site in which the trees had been felled in a conventional manner. Large variations in capture success were observed depending on seasonality and weather conditions with more slugs being trapped during warm weather and less slugs trapped during rainy periods in the surveyed plantation sites. We also evaluated the long-term use of visible implant elastomers which were used to mark the slugs and found them to be durable and easy to use. The *G. maculosus* data were found to be a generally unsatisfactory fit for both the Schnabel and the Jolly-Seber method which is likely due to the trap-response of this species.

Chapter 4 uses Illumina MiSeq sequences from the V3 region of the bacterial 16S rRNA gene to investigate the microbial diversity found within the faeces of *G. maculosus*. This study follows up on chapter 2, which found that the Irish *G. maculosus* populations could not be distinguished using mitochondrial markers. The aims of this chapter were to determine whether the faeces from 30 slugs which were collected from eleven different sites within Ireland possess a site-specific microbial signature and whether a core microbiome was present within the faeces of all sampled slugs. These bacteria often fulfil important functions within their host and could provide information about adaptations of *G. maculosus* to the available food resources within its habitat. Additional sequences were obtained from the faeces of six laboratory reared hatchlings from the same egg clutch which were fed on two different foods to investigate whether diet or kinship had a larger effect on the diversity of their faecal microbiome. Only two operational taxonomic units were found in all of the slugs, one of which has been shown to possess cellulolytic and xylanolytic activity. No clear clustering of the samples by habitat or sample site could be observed and diet was found to have a greater impact than kinship on the diversity of the faecal microbiome, suggesting that there is only little vertical transfer of the parents gut microbiome via the egg in *G. maculosus*.

Chapter 5 summarizes and discusses the main outcomes of the three preceding chapters and highlights areas in need of further research.

Chapter 1:

General Introduction

General Introduction

1.1. Scope and objectives

The preservation of biodiversity is currently one of the biggest challenges facing humankind especially in the light of climate change which is likely to accelerate the rate of extinctions in the future (Walther *et al*., 2002). Every day species are disappearing from the planet, many of them completely unnoticed. Knowledge of the ecological requirements of a species is a key factor for its protection as only then can effective conservation measures be put into place. At the same time, an assessment of the genetic variability of a species provides important information about the health of a population. This is particularly important in the context of small or introduced populations with high levels of inbreeding leading to the loss of genetic variability and reduced fitness which lowers the ability of populations to adapt to environmental changes (e.g. Frankham, 1995).

Ireland has currently over 100 species protected under the Wildlife Act 1979 (as amended) (Ecologists Ireland, 2015) while 50 animal and eleven plant species/groups occurring in Ireland are protected by EU law (NPWS, 2013). As an EU Member State, Ireland is obliged to undertake surveillance of the conservation status of these species, which involves increasing the understanding of their needs.

The aim of this thesis is to further the knowledge about the slug *G. maculosus*, which is protected under Irish and EU law. I am approaching this from three different angles, using both molecular and ecological tools:

1. The range-wide population genetic structure of *G. maculosus* populations was investigated with the aim of providing information about its genetic variability throughout its

distribution area and with a view to determining the origin of the Irish population.

2. Population densities of *G. maculosus* and the sympatric *L. marginata* were determined in the context of variations in habitat structure and weather conditions at six different sites within a conifer plantation using the mark-recapture method. The suitability of marking the slugs with visible implant elastomers as well as the appropriateness of the Schnabel and Jolly-Seber models in obtaining population size estimates for these slug species in forest habitats were evaluated.
3. The microbial diversity within the faeces of *G. maculosus* was explored with the aim of determining whether the microbial signature of the faecal microbiome of *G. maculosus* could be used as a tool for distinguishing between the Irish populations. Core microbiome members were identified and the influence of diet on the microbial community as well as the possibility of a vertical transfer of gut microbes via the egg was investigated.

1.2. Literature review

1.2.1. General information about *G. maculosus*

The slug *G. maculosus* (Gastropoda: Arionidae) is one of only four species within the genus *Geomalacus* and the only one to occur outside of Iberia (Castillejo *et al*., 1994). It was discovered in 1842 beside Caragh Lake in County Kerry, Ireland (Allman, 1843; Allman, 1844; Allman, 1846), resulting in its colloquial name the ‘Kerry slug’. In 1868 the species was reported from northern Spain and in 1873 from northern Portugal (Platts & Speight, 1988), while the sighting of a single specimen in Brittany, France (Mabille, 1867) has since been dismissed as incorrect (Heynemann, 1873; Platts & Speight, 1988;

Falkner *et al*., 2002) which is likely also the case for a record of the species from the Netherlands (Bos, 1914). Within Iberia the distribution of the species stretches along the North-Western coast with the Serra da Estrela in Portugal being the most southerly (Castillejo *et al*., 1994) and Pamplona in Spain the most easterly (Castillejo, pers. com.) known area (Fig. 1). In Ireland the species is found in the south-west of the island in counties Cork and Kerry, as well as more than 200 km further north in county Galway, where it was discovered in a commercial conifer plantation in 2010 (Kearney, 2010) (Fig. 1).

Figure 1. Global distribution of *Geomalacus maculosus* (modified from NPWS, 2010).

Within Ireland *G. maculosus* generally inhabits areas with underlying old red sandstone geology. Here it can be observed on rock outcrops and tree-trunks in oak-dominated and mixed deciduous woodland as well as on sandstone outcrops or boulders on oligotrophic open moor, blanket bog and wet grassland (Platts & Speight, 1988; NPWS, 2010; McDonnell *et al*., 2013). More recently, the species has been reported from tree-trunks in conifer plantations and rock outcrops in clear-fell sites (Kearney, 2010; McDonnell & Gormally, 2011a; E. Johnston, pers. com.). In Spain and Portugal, *G. maculosus* can be found on walls and rocks near houses and gardens as well as in montane forests and chestnut-, oak- and pine-tree-groves (Rodriguez et al., 1993; Castillejo, 1994; Patrão *et al*., 2015).

It is unclear whether *G. maculosus* is originally a species of open-country or woodland (Platts & Speight, 1988) or if it is a generalist, but factors that are of high importance to the slug seem to be humid conditions and a rich cover of moss, lichen and liverworts on either rocks or tree trunks (Platts & Speight, 1988; NPWS, 2010; Reich *et al*., 2012). However, no study has ever validated any of these observations. There is also very little knowledge about the tolerance of *G. maculosus* to habitat factors such as soil pH but its absence from limestone areas suggests a preference for acidic soils.

G. maculosus is generally described as being crepuscular, but can be found feeding during the day on humid and relatively overcast days in Ireland (Platts & Speight, 1988). The species is capable of surviving cold temperatures but is usually inactive after dark in Irish winters when it seeks shelter from the cold (Platts & Speight, 1988). Again, no concrete information is available for the correlation of activity of the slug and climatic variables.

G. maculosus has been observed feeding on a broad range of lichen, liverworts and mosses growing on rocks or trees in the wild as well as on algae or fungi (Boycott & Oldham, 1930; Platts & Speight, 1988; Rosas *et al*., 1992; Rodriguez *et al*., 1993; Speight, 1996; Reich *et al*., 2012). Confirmed food plants include the liverworts *Frullania dilatata* (Taylor, 1907; Reich *et al*., 2012), *Metzgeria furcata* and *Saccogyna viticulos* (Reich *et al*., 2012), the mosses *Campylopus introflexus* and *Pleurozium schreberi* (Reich *et al*., 2012) and a range of *Cladonia* lichens as well as *Parmelia saxatilis*, *Parmotrema perlatum*, *Sphaerophorus globosus* and *Stereocaulon vesuvianum* (Reich *et al*., 2012). Vascular plants like the fern *Blechnum spicant* and heather species *Calluna vulgaris* and *Erica cinerea,* which are frequently found in *G. maculosus* habitat were not consumed when presented to the slug, as were a range of moss species (Reich *et al*., 2012), which is likely due to the large amount of phenolic compounds they produce (Davidson & Longton, 1987; Davidson *et al*., 1989). In captivity the animal can be fed on a range of vegetables including carrots, celery and lettuce, as well as mushrooms, porridge and ready-brek (Boycott & Oldham, 1930; Platts & Speight, 1988; Rosas *et al*., 1992; Rodriguez *et al*., 1993; Speight, 1996) and has also been observed to consume the snail *Vitrina pellucida* as well as other small invertebrate species that have been kept in the same container while in captivity (Taylor, 1907).

There are two distinct colour morphs of adult *G. maculosus* in Ireland, a brown body colour with yellow spots and yellow body mucus or a black body colour with white spots and clear body mucus (Fig. 2). The brown specimens are usually found in woodlands, while the black form is found in open habitats. A range of intermediate or even greyish and orangey forms can be found as well, the latter are prevailing in one site in Kerry (G. Kindermann, pers. com.) and in Santiago de Compostela and surrounding area in Spain (pers. obs.).

Juveniles display two lateral stripes along the full length of their body which become less distinct with age (Platts & Speight, 1988) (Fig. 2).

Figure 2. Left: black and brown adult specimens of *Geomalacus maculosus* (© Inga Reich, slugs from Cloosh Forest); middle: orange tinted Spanish variety (© Inga Reich, slug from Santiago de Compostela); right: juvenile specimen (© Inga Reich, slug from Glanteenassig Forest).

Slugs fulfil important roles within their ecosystems. They are a food source for a range of animals such as birds, hedgehogs, toads or beetles and serve as intermediate hosts for eggs and larvae of a range of invertebrates (e.g. South, 1992). However, extremely little is known about the predators of *G. maculosus* and while Giordani *et al*. (2014) have shown that larvae of *Tetanocera elata* (Diptera: Sciomyzidae) feed on *G. maculosus* in the laboratory, no such reports exist from the wild. Additionally, Kappes (2006) described that slugs contribute to nutrient cycling both directly by feeding on plant matter as well as indirectly by increasing microbial activity on leaf litter through the deposition of their mucus and faeces. As *G. maculosus* feeds on lichens and bryophytes, it might also be responsible for dispersing their propagules (McCarthy & Healy, 1978; Kimmerer & Young, 1995).

1.2.2. Legislation associated with *G. maculosus*

Due to its rarity and limited distribution, *G. maculosus* is listed as a protected species in Appendix II of the Bern Convention and in

Annex II and IV of the EU Habitats Directive 92/43/EC. The directive aims to protect the most vulnerable species and natural habitats across the EU by setting the standard for EU-wide nature conservation and enabling all 28 Member States to work together within the same strong legislative framework (European Union, 2014). EU Member States are required to establish designated core sites for the protection of Annex II and IV species, and consequently *G. maculosus* is listed as a feature of interest and conservation objective for seven Special Areas of Conservation (SACs) in Ireland (Table 1), three SACs in Portugal and 54 in Spain as part of the Natura 2000 network (European Union, 2014).

Table 1. Special Areas of Conservation which list *Geomalacus maculosus* as a feature of interest and conservation objective (NPWS, 2010)

Site number	Site name	County
IE0000090	Glengarriff Harbour and woodland	Cork
IE0000093	Caha mountains	Cork/Kerry
IE0000102	Sheep's Head	Cork
IE0000365	Killarney National Park, Macgillycuddy's Reeks and Caragh River catchment	Cork/Kerry
IE0000370	Lough Yganavan and Lough Nambrackdarrig	Kerry
IE0001342	Cloonee and Inchiquin Loughs, Uragh Wood	Kerry
IE0002173	Blackwater River	Kerry

Species included in Annex IV are under strict protection both inside and outside of Natura 2000 sites, making it an offence to capture, kill or deliberately disturb a species, take or destroy its eggs and damage or destroy a breeding or resting place (European Union, 2014). Under Irish legislation, the slug has further protection under the Wildlife Act 1976 (as amended) (Statutory Instrument No. 112/1990), which prohibits any wilful damage to the species but does not protect it from any indirect harm or activities licensed by other authorities (NPWS, 2010). A number of measures are in place to

ensure the long term conservation of *G. maculosus* in Ireland, including the ’Threat Response Plan – Kerry Slug *Geomalacus maculosus*’ (NPWS, 2010) and the ‘Forestry and Kerry Slug Guidelines’ (Forest Service, 2013) which provides guidelines that have to be adhered to if forestry operations are planned to be conducted in an area where a population of *G. maculosus* is likely to occur.

Geomalacus maculosus is threatened by a number of factors, most of them concerning the destruction of its living space such as the reclamation of land for agricultural use, the construction of new roads, dispersed habituation or the burning of bog (NPWS, 2010). In addition, the use of pesticides, invasive species and general forest management are impacting on the species (NPWS, 2010) and as lichens, which are one of the main food sources of the slug, are very sensitive to atmospheric pollution (Hawksworth & Rose, 1976), this too is a critical factor.

The conservation status of the Irish *G. maculosus* population was classified as favourable in 2012 but in Spain it was deemed unfavourable and nothing is known about the status of the Portuguese populations (Eionet, 2014). Climate change is also likely to impact more on the Iberian populations, as temperatures are predicted to rise (Moreno *et al*., 2005) while in Ireland, the range of *G. maculosus* is likely to expand as winters are predicted to become milder and wetter (Coll *et al*., 2012). In this light, it is especially important to protect the Irish populations of this species as they may be the stronghold of the species in future times.

1.2.3. The Lusitanian question

The term ‘Lusitanian species’ describes an organism which is typically found in the west of Ireland and northern Iberia with no intermediate populations and their disjunct distribution presents an

interesting biogeographical problem. One theory is that these species survived the Last Glacial Maximum (LGM) in an ice-free pocket located in southwest Ireland as well as in refugia in the south of Europe but disappeared elsewhere (Forbes, 1846). However, a recent study suggests that Ireland was completely covered in ice during the LGM (Clark *et al*., 2012), so it is doubtful that temperate species such as *G. maculosus* could have survived those conditions. Thus, the most likely explanation for this disjunct distribution is a post-glacial introduction of species from Iberia to Ireland which is unlikely to have occurred over land via France and Britain unless these intermediate populations subsequently became extinct. However, there has been a long trade history between Spain and Ireland (Corbet, 1961; Cunliffe, 2001) and animals and plants could have been transferred either deliberately or accidentally as cargo on trading ships (e.g. Corbet, 1961; Welter-Schultes, 2008) as has likely been the case for the heather *Erica erigena* (Foss *et al*., 1987) and the snail *Cepaea nemoralis* (Grindon & Davison, 2013). While this snail species is widespread in Europe, Irish specimens were found to belong to the same mitochondrial lineage as those from a specific region of the Eastern Pyrenees (Grindon & Davison, 2013) and the authors speculate it might have been introduced to Ireland by Mesolithic humans.

A widely employed method to investigate the origin of a species is the use of molecular markers (e.g. Pinceel *et al*., 2005a; McDonnell *et al*., 2011; Grindon & Davison, 2013). Observations of genetic variability and population structure as well as the similarity of haplotypes between regions can give insights into the population history of a species: Beatty and Provan (2013) found that the Irish populations of the Lusitanian heath species *Daboecia cantabrica* had a greatly reduced diversity at all three tested loci in comparison with its Spanish populations and only few unique haplotypes were

observed in Ireland. The same was found for the butterwort *Pinguicula grandiflora* (Beatty & Provan, 2014) and the authors conclude that these plants were post-glacially introduced from Spanish refugia into Ireland. To understand the population history of *G. maculosus*, a rangewide investigation into its phylogeographic structure is needed which could also shed light onto the origin of the Irish specimens.

1.2.4. Forestry practices in Ireland and implications for *G. maculosus* in conifer plantations

Forest cover accounts for only about 10.5 % of land usage in the Republic of Ireland (Forest Service, 2013), but is predicted to increase to 17 % by 2030 (DAFF, 1996). About 75 % of these forests are even-aged commercial plantations consisting largely of fast growing, non-native species such as Sitka spruce *Picea sitchensis* or Lodgepole pine *Pinus contorta* (Forest Service, 2013). Commercial afforestation of *G. maculosus* habitats with conifers was listed as one of the main threats to the species in Ireland (Moorkens, 2006; NPWS, 2010). The major reasoning behind this was the reduced light levels within the plantations which, at closed canopy stage, would inhibit the growth of bryophytes and lichens (NPWS, 2010) that the slug depends on for food and shelter (Reich *et al*., 2012). However, certain management practices are also likely to impact on the species. The average forest cycle in Ireland is about 40 years (Coillte 1) and general forestry operations include (re)planting, thinning, and harvesting (Coillte 2) as well as the application of herbicides, insecticides and fertilizers and the drainage of habitats which are to be planted. Planting of open habitats such as blanket bogs, in which *G. maculosus* grazes lichens from boulders, is problematic for the slug as the rocks will become covered in needles and young conifers lack an epiphyte cover on their trunks and hence offer little in terms of food and shelter for *G. maculosus*. In a survey of Cloosh Forest in

the West of Ireland, Reich *et al*. (2012) found that while the species was present in the surrounding mature plantation, it was absent from newly and recently replanted areas. Thinning usually takes place several times throughout the forest cycle and involves the removal of smaller trees (Coillte 2). However this management practice is not applied to forests which are planted on unstable soils such as blanket bog due to the increased risk of windfall (Coillte, 2009). In addition, harvesting in Ireland usually implies clear-felling of an entire stand (Government of Ireland, 2014). The environmental impacts of clear-felling include soil disturbance and compaction, a build-up of debris and alteration of the composition and abundance of litter reaching the forest floor (France, 1997). Additionally, as the epiphytes which grow on the conifer trunks are the main food sources of *G. maculosus* in plantations (Reich *et al*., 2012), the removal of the trees eliminates its substrate and resources.

As the planting of conifer plantations increases and with augmenting records of *G. maculosus* from these habitats (E. Johnston, pers. com.), further investigations into the requirements of the species in commercial plantations and its tolerance to different management practices are urgently needed. The development of a standardised method to estimate population sizes of *G. maculosus* in forest habitats is a vital first step in determining the response of the species to different forest management regimes.

1.2.5. Gut microbial communities of invertebrates

The microbial communities found within the gut of humans and animals are directly related to the health and nutrition of their hosts and their composition is strongly influenced by environmental factors such as diet or social contacts (Amato, 2013). One of the main modes of colonisation of the intestinal tract with microbes is through the environment (e.g. Engel & Moran, 2013; Newton *et al*., 2013) and

thus the microbial community observed in the faeces or gut of an organism should, at least partly, reflect that of its habitat. Hence, host populations inhabiting different sites can have distinct gut microbial communities which has been shown for a number of species such as oysters (King *et al*., 2012) and bees (Moran *et al*., 2012). Diet was found to have such a large effect on the *Drosophila* gut microbiome that samples clustered by diet rather than by host species (Chandler *et al*., 2011). However, not all microbes which are ingested successfully colonise the intestinal tract as the physicochemical conditions of the gut, such as pH or redox potential, only allow certain species to survive (Engel & Moran, 2013). In addition, the innate immune system mediates the intake of symbionts and non-symbionts (Nyholm & Graph, 2012) and there is, at least in some invertebrates such as Hydra, a selective uptake of gut symbionts (Nyholm & Graph, 2012).

Apart from habitat and diet specific microbes the gut harbours a “core microbiome”, members of which have likely co-evolved with their hosts and fulfil important functions including nutrient extraction such as cellulose degradation in termites (Warnecke *et al*., 2007) or aid with the breakdown of toxins which have been ingested with the diet (e.g. Ping *et al*., 2007; Kikuchi *et al*., 2012). These bacteria are often specialized gut symbionts and are transmitted vertically from the eggs, through coprophagy or social interactions and it was found that gut communities of social insects were usually more distinctive and consistent than those of non-social invertebrates (Engel & Moran, 2013). There are also indications that some species are deliberately choosing food items which contain byproducts of desirable bacteria to shape their own gut microbiota (e.g. *Drosophila melanogaster*, Broderick & Lemaitre, 2012).

Studies of the gut and faecal microbiome of gastropods show that these contain microbes that possess cellulolytic activity (Cardoso *et*

al., 2012; Joynson *et al*., 2014) and facilitate the host's digestion of lignocellulose (Joynson *et al*., 2014). This could account for the remarkable efficiency of terrestrial slugs and snails in breaking down plant fibre (Davidson, 1976; Charrier & Daguzan, 1980). Cardoso *et al*. (2012) show that a change in diet causes a shift in the gut microbial community of *Achatina fulica*, similar to that observed in humans and other animals, and the authors suggest that the snail gut microbiota might be able to influence the energy balance equation and affect how much energy is extracted from the diet.

With only a handful of studies investigating the gut microbiome of slugs, more research is needed to determine the influence of environment, diet and vertical transmission on the microbial community of these terrestrial molluscs. In the context of *G. maculosus* it would be especially interesting to explore whether the genetically impoverished Irish populations possess site specific microbial communities and the identification of core microbiome members could allow the investigation of the species' adaptations to its habitat.

1.3. Structure of thesis

The thesis consists of three chapters, preceded by a general introduction and followed by a general discussion.

Chapter 2 investigates the range-wide population genetics of *G. maculosus* employing the mitochondrial markers 16S rRNA and COI and the nuclear marker ITS-1.
This has been published in the Biological Journal of the Linnean Society (2015; 116 (1), 156-168).

Chapter 3 looks at the population densities of *G. maculosus* and sympatric *Lehmannia marginata* in differently managed parts of Cloosh Forest obtained with the mark-recapture method and using visible implant elastomers as markers.

Chapter 4 explores the microbial diversity within faeces samples of Irish *G. maculosus* specimens using bacterial 16S rRNA Illumina MiSeq sequences.

Chapter 2:

Genetic study reveals close link between Irish and Northern Spanish specimens of the protected Lusitanian slug *Geomalacus maculosus*

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Published in: Biological Journal of the Linnean Society, 2015, 116(1):156-168.

Vale do Zêzere (glacial valley), Serra da Estrela, Portugal (© Inga Reich).

Genetic study reveals close link between Irish and Northern Spanish specimens of the protected Lusitanian slug *Geomalacus maculosus*

2.1. Abstract

The slug *Geomalacus maculosus* is a prominent member of the Lusitanian fauna. Since its global distribution is restricted to western Ireland and northern Iberia, it is protected under EU legislation. Nothing is known about the genetic variability and population structure of this species, so, with a special view to shedding light on the origin of the Irish *G. maculosus*, tissue samples from 78 specimens were collected from 13 locations within Ireland and ten locations within Iberia and partial sequences of the mitochondrial 16S rRNA and cytochrome oxidase subunit 1 (COI) and from the nuclear internal transcribed spacer 1 region (ITS-1) were compared. The genetic diversity of the Irish *G. maculosus* was found to be greatly reduced compared to the Iberian populations, with only one (16S rRNA) and two (COI) mitochondrial haplotypes identified respectively. No private Irish ITS-1 haplotype was found. Based on the COI sequences, the Irish specimens clustered closest to Spanish specimens from Northern Asturias and Cantabria, and the bGMYC analysis identified five further Iberian clades that were highly genetically differentiated suggesting long-term allopatric divergence.

2.2. Introduction

The present-day distribution and population structure of plant and animal species has been influenced greatly by climate changes occurring during the Quaternary (Hewitt, 1999, 2000). During the cold cycles, parts of southern Europe such as the Iberian Peninsula, the

Balkans and Italy have served as refugia for temperate species and while many of these expanded their range northwards as the climate warmed, some are still only found in the southern areas. The Iberian Peninsula in particular has a large number of endemic plants and animals, populations of which are often highly structured (Gómez & Lund, 2007). It is also home to a number of species belonging to the so-called 'Lusitanian' flora and fauna which demonstrate highly disjunct distributions. While their occurrence is typically limited to south-western Ireland and northern Iberia without any intermediate populations, some species such as the Pyrenean glass snail (*Semilimax pyrenaicus*) are also found in Brittany and the Lower French Pyrenees (e.g. Praeger, 1932/1933, 1939).

Several theories have been proposed to explain this mysterious distribution. Forbes (1846) stated that the Lusitanian species survived the Last Glacial Maximum (LGM) in an ice-free pocket located in southwest Ireland and in the southern European refugia but disappeared elsewhere. However, a recent study suggests that Ireland was entirely covered by ice during the height of the LGM (Clark *et al.*, 2012). Alternative hypotheses are a post-glacial colonisation of Ireland via land-bridges from Britain (e.g. Charlesworth, 1930) and a human mediated introduction (Corbet 1961, 1962; O'Rourke, 1970). While the existence of land-bridges between Britain and Ireland after the LGM is doubtful (Edwards & Brooks, 2008), the question of why the Lusitanian species became extinct in intermediate countries but survived in Iberia and Ireland would still remain. Scenarios for the human introduction of species (either intentionally or accidentally) seem more likely, especially since there is evidence of trade between Iberia and the southwest of Ireland extending back to Mesolithic times (Corbet, 1961) and the genetic similarity of people from these areas proves that cultural links existed for more than 2,000 years (Hill *et al*., 2000).

Phylogenetics of *G. maculosus*

Recent studies by Beatty and Provan (2013, 2014) and Beatty *et al*. (2015) on four Lusitanian plant species indicate that these were most likely post-glacially re-introduced to Ireland from a southern refugium. Another study shows that even animals that are more widespread within Europe might have a cryptic 'Lusitanian element': Grindon and Davison (2013) found that most Irish specimens of the snail *Cepaea nemoralis* belong to the same mitochondrial lineage as snails from a specific region of the Eastern Pyrenees and suggest that the species might have been introduced to Ireland by Mesolithic humans more than 8,000 years ago.

We investigated the phylogeographic structure of one of the most famous Lusitanian species, the arionid slug *Geomalacus maculosus* Allman, 1843. This species occurs only in Northern Iberia and Ireland where it is protected by EU as well as national laws. Within Iberia the species' distribution stretches along the North-Western coast as far south as the Serra da Estrela in Portugal (Castillejo *et al*., 1994) and as far east as Pamplona in Spain (J. Castillejo, pers. com.) and is predominantly found in montane forests and chestnut- and oak-tree-groves as well as on different types of rock outcrops and walls (Rodriguez *et al*., 1993; Castillejo *et al.*, 1994). Within Ireland its distribution was believed to be restricted to woodland and blanket bog habitats in the southwest of the country, until the slug was recently discovered in a coniferous plantation about 200 km north of its previously known range (Kearney, 2010). However, this seems to be a recent introduction, with commercial forestry being the most likely vector (Reich *et al*., 2012).

To assess the genetic variation and population structure throughout the species' range with a view to determining whether the Irish population was introduced from Iberia after the LGM, we compared sequences from 78 specimens sampled from 13 Irish and ten Iberian localities using two mitochondrial markers (16S rRNA and COI

genes) and one nuclear marker (ITS-1 region). The implication of the results of this study to the future conservation of the species is also discussed.

2.3. Materials and Methods

2.3.1. Sampling

In June and July 2012, we collected 50 *G. maculosus* from 13 different locations within Ireland (Fig. 3a) and in April 2013, 82 specimens were sampled from ten sites in Northern Spain and Portugal (Fig. 3b).

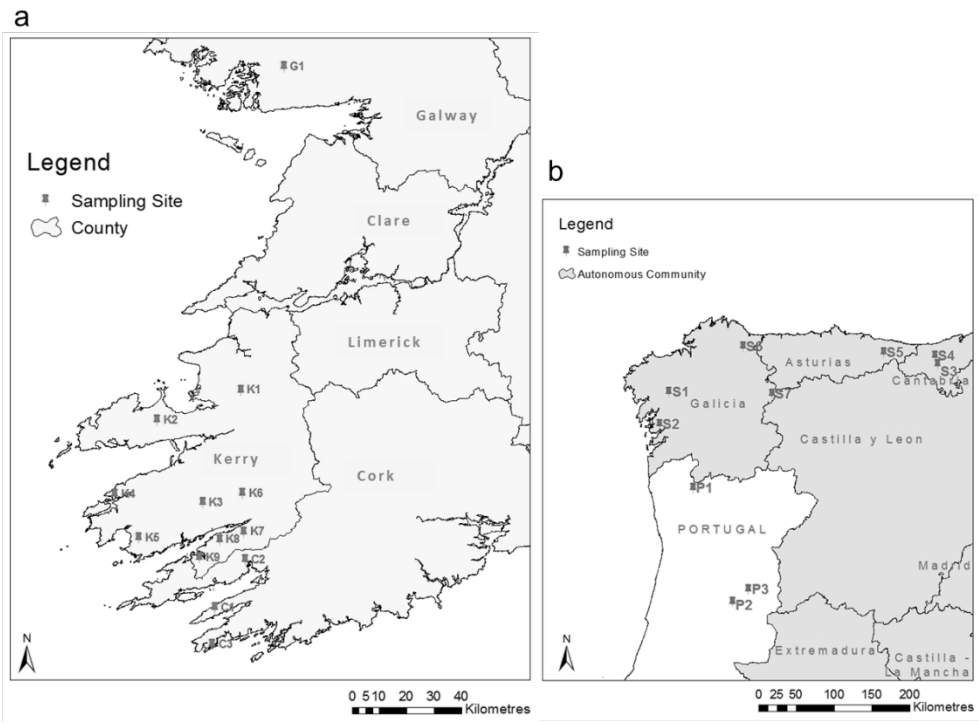

Figure 3. Sampling locations of *Geomalacus maculosus* in Ireland (**a**) and Iberia (**b**). Site identifications: **a** G1: Cloosh Forest; K1: Tursillagh; K2: Glanteenassig Forest; K3: Ballaghbeama Gap; K4: Ballycarbery; K5: Lough Currane; K6: Derrycunihy Wood; K7: Barraduff; K8: Gleninchiquin; K9: Derreen Forest; C1: Sheep's Head; C2: Glengarriff; C3: Crookhaven; **b** S1: Santiago de Compostela; S2: Armenteira River; S3: Barcena Mayor; S4:

Ucieda; S5: Covadonga; S6: Mondoñedo; S7: Serra dos Ancares; P1: Gerês; P2: Manteigas; P3: Guarda.

The specimens were collected by hand from a range of habitats, including blanket bog, coniferous and deciduous forests in Ireland while in Iberia specimens were collected from stone walls, tree trunks in deciduous forests and parks and from rock faces. A piece of tail tissue (~ 0.5 g) was snipped from the collected specimens (after McDonnell *et al*., 2011) and stored in 95 % ethanol at -20 °C.

2.3.2. DNA extraction, PCR and sequencing

DNA was extracted from the tail tissue using the DNeasy blood and tissue kit (Qiagen, Limburg, Netherlands). Polymerase chain reaction (PCR) was used to obtain a 658bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) with the universal primers LCO1490 and HCO2198 (Folmer *et al*., 1994) from 73 specimens and a 287bp fragment of the mitochondrial 16S rRNA gene from 77 specimens. For the 16S rRNA gene target, a *G. maculosus* specific primer set was developed by modifying the primers 16SAR and 16SBR (Palumbi & Benzie, 1991). Additionally, a 757bp fragment of the nuclear marker ribosomal internal transcribed spacer 1 region (ITS-1) was amplified from 54 specimens, using the universal primers ITS1L and 58C (Hillis & Dixon, 1991). 2 µl of purified DNA were added to a 48 µl PCR mixture containing 1 x PCR buffer (Sigma) (1.5 mM $MgCl_2$ included), 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 µM of each primer and 2.5 units of *Taq* polymerase (Sigma). Primer details and cycling conditions are given in Table 2. Amplified PCR products of the correct size were purified with the QIAquick PCR Purification Kit (Qiagen, Limburg, Netherlands) or with the reSource PCR Purification Kit (Source BioScience, Nottingham, UK). PCR products were submitted to a commercial facility (Source BioScience, Dublin, Ireland) for gene sequencing. Sequences were

deposited in the GenBank database under accession numbers KM102774-KM102851 (16S rRNA), KM102852-KM102905 (ITS-1) and KM102906-KM102978 (COI).

Table 2. Primers and cycling conditions used for *Geomalacus maculosus* PCR amplifications.

Marker	Primer	Primer sequence 5'-3'	Cycling conditions
COI	LCO1490[a]	GGTCAACAAATCATAAAGATATTGG	5 min @ 95 °C; (1 min @ 95 °C, 1 min @ 46 °C, 2 min @ 72 °C) x 30; 5 min @ 72 °C
	HCO2198[a]	TAAACTTCAGGGTGACCAAAAAATCA	
16S rRNA	16SfGM[b]	CCGCAGTACTTTGACTGTGC	5 min @ 95 °C; (1 min @ 95 °C, 1 min @ 55 °C, 2 min @ 72 °C) x30; 5 min @ 72 °C
	16SrGM[b]	AATTATGCTGTTATCCCTCAGGTA	
ITS-1	ITS1L[c]	TCCGTAGGTGAACCTGCGGAAGGAT	5 min @ 95 °C; (1 min @ 95 °C, 1 min @ 55 °C, 2 min @ 72 °C) x 35; 5 min @ 72 °C
	58C[c]	TGCGTTCAAGATATCGATGTTCAA	

a: Folmer *et al*., 1994; b: this study, c: Hillis & Dixon, 1991

2.3.3. Alignment and sequence analyses

All sequence chromatograms were examined visually and trimmed in CHROMAS PRO 1.7.1 (McCarthy, 1996), subsequently aligned with CLUSTAL X 2.1 (Thompson *et al*., 1997) and edited in BIOEDIT

7.2.0 (Hall, 1999). The size of the alignments used for further analyses was 628bp for COI, 255bp for 16S rRNA and 725bp for ITS-1. Haplotype diversity (h) and nucleotide diversity (π) were estimated for all markers with DNASP 5 (Librado & Rozas, 2009), which was also used to infer variable sites and to determine the amount of synonymous and non-synonymous changes at different codon positions for COI. The genetic diversity within regional populations, within the entire population and the mean interpopulation diversity as well as mean p-distance between and within regional populations were calculated in MEGA 5 (Tamura *et al*., 2011) using the p-distance method with transitions and transversions included. Substitution saturation was tested with the index of Xia *et al*. (2003, 2009) in DAMBE 5 (Xia, 2013); first, second and third codon positions were analysed separately and combined for COI. To partition the genetic variation among groups (Ireland and Iberia), among populations (S1, S2, S3, S4, S5, S6, S7, P1, P2, P3, K1-9, C1-3, G1) and within those populations, an Analysis of Molecular Variation (AMOVA; Excoffier *et al*., 1992) was carried out in ARLEQUIN 3.5 (Excoffier & Lischer, 2010) using a concatenated mitochondrial data set.

To resolve the relationships within clades, statistical parsimony networks were created with TCS 1.21 (Clement *et al*., 2000) for all markers. The connection limit was set at 95 % and gaps were treated as a fifth character state. One additional sequence from GenBank (accession number AY947384) of a *G. maculosus* specimen from Santiago de Compostela was included in this analysis.

2.3.4. Phylogenetic analyses

The optimal model of DNA substitution was estimated using JMODELTEST 2.1.4 (Posada, 2008) for all markers and for the concatenated mitochondrial data set (Table 3), *Arion lusitanicus* was

used as outgroup (GenBank accession numbers: AY947369 (16S rRNA), EF520642 (COI)).

Eighty-eight substitution models with full likelihood optimisation were evaluated under the Akaike Information Criterion corrected for small sample sizes (AICc). As AICc converges towards the AIC for larger sample sizes, it should be preferred over AIC regardless of sample size (Burnham & Anderson, 2004). Based on these models, a Bayesian analysis was carried out in MRBAYES 3.2.2 (Ronquist *et al*., 2012) for a partitioned dataset of the mitochondrial markers using a four chain Markov Chain Monte Carlo (MCMC) algorithm. Only non-identical concatenated haplotypes were used, resulting in a total of 28 sequences being used for the analysis. The two distinct Irish haplotypes were labelled IRE_1 (specimen 2 from site K8) and IRE_2 (all other Irish specimens). The analysis was run for an initial 20,000 generations, sampling every 100 generations, and then run for another 600,000 generations with the same sampling frequency, until the average standard deviation of split frequencies was below 0.01, indicating sample convergence.

Table 3. Optimal DNA substitution models under the AICc for all markers for *Geomalacus maculosus*

Marker	Model	Proportion of invariable sites	Gamma correction
16S rRNA	F81+G	/	0.135
COI	HKY+I+G	0.7	1.794
ITS-1	HKY	/	/
16S rRNA + COI	TrN+I+G	0.6630	1.086

As the p-distances between regional populations were high for the mitochondrial markers (> 0.1 in many cases), we also applied the Bayesian implementation of the generalised mixed Yule-coalescent (bGMYC) approach (Pons *et al*., 2006; Reid & Carstens, 2012) to our

COI data set. This method has been found to be a robust tool for species delimitation, even when only single-locus information is available (Fujisawa & Barraclough, 2013). To prevent the model from over-partitioning the dataset (Reid & Carstens, 2012), all identical Irish haplotypes were omitted, resulting in a total of 44 sequences being included in the analysis. An XML input file for BEAST 2.1.3 (Bouckaert *et al*. 2014) was prepared in BEAUTI 2.1.3 (Bouckaert *et al*. 2014). As the majority of variability occurred at the third codon position, the data were partitioned into codon positions ((1+2), 3), and a HKY (Hasegawa *et al*., 1985) substitution model with a gamma rate distribution (four rate categories) plus a proportion of invariant sites (see Table 3) was applied with estimated kappa and base frequencies. We used path-sampling to determine the best fitting tree and clock prior combination, which was the coalescent tree prior with exponential population growth plus a log-normal relaxed clock. The gamma shape and kappa were given a gamma prior while all other priors were left at their default settings. Two independent MCMC analyses of the data plus one of an empty alignment (to determine the effects of the priors on the results) were run in BEAST 2.1.3 (Bouckaert *et al*. 2014) for 10,000,000 generations, sampling every 1,000 generations. The results were reviewed in TRACER 1.6 (Rambaut *et al*. 2014) and then combined with LOGCOMBINER 2.1.3 (Bouckaert *et al*. 2014), removing 20 % as burnin, to produce two output files. One contained 16,002 ultrametric trees, which were subsequently reduced to a single ultrametric tree with posterior probabilities on nodes using TREEANNOTATOR 2.1.3 (Bouckaert *et al*. 2014), the other contained 100 ultrametric trees selected by subsampling the last 50 % of trees from each of the initial runs. GMYC analyses were run in R 3.0.2, using the packages ‘splits’ (Fujisawa & Barraclough, 2013), which requires a single ultrametric tree as input, and ‘bGMYC’ (Reid & Carstens, 2012), which requires as input a sample from the posterior distribution trees, thus allowing

the analyses to take uncertainty in tree topology and branch lengths into account.

BEAST 2.1.3 (Bouckaert *et al*. 2014) was also used to estimate the divergence times of the sampled populations. Results from a previous study on the closely related slug *Arion subfuscus* suggest that this species' 16Sr RNA evolved with a rate of about 5.4 % divergence per Myr (Pinceel *et al*., 2005b). Other gastropods, such as *Mandarina* land snails were found to have accelerated rates of up to 10 % divergence per Myr (Chiba, 1999), while the 16S rRNA gene of *Albinaria hippolyti* and the entire mitochondrial genome of *Partula* land snails evolved at slower rates of 1-1.2 % and 2.8 % per Myr respectively (Douris *et al.*, 1998; Murray *et al*., 1991). To allow for each of these scenarios, three different clock rates (0.01, 0.027 and 0.05 substitutions per site per Myr) were used with a strict clock and the coalescent tree prior for constant population size (which were found to be the best fit as established by path-sampling) on our 16S rRNA dataset to which we applied a HKY (Hasegawa *et al*., 1985) substitution model with a gamma rate distribution (see Table 3) and estimated kappa and base frequencies. TREEANNOTATOR 2.1.3 (Bouckaert *et al*. 2014) was used to create a single maximum clade credibility tree, and node ages as well as the 95 % highest posterior density (HPD) intervals were subsequently obtained from FIGTREE 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Results

2.4.1 Sequence analyses

Only a single 16S rRNA haplotype and two COI haplotypes were found for all sequenced Irish specimens, while 22 16S rRNA and 24 COI haplotypes were found in Iberia. For the combined Irish and

Iberian datasets, the 16S rRNA gene amplicon contained 55 variable sites, 51 of which were parsimony informative. For the COI fragment, 135 variable sites were found, 129 of them parsimony informative. Out of the variable sites, 109 were third codon positions resulting in twelve non-synonymous changes in amino-acid sequences. The 26 variable sites at the first and second positions resulted in eight non-synonymous changes. Ten haplotypes were found for the ITS-1 fragment, four of which were found in both, Ireland and Iberia. It contained a total of 17 variable sites, all parsimony informative (Table 4).

Table 4. Diversity statistics for all markers for Irish and Iberian *Geomalacus maculosus*

	16S rRNA		COI		ITS-1	
	Ireland	Iberia	Ireland	Iberia	Ireland	Iberia
N	35	42	31	42	14	40
H	1	22	2	24	4	10
h_d	0	0.959	0.065	0.966	0.396	0.906
π	0	0.061	0.0001	0.079	0.002	0.005
k	0	19.62	0.065	49.33	1.35	3.578
N_{vs}/l	0/255	54/252	1/628	132/628	4/717	17/717

N: number of individuals, H: number of haplotypes, h_d: haplotype diversity, π: nucleotide diversity, k: average number of nucleotide differences, N_{vs}: amount of variable sites, l: sequence length excluding gaps and missing data.

The mean p-distance between specimens sampled from Ireland was smallest compared to specimens obtained from site S3 for 16S rRNA (0.037), and to specimens from site S4 for COI (0.025); for ITS-1 the mean p-distance was smallest at both these sites (0.004). Within Iberia, the smallest p-distances found for 16S rRNA and COI were between populations sampled from sites P2 and P3 (0.012 and 0.008 respectively) and for ITS-1 between populations from sites S3 and S4 (0.000). Large mean p-distances were observed between

individuals sampled from site S7 and all other sites except P2 and P3 for 16S rRNA (> 0.105) and between specimens from site S6 and sites S1, S2, S7, P2 and P3 for COI (> 0.110). While mean p-distance was generally ≤ 0.005 for ITS-1, individuals sampled from site P2 had a higher mean p-distance than all other sites (≥ 0.016). Mean p-distances within the regional populations were largest at site S1 (0.052 for 16S rRNA, 0.037 for COI) and site S7 (0.014 for 16S rRNA and 0.047 for COI) and smallest at sites P1, P2, P3 and Ireland for 16S rRNA (0.000) and at site P3 for COI (0.000) (Appendix 1).

The results of the AMOVA show that the majority of the variation (significant at $P < 0.05$) is caused by differences among and within populations. The variation between Iberian and Irish groups is low and non-significant (Table 5).

Table 5. Results of the Analysis of Molecular Variance (AMOVA) from the concatenated mitochondrial data set (16S rRNA + COI) of *Geomalacus maculosus* (n = 73)

Source of Variation	d.f.	Variance components	Variation (%)	ϕ-Statistic	*P*-value
Among groups	1	$\sigma^2_a = 5.35956$	6.94	$\phi_{CT} = 0.06936$	0.169
Among populations	12	$\sigma^2_b = 66.10088$	85.54	$\phi_{SC} = 0.91918$	<0.0001
Within populations	72	$\sigma^2_c = 5.81182$	7.52	$\phi_{ST} = 0.92479$	<0.0001

d.f.: degrees of freedom, σ^2_a: variance among groups, σ^2_b: variance among populations, σ^2_c: variance within populations, ϕ_{CT}: differentiation among groups, ϕ_{SC}: differentiation among populations, ϕ_{ST}: differentiation within populations.

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Due to the large numbers of mutational steps between clades, no single haplotype network could be created using the 95 % connection limit for both the 16S rRNA and the COI gene, so the limit was increased to 19 steps (16S rRNA, not shown) and 55 steps respectively (COI, not shown), at which point all haplotypes were connected. The majority of haplotypes are grouped in the same way for COI and 16S rRNA, with specimens from Ireland being closest to the 'North Asturias/Cantabria' cluster (specimens from sites S3, S4 and S5). Other clusters that can be found for both markers are the 'West Galicia/North Portugal' cluster (specimens from sites P1, S2 and specimen S1-3) and the 'East Portugal/Galicia' cluster (specimens from P2, P3 and S7). Slugs from sites S1 (except for one specimen clustering with specimens from site S2) and S6 are not clustering with any other group.

Ten haplotypes were found for the ITS-1 gene, four of them shared between Ireland and Iberia. ITS-1 haplotype 'A' was most frequently found in Irish *G. maculosus* (11 specimens) but one specimen with this haplotype was also found in site S2 and one in site S7. Other haplotypes found in Ireland were haplotype D (also found at sites S3 and S4), G (also found at site S7) and H (prevalent in Portuguese populations from sites P1 and P3). Haplotype I+J were exclusively found at site P2 and were also the ones furthest removed from central haplotype G (Fig. 4).

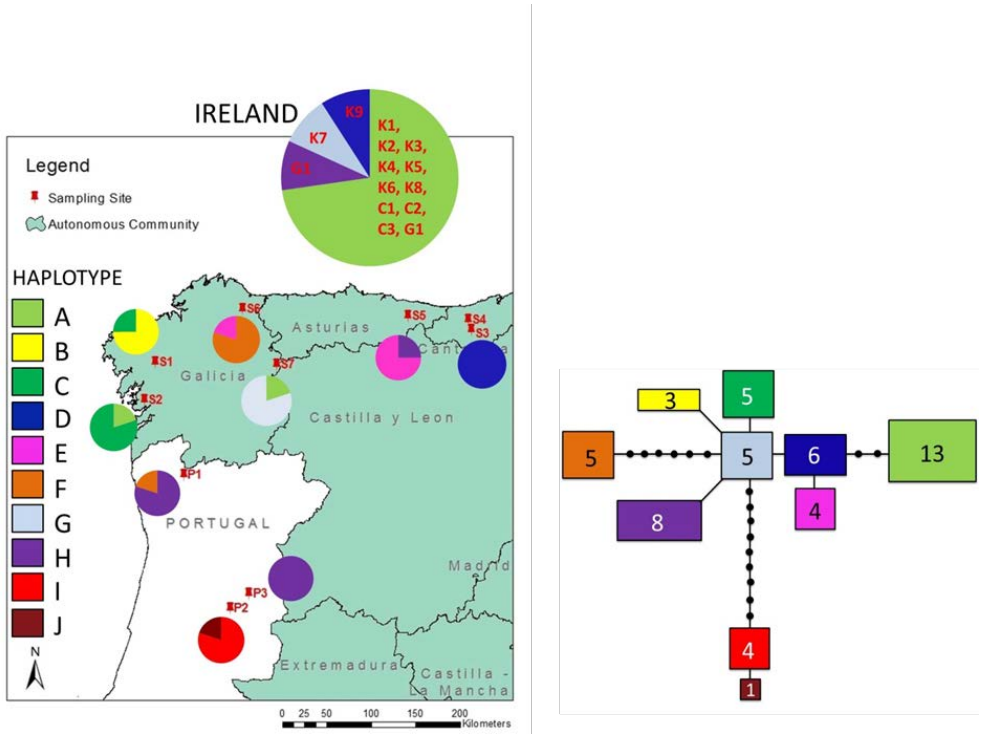

Figure 4. Distribution of ITS-1 haplotypes (left) and ITS-1 haplotype network (right, connection limit: 95 %) for *Geomalacus maculosus*. Dots represent missing haplotypes and numbers indicate the number of specimens per haplotype.

2.4.2 Phylogenetic analyses

The bGMYC analysis identifies six clades with high species level probabilities and Ireland clusters monophyletically with specimens from sites S3, S4 and S5, as well as with one specimen from site S7 (Fig. 5). This could not be observed in our analysis of the concatenated mitochondrial dataset, where Ireland clusters with specimens from site S6, however, this clade is very poorly supported (posterior probability 0.55; Fig. 6). The 'West Galicia/North Portugal', Santiago de Compostela, North Asturias, North Galicia and Ireland clades are strongly supported (posterior probability values > 0.95) in both analyses, as is the 'East Portugal/Galicia' clade, but this excludes specimen S7-6 in Figure 6 (posterior probability for the

entire clade is 0.66). The North Cantabria clade is not supported in the analysis of the concatenated mitochondrial dataset.

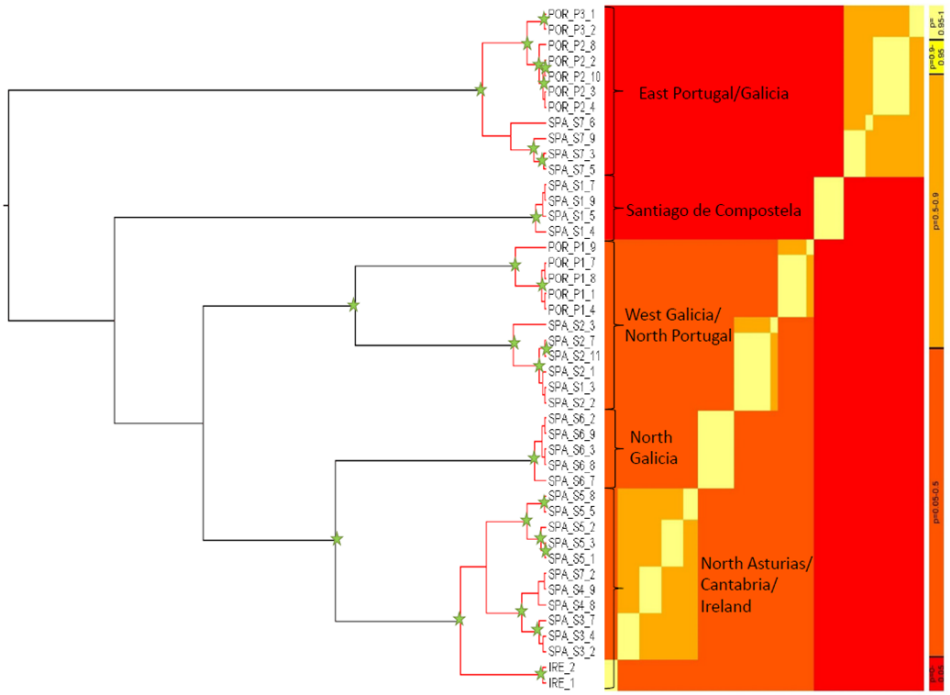

Figure 5. Maximum clade credibility COI gene tree for *Geomalacus maculosus* (left, from BEAST analysis). Stars indicate nodes with posterior probability > 0.95. Red highlighted clades represent the Maximum Likelihood (ML) threshold of species limits while the sequence by sequence matrix heat-map (right, from bGMYC analysis) shows the posterior probability that corresponding sequences are conspecific, with high probabilities coloured bright yellow ($1 \geq p > 0.95$).

The results from the divergence time analysis show that node ages for the most recent common ancestor (MRCA) of the Irish population range from 430,000 years under a slow rate (2 % per Myr) over 160,000 years (5.4 % per Myr) to 90,000 years with an accelerated rate (10 % per Myr), while the time that the Irish population coalesce with the closest sampled ancestor is either around 2.2 Myr, 810,000 years or 430,000 years ago. The mean age of the terminal node between the MRCA of populations from sites S7, P2 and P3 and the

MRCA of all other populations is estimated to be between 13.6 million (slow rate) and 2.7 million years (fast rate) (Appendix 2).

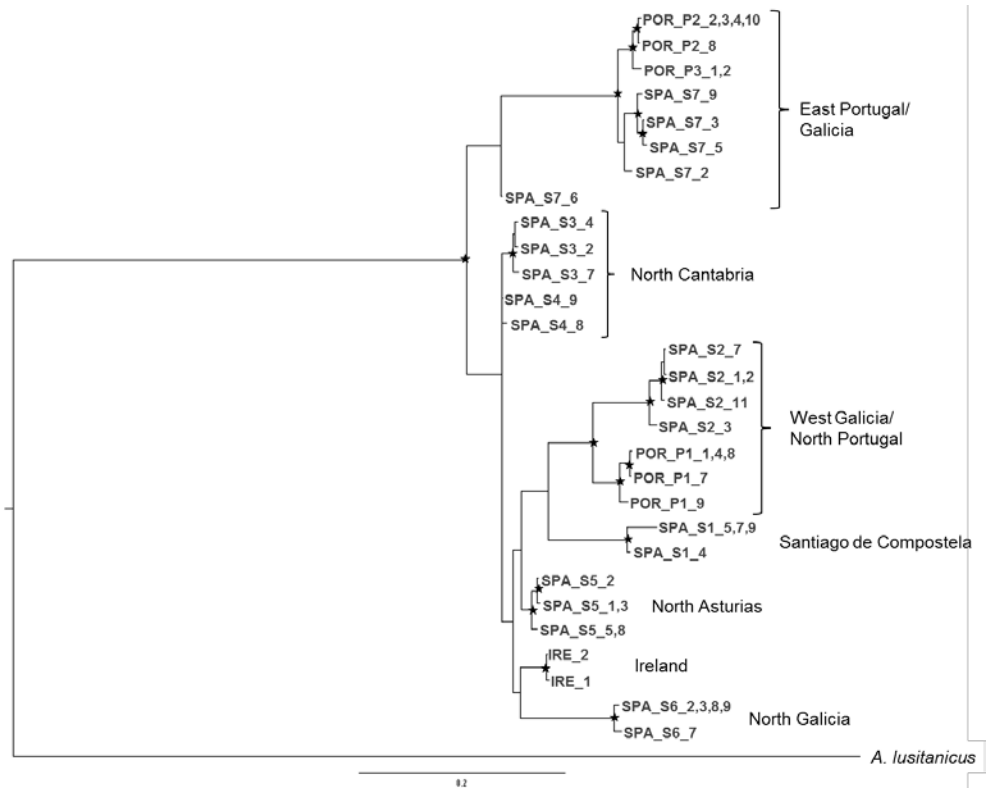

Figure 6. 16S rRNA + COI tree for *Geomalacus maculosus* from MRBAYES analysis. Stars indicate nodes with posterior probability > 0.95.

2.5. Discussion

2.5.1 The Iberian population of *G. maculosus*

There was high genetic differentiation among regional populations for both 16S rRNA and COI in Iberia, usually exhibiting low levels of within-group diversity (mean diversity within regional populations = 0.01 for both markers). Combined with the high mean diversity between some Iberian populations (Appendix 1) and the highly significant ϕ_{ST} value (Table 5), these results suggest long-term

allopatric divergence with restricted gene flow between populations. This might be due to the low dispersal abilities and patchy population structures of pulmonate gastropods, which would facilitate the maintenance of genetic differences among the groups (Thomaz *et al*., 1996). The results from the bGMYC analysis suggest that the genetic divergence between some groups might be so large that they could in fact be recognised as different species (Fig. 5). However, as extreme intraspecific mitochondrial DNA differences are not unusual in pulmonate gastropods (Pinceel *et al*., 2005b, Quinteiro *et al*., 2005), analyses with a range of other genes as well as morphological comparisons would be necessary to determine whether speciation has already taken place between some populations. The observed deep divergences are most likely consequences of cyclic population events such as dispersion and extinction that took place due to climatic oscillations (Hewitt, 1999, 2000, 2001). Although it is not known where in Iberia this species survived during the ice ages, the current distribution of *G. maculosus* indicates that during the warming phases southern Iberia was an unlikely refugium for this species. So if the slug occurred further south during the cold cycles, specimens likely migrated north during the warming cycles. Hewitt (1996) also suggested that populations in southern refugia could have survived through several glacial cycles by moving up and down mountains. Six of our Iberian sampling sites were located within major mountain ranges with elevations of more than 1,500 meters (Central Cantabrian Mountains (sites S3, S4 and S5), Serra dos Ancares (site S7), Serra do Gerês (site P1) and Serra da Estrela (sites P2 and P3)), many peaks of which were covered with ice sheets during the Pleistocene (Enamorado, 1997). At these sites it is likely that *G. maculosus* would have only survived in the valleys during the ice ages. As a result the populations may have been segregated by the mountains which consequently facilitated allopatric divergence. The divergence time analysis suggests that even if the 16S rRNA evolved

with a fast rate of 10 % per million years, the populations from sites P2, P3 and S7 have been separated from the other populations since the Pliocene more than 2.5 million years ago (Appendix 2). While sites P2 and P3 are located south of the river Douro, which is likely to have acted as a natural barrier for gene flow, site S7 is located north of the river and more than 250 km away from these two sites. The close genetic similarity of these populations is hence easiest explained by a secondary introduction event from the Serra da Estrela to the Serra dos Ancares (the divergence time analysis suggests a separation of these populations of between 300,000 to 1.52 Myr ago) but sampling of intermediate populations would be necessary to confirm this theory.

The results of the ITS-1 gene analysis provide conflicting results, as the populations are not clustering in the same way as the mitochondrial genes, and several haplotypes are shared between populations (Fig. 4). While most of the haplotypes only differ by one or two nucleotides, the haplotypes F, I and J are clearly set apart. Haplotype F is found within two populations (S6 and P1), but haplotypes I and J are unique to site P2 and differ by ten/eleven nucleotides from haplotype allele H, found in nearby site P3. In contrast, the mitochondrial haplotypes of specimens from sites P2 and P3 were very similar. This discrepancy could be due to unsampled allele variation of intra-individual multiple ITS-1 copies, or the small effective population size of the mitochondrial genome and its fast mutation rate, resulting in rapid mtDNA lineage sorting (Neigel & Avise, 1986), compared to the rather slow mutation rate of the nuclear ITS-1 gene. Hence the lineage sorting of the ITS-1 gene might be incomplete and the haplotype distribution could reflect more ancient distributions in which populations might have mixed more freely. Human assisted dispersal, as observed in Ireland recently (Kearney, 2010; Reich *et al.*, 2012), could have also contributed to

the spread of slugs and/or their eggs to previously uninhabited areas or areas inhabited by established populations, leading to a mixing of gene-pools.

2.5.2. The Irish population of *G. maculosus*

The data presented here show that the Irish population of *G. maculosus* has a greatly reduced genetic diversity compared to the Iberian populations, especially within the mitochondrial genes, where only one (16S rRNA) or two haplotypes (COI; one of which was due to one individual that differed by a single nucleotide from the others) were found (Table 4). Shared ITS-1 haplotypes of Irish and Iberian specimens indicate a recent common ancestor and the AMOVA results show that variation among populations is larger than among groups for the tested mitochondrial markers 16S rRNA and COI (Table 5). This suggests that the reduced diversity in Ireland is the result of a founder effect as opposed to a past bottleneck in a putative native Irish population that might have occurred during the last ice age. Beatty and Provan (2013) found similar results while investigating the distribution of the Lusitanian heather *Daboecia cantabrica*, proposing a leading-edge colonisation scenario. In addition, phylogenetic relationships monophyletically grouped the Irish haplotypes with those sampled from Cantabria and Northern Asturias (S3, S4 and S5, Fig. 5), which exhibit lower p-distance in respect to the Irish specimens than to some of the Iberian specimens (Appendix 1). This also highlights the close link between these Irish and Iberian populations. However, the 16S rRNA and COI haplotypes we found in Ireland were not recorded in any of the Iberian populations that we sampled. As the divergence time analysis suggests that the age of the Irish cluster might be between 430,000 and 90,000 years, the mitochondrial haplotype of the Irish population should still be found in its Iberian source population, if the species was introduced after the LGM. We propose that this unsampled

source is likely located along the Spanish North Atlantic Coast, as specimens sampled from sites S3, S4 and S5 grouped very closely to the Irish specimens.

The ITS-1 haplotype A that was observed most frequently in Ireland (in 78.6 % of sequenced specimens) was found in only 5 % of the 40 sequenced Iberian specimens, one from site S2 (West Galicia) and one from site S7 (East Galicia) (Fig. 4). The other three haplotypes present in Ireland (D, G & H) were found in a variety of sites (S3, S4, S7, S5, P1 and P3). This also suggests that the putative source location for Ireland might be yet unsampled, as in none of the above mentioned sites do all of these haplotypes occur together.

2.5.3. Possible ways of introduction

A known pathway of introduction for gastropods is their deliberate introduction as a food source as was discussed for *C. nemoralis* (Grindon & Davison, 2013) but this is unlikely to be the case for *G. maculosus* as there are no records of it ever being consumed. It is more likely that individual slugs were introduced to Ireland as 'blind passengers' on cargo-ships as has been the case for other gastropods. Snail shells have been found in a late bronze age ship wreck in southern Turkey and Welter-Schultes (2008) suggests that these land snail species were brought accidentally on board attached to the packaging material (e.g. bushes) of breakable goods, in which they were able to survive the long journeys on the boat. Equally, fragile goods such as amphorae of wine, that were transported from the ports of Bilbao and Santander in Spain to Ireland during the middle ages (Cunliffe, 2001) were probably also protected by layers of heath or other vegetation which may have contained *G. maculosus* specimens and/or eggs.

There is still uncertainty about what limits the distribution of *G. maculosus*, e.g. why there are no records of the species elsewhere in

Europe except for a most likely erroneous (Heynemann, 1873; Platts & Speight, 1988; Falkner *et al*., 2002) report from Brittany in France (Mabille, 1867). Unravelling this question will aid in determining whether the species is absent elsewhere because of unsuitable living conditions, or whether its current distribution is solely because it has not spread from Iberia into the rest of Europe. Interestingly, the three other known *Geomalacus* species are endemic to Iberia, found only in central (*G. oliveirae*) and southern (*G. anguiformes, G. malagensis*) Portugal and Spain as well as Gibraltar (*G. malagensis*) (Castillejo *et al.*, 1994).

2.5.4. Implications

As *G. maculosus* is an EU-protected species, Ireland, Spain and Portugal are legally bound to ensure its conservation. The observed low genetic variability of the Irish specimens might prove to be a threat to the survival of this population because of its subsequent inability to adapt to environmental changes. While there is currently no evidence of a decline of *G. maculosus* in Ireland and a report on the predicted impact of climate change on this species in Ireland found that it is likely to be positive for the slug (Sweeney *et al.*, 2006), habitat management in areas inhabited by this species must take its vulnerability into account. In this context, the Iberian population will play a critical role in protecting the genetic diversity of this species.

2.5.5. Conclusions and future work

While we cannot pin-point the exact origin of the Irish *G. maculosus* population, our results show that there are strong links to Spain, especially to specimens sampled from Northern Asturias and Cantabria. The extremely low genetic diversity of the Irish specimens suggests an introduction by only a few founding individuals, which was probably accidental and human assisted. The Iberian populations of *G. maculosus* are highly structured, which is typical for

species with low dispersal abilities in mountainous habitats, and the large mitochondrial sequence divergences of often more than 10 % between populations indicate long-term allopatric divergence.

Future research should focus on determining whether these demes are reproductively isolated. Additionally, morphological and behavioural studies of specimens from different populations and habitats should be carried out to investigate adaptation and speciation processes. The identification of ecophysiological factors that limit the distribution of *G. maculosus* will also be important, as this will inform effective habitat management for conserving this species. Furthermore, sampling of slug populations from additional locations within Iberia will be essential to discover the Irish source population.

Chapter 3:

EU-protected slug *Geomalacus maculosus* and the sympatric *Lehmannia marginata* in commercial forests: What can the mark-recapture method tell us about their population densities?

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View of the partial clear-fell site in Cloosh Forest, County Galway (© Inga Reich).

EU-protected slug *Geomalacus maculosus* and the sympatric *Lehmannia marginata* in commercial forests: What can the mark-recapture method tell us about their population densities?

3.1. Abstract

Geomalacus maculosus Allman is an EU protected slug species which is only found in the West of Ireland and Northern Iberia. There is little knowledge of its population sizes throughout its range and no long term studies have been conducted to calculate estimates. Localised populations of *G. maculosus* and the sympatric slug *Lehmannia marginata* Müller at five different sites within a commercial conifer plantation in Ireland were monitored for one year with the mark-recapture approach using visible implant elastomers, which were injected into the foot as a marker. Population size estimates were calculated using the Schnabel and Jolly-Seber methods and densities of up to 23.1/ m^2 and 5.2/ m^2 (Schnabel) and 37.2/ m^2 and 5.3/ m^2 (Jolly-Seber) for *G. maculosus* and *L. marginata* respectively were observed but these varied greatly throughout different areas of the plantation and throughout the year. Temperature was correlated with capture success, with fewer captures throughout the colder months. This is the first long-term study that uses visible implant elastomers as markers on slugs and we evaluate their use, provide guidelines for trapping intensity and advise on the optimal survey time and conditions.

3.2. Introduction

The abundance of a species in its habitat depends on a variety of factors such as abiotic conditions or the presence/absence of food plants, prey and predators as well as other species competing for the same resources. Knowing the population densities of a species within a range of sites can help to determine the key factors

responsible for maintaining robust populations. This is especially important for the conservation of endangered and protected species and finding a reliable protocol for determining population sizes is crucial for their assessment: Direct counts are suitable for some species, but many organisms are easily overlooked (Greenwood, 1996), making this an inadequate approach especially for small and very mobile animals. Trapping and subsequent mark-recapture techniques are likely to be more suited to these species and provide a continuous assessment of a population throughout a certain time period. However, mark-recapture studies are time intensive and their accuracy is based on a range of assumptions about the studied population (Krebs, 1999).

The suitability of mark-recapture studies has been explored as a measure to provide population size estimates for the red-listed terrestrial snail *Prestonella bowkeri*, (Janks & Barker, 2013) but research on slugs has usually focused on the population dynamics of ground dwelling pest species (e.g. Grimm *et al*., 2000; Grimm & Paill, 2001; Ryser *et al*., 2011; Knop *et al*., 2013), and only one study to date (McDonnell & Gormally, 2011a) has used this method to investigate mobility and population density of the EU-protected slug *Geomalacus maculosus* Allman. However, their study only covers periods of two and three months and consequently does not provide data on seasonal fluctuations in populations. Our study is the first year-long mark-recapture investigation undertaken for *G. maculosus*, and the sympatric *Lehmannia marginata* Müller, essential for determining the influence of seasonality on trapping success and for finding the optimum time for future monitoring of these species. It is also the first long-term study that uses visible implant elastomers (Northwest Marine Technology, Shaw Island, Washington, USA) on slugs and we evaluate the longevity and practicality of these markers. While visible implant elastomers have been shown to be an effective marking method for slugs (Wallin & Latty, 2008; McDonnell

& Gormally, 2011a), they have never been tested for a period longer than three months.

Geomalacus maculosus is a Lusitanian slug species, which only inhabits the West of Ireland and Northern Iberia. In Ireland this species has been generally found in deciduous, often oak dominated woodland, blanket bog or unimproved oligotrophic open moor in the south-west of Ireland (Platts & Speight, 1988; NPWS, 2010; McDonnell *et al*., 2013), while in Iberia, it is most frequently associated with montane forests and chestnut- and oak-tree groves as well as on stone walls an rocks near houses and gardens (Rodriguez *et al*., 1993; Castillejo *et al*., 1994; Patrão *et al*., 2015), where it feeds predominantly on lichens, liverworts, fungi and algae (Boycott & Oldham, 1930; Platts & Speight, 1988; Rodriguez *et al*., 1993; Speight, 1996; Reich *et al*., 2012). Only recently has this species been recorded from commercial conifer plantations in Ireland (Kearney, 2010; McDonnell & Gormally, 2011a), which was surprising given that this habitat would have previously been considered unsuitable for *G. maculosus* (NPWS, 2010). Even more surprising was the recent discovery of the species in a commercial conifer plantation in western Ireland (Cloosh Forest) 200 km north of its previously known distribution area (Kearney, 2010). This brings into question the potential role of commercial forestry practices in aiding the spread *G. maculosus* in Ireland (Reich *et al*., 2015). Given the inclusion of *G. maculosus* in Annex II and IV of the Habitats Directive, commercial forestry management practices in plantations where the species occurs, must now take the protection of the species into account. With conifer plantations already accounting for over 90 % of Ireland's forested area and commercial afforestation likely to increase in the future (DAFF, 1996), this is especially important as plantation forests, if managed properly, could serve as valuable habitats for the species. However, there is currently a dearth of information on the population densities of *G. maculosus* in its

associated habitats including conifer plantations and the provision of these data is a crucial step in effectively conserving the species. This is especially critical in the context of climate change where predicted temperature rises (Moreno *et al*., 2005) could threaten Iberian *G. maculosus* populations while in Ireland, the range of *G. maculosus* is likely to expand with predicted milder and wetter winters (Coll *et al*., 2012).

The slug *L. marginata* is frequently found in the same habitats as *G. maculosus*, inhabiting woodlands but also open areas where it is found on stone walls or other rocky surfaces (Rowson *et al*., 2014). In a survey of Irish conifer plantations from which *G. maculosus* was found to be absent, *L. marginata* was present in all sites, and was usually the dominant slug species (Reich *et al*., 2012). However, in certain parts of Cloosh Forest where *G. maculosus* is present, *L. marginata* abundances were less than those of *G. maculosus* (Reich *et al*., 2012). This is especially interesting, as *G. maculosus* was likely accidentally introduced into Ireland from Spain (Reich *et al*., 2015) with the possibility that it could compete with 'native' slugs occupying a similar niche. Since *L. marginata* and *G. maculosus* both feed on bryophytes and lichens (Rowson *et al*., 2014) and shelter below bark or moss on tree trunks or rocks (Reich *et al*., 2012), *L. marginata* was included in this study to investigate how the abundances of both species relate to each other.

The aims of this study were to:

- Assess the suitability of the mark-recapture method using visible implant elastomers for the two slug species
- Estimate population sizes of *G. maculosus* and *L. marginata* from a range of management compartments within a commercial conifer plantation
- Relate capture success to seasonality and weather conditions

Population densities of *G. maculosus* and *L. marginata*

3.3. Materials and Methods

3.3.1. Study area

Our study area is located within Cloosh Forest, County Galway, in the west of Ireland (Fig. 7a). This was the first conifer plantation from which *G. maculosus* had been reported (Kearney, 2010) and to date its presence has only been confirmed in small areas of this 7,000 hectare property, including the Lettercraffroe compartment (Kearney, 2010; Reich *et al*., 2012; McDonnell *et al*., 2013), where this study was undertaken (Fig. 7b). This compartment consists mostly of mature *Picea sitchensis* and *Pinus contorta*, planted on blanket peat mainly in the 1960s and 1970s (Coillte, 2009) and interspersed with clear-felled areas.

3.3.2. Experimental design

Our study was undertaken in two different periods between August 2012 and August 2014 during which five different sites were surveyed (Fig. 7b). The first part of the study was used as a pilot to test the general methodology and was undertaken from August 2012 to July 2013 at site P1, located in a mature plantation plot. As the marking procedure and setup were successful (with sufficiently large numbers of *G. maculosus* specimens captured and re-captured to calculate reliable population size estimates), the same methodology was used again from September 2013 until August 2014 at four additional sites, two of which (P2 and P3) were located within planted forestry (Fig. 8a) while two (C1 and C2) (Fig. 8b-d) were located in clear-felled areas (Table 6). While the sites were close to each other, they differed in a range of factors: site P2 is located about 50 meters inside the plantation and site P3 is located near the edge of a plot which is surrounded by paths on two sides and experiences higher light levels than site P2 as the edge trees are missing outer branches. Since *G. maculosus* was discovered at site C1 prior to

scheduled felling operations in summer 2011, forest managers conducted a partial clear-fell (Fig. 8b) at this site. This involved retaining three meter high (approx.) tree trunks during felling in an attempt to mitigate the immediate negative impacts of conventional clear-felling on the species i.e. felling trees at the base of the tree trunk and stripping off side branches. Site C2 is located in a conventional clear-fell in which trees were cut at the base in the standard fashion (Fig. 8c).

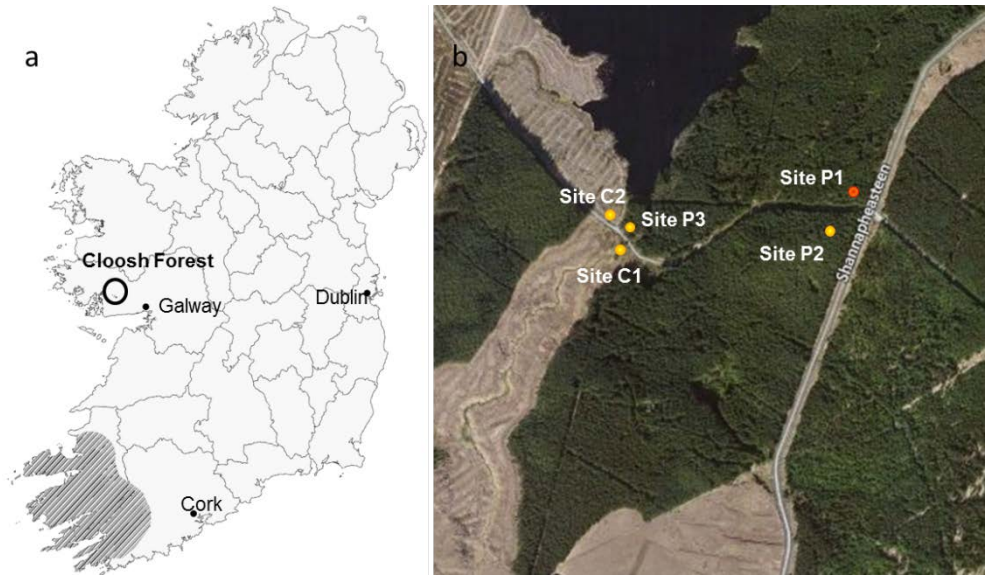

Figure 7. (**a**) The Irish distribution area of *Geomalacus maculosus* (shaded) and the location of Cloosh Forest (encircled) (map modified from G. Kindermann); (**b**) Aerial photograph (© Bing Maps) showing the location of the mark-recapture sites. The orange dot indicates the site that was sampled from August 2012 to July 2013, the yellow dots indicate sites sampled from September 2013 to August 2014.

To quantify the differences between the plantation sites P1, P2 and P3, the circumference at breast height (CBH) and the thickness of the epiphyte layer were measured and overall bark structure as well as epiphyte cover were estimated at each tree (Appendix 3). Bark structure was chosen as a measurement of the availability of crevices (shelter for the slugs) on the trunk and was divided into

three categories (smooth, some cracks/flaking bark and many cracks/flaking bark). Epiphyte cover was measured as a percentage of the visible trunk (ground to 4m height approx.) that was covered with moss, lichen, liverworts etc. and was also split into three categories (< 30 %, 30 – 60 %, > 60 %) while the thickness of the epiphyte layer was measured with a ruler just above the ground and just above the traps, averaged and assigned to one of three categories (< 0.5 cm, 0.5 cm - 1 cm, > 1cm) (Appendix 3).

Table 6. Summary of the sites used in this study

Site	Type	Sampling period	Plant/fell* year	Main tree species
P1	Conifer plantation	2012/13	1960	*P. sitchensis*
P2	Conifer plantation	2013/14	1960	*P. sitchensis*
P3	Conifer plantation	2013/14	1960	*P. sitchensis*
C1	Clear-fell (partial)	2013/14	2011*	*P. sitchensis* + *P. contorta*
C2	Clear-fell (conventional)	2013/14	2008*	*P. sitchensis*

3.3.3 Methodology

Setup and marking

Refuge traps (*De Sangosse*, Pont du Casse, France) were installed two weeks before the start of the survey and left in place for the duration of the year. The traps consist of a 50 x 50 cm piece of absorbent material, covered with a reflective upper surface and a perforated plastic underside and had been previously shown to be an effective method for catching *G. maculosus* (McDonnell & Gormally,

2011b) and *L. marginata* (Reich *et al*., 2012). Nine adjacent trees that were standing in a rough square (measuring between 6 m^2 (site P3) and 50 m^2 (site P2)) were chosen at each site and the refuge traps were installed on each tree, covering the entire circumference of the trunk at breast height (Fig. 8a). Depending on the circumference of the trunk one to four traps per tree were used. In the conventional clear-fell, a single trap was nailed to the top of a tree stump (Fig. 8d) as these were covered with a denser layer of epiphytes than the sides of the stumps. To adjust for unequal tree circumferences within the sites, population density was calculated by dividing the population size estimate by the area of the traps (i.e. tree circumference x trap width (50 cm)) of all nine trees in every site.

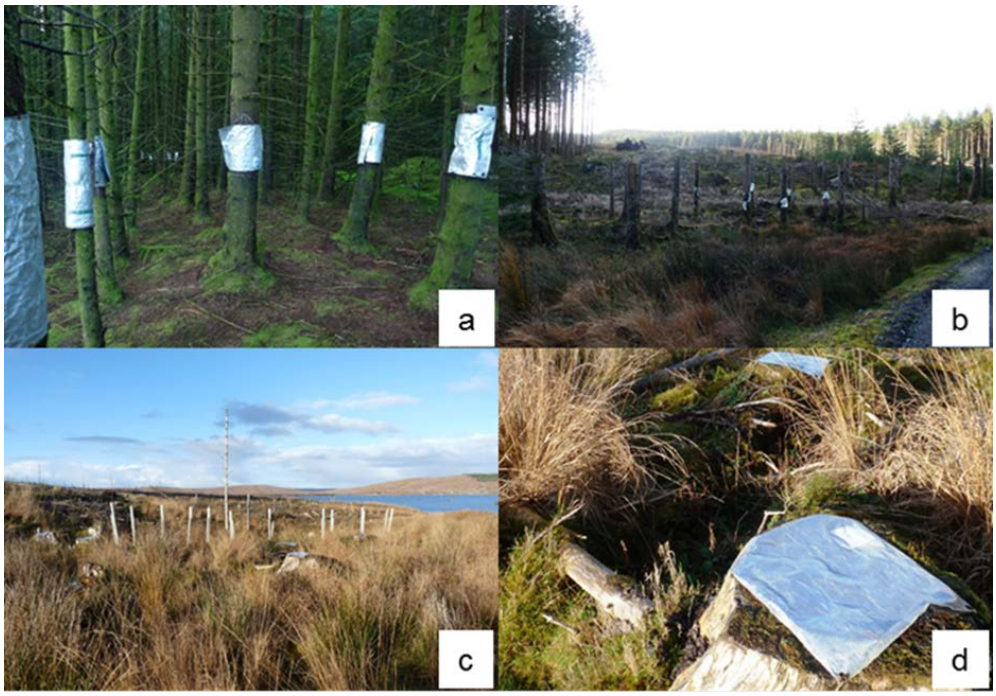

Figure 8. Refuge traps installed in the plantation site P1 (**a**), the partial clear-fell site C1 (**b**) and the conventional clear-fell site C2 (**c**). **d** shows a close-up of a tree-stump trap in site C2.

Traps were not baited to reduce possible 'trap-happiness', meaning that animals which encountered the trap once are likely to return and

be caught over and over again. This would violate the assumption of mark-recapture models that the proportion of marked animals below the traps corresponds to the proportion of marked animals in the wild (Greenwood, 1996) and result in the underestimation of population sizes. A robust design (Pollock, 1982; Pollock *et al*., 1990) was used, consisting of primary and secondary periods. Ten primary periods were chosen (once per month), made up of five consecutive days as the secondary periods. On the first day of each sampling period, the number of slugs under each trap was recorded and the slugs were injected with a visible implant elastomer just below the surface of their foot (Fig. 9) using a 1 ml syringe. To prolong the usability of the elastomer on a sampling day, we did not use the supplied curing agent (McDonnell & Gormally, 2011a). Slugs were split up into three age categories: adults (> 2 cm length; Oldham, 1942), sub-adults (≤ 2 cm length) or juveniles (< 0.5 cm length). While the latter were too small to be effectively marked, they were still counted to compare when the juveniles of each species were present over the year. Before placing the slugs back underneath the traps from which they had been removed, tags were checked to ensure they were clearly visible, and if necessary, additional dye was injected. Due to the elastomer moving slightly over time within the foot of the slug, individual animals could not be distinguished by their marks. On the subsequent four days, marked and unmarked slugs were counted separately and unmarked slugs were injected with the relevant colour for each month. All slugs were subsequently placed back beneath the trap from which they had been removed. A different coloured elastomer was used in each primary sampling period and at each capture it was noted whether:

- The slug had been marked previously during this period;
- Colour tags from previous periods were present and if so, which colours;

- The slug was an adult or sub-adult specimen.

As there were only ten colours of elastomer available, data were collected for ten months of each year. No data were collected in February because slug numbers were likely to be small due to low temperatures and in April due to the absence of the recorders in this month in 2013. For consistency, these months were not surveyed in 2014 either.

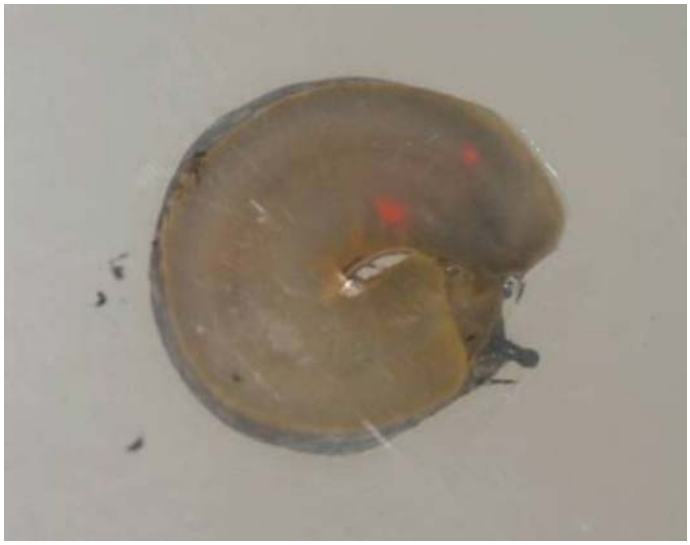

Figure 9. *Geomalacus maculosus* marked with a red elastomer in two places on its foot (© Rory McDonnell).

Population size estimation

The population sizes at each of the sites were estimated using the Jolly-Seber method as well as the Schnabel method (Appendix 4). While the latter implies that the population in question is closed (e.g. no immigration, emigration, births or deaths), the Jolly-Seber model assumes a, generally more realistic, open population scenario in which the number of animals varies over time (Krebs, 1999). Other underlying assumptions for both methods include: (1) Marking of animals does not affect their survival or probability of capture; (2) marks are not lost between sampling periods and are recorded at

each sampling event; (3) all animals (e.g. males and females or different life stages) have the same chance of getting caught; (4) there is no trap response. McDonnell and Gormally (2011a) and Wallin and Latty (2008) found that the elastomer injection of non-anaesthetized slugs did not result in any mortality or altered behaviour and injected animals also successfully oviposited. An increased risk of predation of marked slugs was deemed unlikely, as only the underside of the slug was marked (Fig. 9). To test for equal catchability, the recapture rates of adult and sub-adults were compared. They were also used to estimate the trap response of the slugs with high recapture rates indicating 'trap-happiness'. Goodness-of-fit tests were calculated for the Schnabel and the Jolly-Seber method (Greenwood, 1996) to test the general suitability of our data to the applied models (Appendix 5). The Schnabel goodness-of-fit test checks whether the obtained data fit a regression line connecting the point at which the proportion of marked animals in a sample is zero with the point at which the proportion of marked animals is one, assuming that the true population size equals the total number of animals marked at this point (Greenwood, 1996). The Jolly-Seber goodness-of-fit test checks whether the capture probability of animals which had never been captured is equal to the capture probability of animals which had been captured in a previous sample (Greenwood, 1996).

Statistical analysis

Population size estimates and associated tests were calculated using Microsoft Excel and the confidence intervals were obtained from the Poisson distribution (Krebs, 1999) (Appendix 6). All other analyses were carried out using SPSS version 21. Since site P1 was sampled in a different year, it was not included in the statistical analysis with the other sites.

Population densities of *G. maculosus* and *L. marginata*

To assess how weather conditions influenced capture success, hourly data were obtained from the nearest Met Éireann (Irish National Meteorological Service) stations at Mace Head and Claremorris (www.met.ie). These are situated about 32 km west and 45 km north of Cloosh Forest as the crow flies and data from both stations were combined and averaged for the analysis. Temperature and rainfall data recorded by the meteorological stations in the 24 hour period prior to recording catches under traps were averaged and Spearman rank correlations were undertaken between these weather variables and trapping success.

3.4. Results

A total of 368 *G. maculosus* specimens were captured over the two sampling years with the greatest number (131) found at site P1 and the least (15) at site C2 (Table 7). In comparison, 86 *L. marginata* specimens were caught with the most (40) at site P2 and the least (1) at site C2 (Table 7). Due to the low number of captures no population size estimates could be calculated for *L. marginata* at sites P3 and C2. Capture success varied considerably during the year, with comparably low numbers caught from November to March at most sites (Fig. 10). Significantly more *G. maculosus* than *L. marginata* were captured at sites P1, P3, C1 and C2 (Mann Whitney U test, $P = 0.005$, $P = 0.002$, $P < 0.001$, $P = 0.002$ respectively).

Juvenile *G. maculosus* smaller than 0.5 cm were found during all months except September, with the largest number trapped in July 2013 where a maximum of 19 juveniles was discovered on a single day. Abundances were also high in May and June of the same year when 13 and 14 juvenile slugs were captured on a single day respectively while only two individuals were trapped in November 2013. Generally, fewer juveniles of *L. marginata* were discovered,

with a maximum of four on a single day in August 2012 and one or two in the remaining months except for the period between November and March when no juveniles were found.

Table 7. Total number of slugs (**a**) and slugs/ m^2 (**b**) of *Geomalacus maculosus* and *Lehmannia marginata* captured at the five sites

	Site P1	Site P2	Site P3	Site C1	Site C2
	a/b	**a / b**	**a / b**	**a / b**	**a / b**
G. maculosus	131 / 46	85 / 19	41 / 17	96 / 25	15 / 7
L. marginata	26 / 9	40 / 9	2 / 1	17 / 4	1 / 0

No significant difference was found between the capture probability of adults and sub-adults in either *G. maculosus* or *L. marginata* (Mann-Whitney U, $P = 0.574$ for both) and they were subsequently pooled for the calculation of the population size estimates. The mean recapture rate of *G. maculosus* ranged from 39 % at site C2 to 77 % at site P1. The mean recapture rate for *L. marginata* was found to be generally slightly but not significantly (t-test, $P = 0.071$) higher than that of *G. maculosus*, ranging from 52 % at site C1 to 82 % at site P1.

3.4.1. Population density estimates

Due to the low capture numbers, the goodness-of-fit tests for the Schnabel and Jolly-Seber methods could not be carried out for all sampling occasions. For the remaining periods the Schnabel model was found to be a good fit to the data obtained from both species on the majority of sampling occasions, however, the overall fit was unsatisfactory for *G. maculosus* while it was satisfactory for *L. marginata* (Appendix 5). The Jolly-Seber model was found to be a good fit on only half the sampling occasions for *G. maculosus* and the overall fit was also unsatisfactory for this species but satisfactory for *L. marginata* (Appendix 5).

Population densities of *G. maculosus* and *L. marginata*

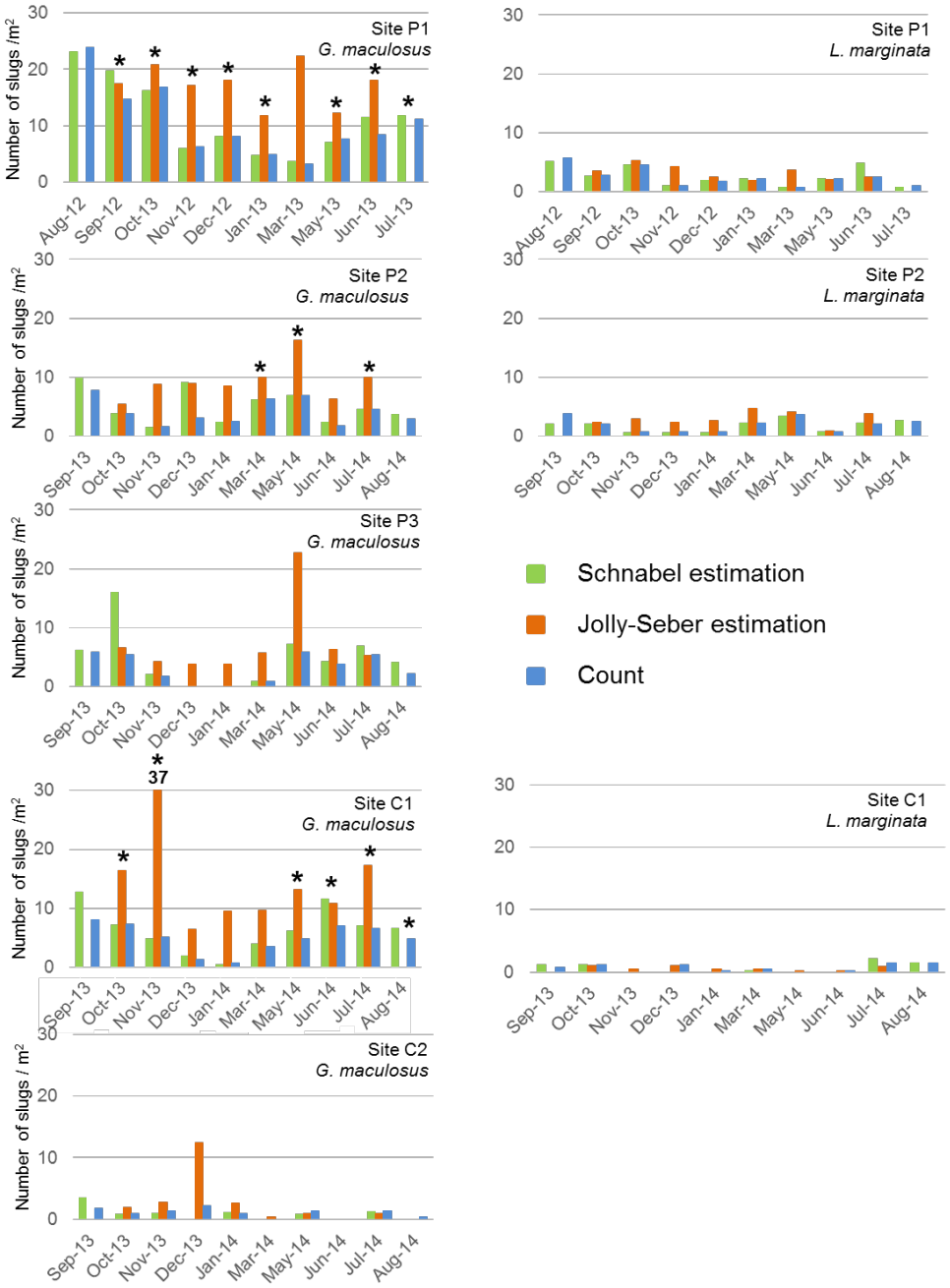

Figure 10. Monthly Schnabel, Jolly-Seber and count population density estimates for *Geomalacus maculosus* and *Lehmannia marginata*. Jolly-Seber estimates cannot be calculated for the first and final sampling periods, as data collected from both the previous and the following sampling period are needed. * indicates months with more than ten recaptures. Results from sites P3 and C2 are not shown for *L. marginata* due to low capture numbers.

Population densities of *G. maculosus* and *L. marginata*

The mean Schnabel density estimates and standard deviations ranged from 0.9/ m^2 ± 1.1 (site C2) to 11.2/ m^2 ± 6.6 (site P1) for *G. maculosus*, and from 0.6/ m^2 ± 0.8 (site C1) to 2.6/ m^2 ± 2.2 (site P1) for *L. marginata* (Fig. 11). The highest Schnabel estimate for *G. maculosus* was 23.1/ m^2 in August 2012 (site P1) and for *L. marginata* 5.2/ m^2 in August 2012 (Fig. 10).

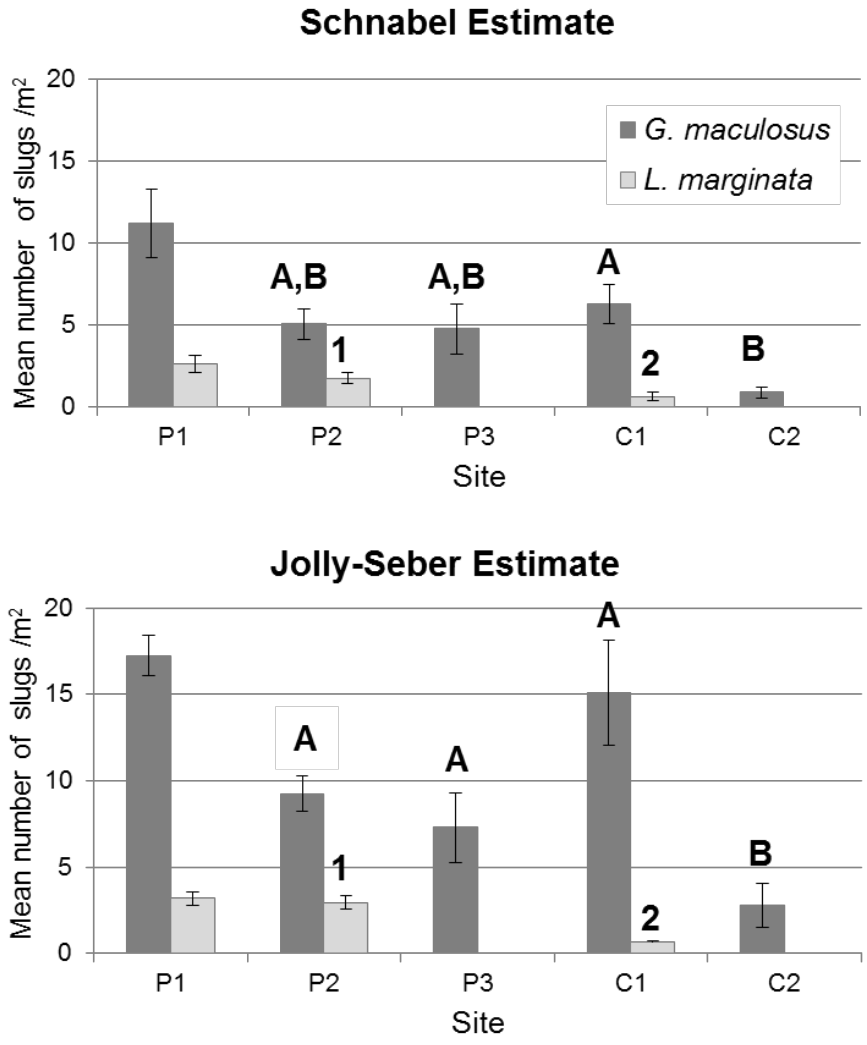

Figure 11. Mean Schnabel and Jolly-Seber population density estimates and standard errors for *Geomalacus maculosus* and *Lehmannia marginata* at each site (N = 10). Different letters (*G. maculosus*) and numbers (*L. marginata*) indicate the sites where the estimated mean densities were significantly different (Tukey's HSD & t-test, $P < 0.05$; the Jolly-Seber estimates were $Log_{10}+1$ transformed prior to the ANOVA).

Population densities of *G. maculosus* and *L. marginata*

The mean population density estimates and standard deviations following the Jolly-Seber method ranged from 2.8/ m^2 ± 4 (site C2) to 17.3/ m^2 ± 3.7 (site P1) for *G. maculosus* and from 0.6/ m^2 ± 0.3 (site C1) to 3.2/ m^2 ± 1.2 (site P1) for *L. marginata* (Fig. 11). The highest Jolly-Seber estimate for *G. maculosus* was 37.2/ m^2 in November 2013 (site C1) and for *L. marginata* 5.3/ m^2 in October 2012 (Site P1) (Fig. 10). Jolly-Seber estimates are only considered precise when the number of recaptured animals is more than ten (Greenwood, 1996) and while this requirement was frequently met for *G. maculosus*, it was never met for *L. marginata* (Fig. 10).

If using the number of captured specimens per m^2 rather than the Schnabel and Jolly-Seber density estimates, the number of *G. maculosus* per m^2 was significantly higher at sites P2 and C1 compared to site C2 (Tukey's HSD, $P < 0.05$). The $Log_{10}+1$ transformed number of captured *L. marginata* per m^2 was significantly higher at site P2 compared to all other sites (Tukey's HSD, $P < 0.01$) and significantly higher at site C1 compared to sites P3 and C2 (Tukey's HSD, $P < 0.05$).

3.4.2. Weather conditions and trapping success

The number of slugs captured per sampling day was positively correlated with the mean temperature during the 24 hour period prior to sampling in sites P1, P3 and C1 for *G. maculosus*, while for *L. marginata* a positive correlation was found with mean temperature at site C1 (Table 8). Rainfall was negatively correlated with the number of slugs captured in the plantation sites (P1, P2 and P3) for *G. maculosus*, and for *L. marginata* at site P2 (Table 8). Too few *G. maculosus* were captured at site C2 and too few *L. marginata* were captured at sites P3 and C2 to correlate their abundance with the weather data.

Table 8. Spearman rank correlations for the weather data from the Claremorris and Mace Head weather stations with the number of *Geomalacus maculosus* (**a**) and *Lehmannia marginata* (**b**) trapped at each site during the duration of the study. No correlations were carried out for *L. marginata* at site P3 due to low capture numbers.

a	Weather variables	**Site P1** Rho, *P*	**Site P2** Rho, *P*	**Site P3** Rho, *P*	**Site C1** Rho, *P*
	Temperature	0.42**, 0.003	-0.04, 0.801	0.64**, 0.000	0.44**, 0.001
	Rainfall	-0.36*, 0.011	-0.38**, 0.006	-0.33*, 0.02	-0.02, 0.916
b	Weather variables	**Site P1** Rho, *P*	**Site P2** Rho, *P*	**Site P3** Rho, *P*	**Site C1** Rho, *P*
	Temperature	0.25, 0.088	0.24, 0.088	n/a	0.43**, 0.002
	Rainfall	-0.24, 0.104	-0.3*, 0.033	n/a	-0.27, 0.063

* significant at 0.05 level, ** significant at 0.01 level.

3.5. Discussion

3.5.1. Population density estimates

The population density estimates of both species were found to vary considerably between the five sites with the largest numbers captured at site P1 (*G. maculosus*) and site P2 (*L. marginata*) and the smallest numbers captured at sites P3 and C2 (both species). While the trees in the plantation sites were all planted in the 1960s, those in site P3 had a significantly lower CBH than those at site P2 (Tukey's HSD, $P = 0.004$) possibly due to poorer growth conditions connected with the close proximity of site P3 to the lake (Fig. 7b). The epiphyte cover of the trunk was also found to be significantly lower at this site compared to site P2 (Mann-Whitney U, $P = 0.014$) and the depth of the epiphyte layer was significantly lower at site P3 compared to both site P1 and P2 (Mann-Whitney U, $P < 0.001$). In a

previous study (Reich *et al*., 2012), *G. maculosus* abundance was found to be positively correlated with epiphyte cover of the trunk and CBH, which were found to be inter-correlated with more mature trees having a greater epiphyte cover. The lower abundance of both species at site P3 compared to sites P1 and P2 is likely connected to thickness and percentage cover of the epiphyte layer which plays an important role for the slugs as it provides both shelter and food (Reich *et al*., 2012).

Both species were found in significantly greater numbers at site C1 (partial clear-fell) compared to site C2 (conventional clear-fell). This could be due to a variety of factors, including the position of the trap on top of the stump which might not be utilised by the slugs, the time since the clear-felling operation (Table 6) or the difference in the felling method. The remaining large tree stumps in site C1 were still covered with epiphytes which could contribute to the remaining of the slugs at this site and the comparably large population sizes we encountered in this site indicate that, at least in the short term, this modification of a traditional clear-fell can offer suitable conditions for forest slugs.

Geomalacus maculosus was consistently found in higher numbers than *L. marginata* in this study, which was significant for all sites except site P2. This could suggest that *G. maculosus* is better suited to the conditions within this plantation than *L. marginata*, in particular to the traditional clear-fell site where only one specimen of *L. marginata* was captured during the entire survey. Another possibility is that *G. maculosus* responds better to the trapping method used, although results from other studies (McDonnell & Gormally, 2011a; Reich *et al*., 2012) which show large numbers of *L. marginata* being captured using the same traps suggest otherwise. The presence of *G. maculosus* juveniles in Cloosh Forest throughout most of the year

could indicate that this species might have a reproductive advantage over *L. marginata*, the juveniles of which were only captured from May to September. South (1992) states that competition between slugs in the wild is generally rare, as even sympatric species still occupy slightly different niches and/or differ in their activity times or life cycles. However, *Arion lusitanicus* (now *vulgaris*) had been found to replace *Arion rufus* Linnaeus in Austria (Fischer & Reischütz, 1998) and it is not impossible that *G. maculosus* could pose a threat to *L. marginata* populations. More replicates are needed across additional sites where both species are known to co-occur to further investigate the relationship between these two slug species.

3.5.2. Weather conditions and trapping success

Population densities for both species varied with season with the least number of slugs overall caught during the colder months (November until March) and the greatest numbers caught between August and October. Fluctuations in temperature are generally found to have a major effect on slug activity (South, 1992) which is reflected in our results: Temperature was positively correlated with capture success for *G. maculosus* in both the clear-fell and plantation sites (except P2) and at site C1 for *L. marginata*. McDonnell and Gormally (2011a) found no correlations between temperature and *G. maculosus* abundance at a number of forest and bog sites in counties Cork and Kerry. However, their studies were undertaken from August to October and not over a full year so fluctuations in temperatures were unlikely to be at the scale observed in this study.

Capture success of *G. maculosus* was negatively correlated with rainfall in the plantation sites, while the capture success of *L. marginata* seemed to be less influenced by rainfall. According to Dainton (1989) and Barnes and Weil (1945) slug activity is reduced during heavy rainfall and this probably affects slugs in open areas

more than in forests. While McDonnell and Gormally (2011a) also observed a negative correlation of capture success with rainfall in forests, they found a positive correlation with trapping success and rainfall at a blanket bog site. This suggests that in open areas *G. maculosus* uses the traps for shelter during periods of heavy rain, while in the more sheltered forest it can remain active and continue foraging. Future surveys in planted areas should hence take place during dry and warm weather to ensure maximum capture success, while in open areas trapping during periods of rain would be recommended.

3.5.3. Assessment of the methodology

The observed high recapture rates, which can be used as a measure of dispersal and mortality (with higher recapture rates indicating low dispersal and/or low mortality and vice versa), indicate that the slugs are likely to remain below the traps. This is supported by the Jolly-Seber goodness-of-fit test which found that the capture probability of *G. maculosus* specimens that had been previously captured was larger than that of slugs which had never been captured. This could pose a problem to the unbiased calculation of population density estimates using either the Schnabel or the Jolly-Seber method. The general low mobility of slugs and the elimination of juvenile slugs from the survey could also pose problems when applying an open population model which implies birth, death, immigration and emigration. Limits to both methods were the often small numbers of captures and recaptures, which is especially critical for the Jolly-Seber method, where the number of recaptures should be more than ten to produce reliable estimates (Greenwood, 1996). While nine traps were adequate to achieve sufficient recaptures for *G. maculosus* at site P1, the amount of recaptures for certain months at other sites were too small. With three or less recaptures at the

majority of sampling occasions, it is estimated on the basis of this study (where nine traps were used) that 30 traps would have been needed at site P3 (the site with the fewest captures) to produce reliable estimates for the species. For *L. marginata*, 20 traps should have produced a sufficient amount of recaptures in sites P1 and P2 (the mean amount of recaptured slugs was 4.7 using nine traps, using 20 traps would yield 10.4), while at site C1 recaptures were usually below three so more than 30 traps would have been necessary. For future studies we recommend the installation of 30 traps for a pre-survey the length of one secondary period i.e. five days, to indicate how many traps should be used in that particular site keeping in mind that capture numbers will vary considerably depending on weather conditions. This should help ensure that reliable estimates can be calculated on most sampling occasions during the survey period.

The visible implant elastomers were a generally quick and easy method to mark the slugs of both species. The dyes were long-lasting and animals marked in the first period retained the mark after one year. However, as the slugs usually ejected a certain amount of elastomer after the injection, it is recommended to ensure that the marking is still visible before returning them to the traps. Limits to the method are that only ten colours are available and that the orange, red and pink colours can be easily confused. While in our case it was possible to eliminate the mis-identifications (about 5 % of all marks) that occurred by comparing the marking history of the captured slugs, in a larger study this might not be feasible. Due to its light coloured sole, the colours were more easily identified in *L. marginata*, especially when compared to some woodland individuals of *G. maculosus* which can have tougher, darker and slightly ‘grainy’ soles. It is also advisable to have at least two different people to check all marks and then to compare the findings.

3.5.4. Conclusions

This study indicates that using visible implant elastomers and *De Sangosse* refuge traps which are wrapped around tree trunks is a suitable approach to estimate the population densities of *G. maculosus* and *L. marginata* in forest habitats, in particular if comparative rather than exact densities are needed. The mark-recapture approach allows forestry managers to compare population densities of the species pre- and post- management operations and the impact of these operations on resident populations can be estimated by observing the amount of marked individuals which remain after forest practices such as felling take place. When using the Schnabel or Jolly-Seber methods, it should be kept in mind that the resulting population size estimates are very likely underestimations of the actual population sizes due to the trap-happiness of the slugs. The use of at least 30 trapping trees in a commercial conifer plantation is likely to provide sufficient numbers of captures to calculate reliable population density estimates even in sites with small slug populations. Since it can sometimes be difficult to separate orange, red and pink elastomers in the field, it is recommended to not use these three colours together in the same survey.

Capture success was significantly greater in warmer temperatures so future surveys should ideally take place between May and October when capture numbers were found to be greatest. Surveying within the forest should be avoided during heavy rainfall as capture success was negatively correlated with rainfall.

In a wider context, it is hoped that this study will encourage the use of mark-recapture methods to inform population estimates for other terrestrial mollusc species. This is particularly important given that over 20 % of 1,138 terrestrial mollusc species in the EU alone are

currently classified as threatened (IUCN Red List Status) and require further study to ensure their future survival.

Chapter 4:

Microhabitat is likely to be the major determinant of the highly diverse microbiome found within faecal samples of the EU protected slug *Geomalacus maculosus*

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Geomalacus maculosus on lichen covered rock in Dingle, County Kerry (© Inga Reich).

Microhabitat is likely to be the major determinant of the highly diverse microbiome found within faecal samples of the EU protected slug *Geomalacus maculosus*

4.1. Abstract

The EU-protected slug *Geomalacus maculosus* Allman is a Lusitanian species occurring only in the West of Ireland and in northern Spain and Portugal. While the Iberian populations are genetically highly structured, Irish specimens are indistinguishable using mitochondrial markers. In this study we explored the microbial community found within the faeces of Irish *G. maculosus* with a view to determining whether a core microbiome existed among geographically isolated slugs. These bacteria potentially fulfil important functions within the host and could give insight into the adaptations of *G. maculosus* to the available food resources within its habitat. Using laboratory reared hatchlings from the same egg clutch we also investigated if a vertical transfer of microbes occurred. For the first time, faecal samples of 30 wild and six reared *G. maculosus* specimens were collected throughout the slug's Irish range and the V3 region of the bacterial 16S rRNA gene was sequenced using Illumina MiSeq. We found a widely diverse microbiome dominated by Proteobacteria. Only two core operational taxonomic units were shared between all specimens, indicating that the faecal microbiome of *G. maculosus* is likely dependent on the microhabitat of the individual slugs rather than being selected for by the host. For the laboratory reared slugs diet was found to have a greater impact than kinship on the diversity of the faecal microbiome, suggesting that very little vertical transfer of the parents gut microbiome via the egg takes place in *G. maculosus*.

4.2. Introduction

While the study of gut microbial communities is becoming increasingly popular, there is still a dearth of research focusing on those of wild animal populations (Amato, 2013). This is despite the large influence that factors such as habitat and food availability are likely to have on the gut microbial composition. In fact, it has been shown that captive animals have a distinctly different gut microbiome than those from the wild (e.g. Nakamura *et al*., 2011; Nelson *et al*., 2013) which is hardly surprising, as a main mode of colonisation of the intestinal tract with microbes is through the environment (e.g. Engel & Moran, 2013; Newton *et al*., 2013). Hence, the gut microbiome of a species should reflect, at least to an extent, the bacteria which can be found associated with the food or water it ingests in its habitat. The food availability within the habitats is, among others, dependent on abiotic factors as well as seasonality and it has been shown that the composition of the gut microbiome of some animals differs between sites and season (e.g. Kobayashi *et al*., 2006; King *et al.*, 2012; Moran *et al*., 2012). Additionally, geographical patterns of enteric microbial communities have been discovered in Galapagos iguanas by Lankau *et al*. (2012) with the microbiota being more distinct the further the islands were separated from each other. While the authors suggest that the dominant drivers of the observed differentiation are host-bacterial interactions and differences in diet, historical and contemporary processes of ecological drift could have also been a factor. The gut microbiome is usually not only horizontally transmitted from the environment but a vertical transfer of microbes between specimens is possible through processes such as birth or hatching, social interactions or coprophagy. This is seen, in particular, among social insects that possess distinctive and consistent gut microbial communities (Engel & Moran, 2013).

Gut microbiome of *G. maculosus*

Geomalacus maculosus Allman is an EU protected slug species which is found only in the West of Ireland and the North of Iberia. Recent research has shown that the Irish population was likely introduced from Iberia sometime after the last glacial maximum (LGM) and that specimens from different locations within Ireland could not be distinguished using the mitochondrial markers 16S rRNA and COI (Reich *et al*., 2015). While the species' Irish distribution was generally believed to be confined to counties Kerry and Cork in the Southwest of the island, a population was recently discovered in Cloosh Forest, County Galway (Kearney, 2010) more than 200 km to the North. As Cloosh forest is a commercial plantation, it is likely that the slug has been accidentally introduced there through forestry operations (Reich *et al*., 2012). Apart from coniferous and deciduous forests, the Irish *G. maculosus* inhabits a range of open habitats including blanket bogs and wet grasslands where it feeds on lichens, liverworts, bryophytes and fungi which it grazes from rocks or the bark of trees (e.g. Platts & Speight, 1988; Reich *et al*., 2012; McDonnell *et al*., 2013). As no records of this species feeding on agricultural crops or other vascular plants exists, its gut microbiota might be highly adapted to aid the digestion of non-vascular plants which are staples of its diet. *Geomalacus maculosus* is a hermaphrodite which is capable of self-fertilisation (Oldham, 1942). Eggs are laid in clutches of up to 18 to 30 eggs and juveniles take between six and eight weeks to hatch (Rogers, 1900). While it lacks parental care and sociality, a vertical transmission of microbes could occur via the egg. While hatching slugs do not consume their eggs, even if these are left within the same container for a few days (pers. obs.), they do eat a tiny hole into their egg before emerging which might be sufficient for microbial transfer.

This study is the first to use next generation sequencing to assess the diversity of bacteria found within faeces of the protected slug G.

maculosus. We employed a two-pronged approach, utilising faeces samples from slugs that were collected from the wild as well as from laboratory hatched specimens to address our aims:

1. To determine whether the slug is a major selector of its microbiome, in which case we would expect a high amount of 'core microbiome members' shared by all specimens, or if their gut microbes are more reflective of their environment. If the latter is the case, we hypothesize that only few, if any, microbes are shared between all specimens and that the microbial signatures of slugs collected from the same site/habitat will be more similar than those collected from different sites/habitats.

2. To explore the possibility of a vertical transmission of microbes using laboratory reared slugs from the same egg clutch which were fed on two different diets. We hypothesize that if the vertical transmission of microbes was the major determinant of the gut microbiome composition, a great number of shared phylotypes between the two groups should be observed irrespective of diet.

4.3. Materials and Methods

4.3.1. Sampling

Wild specimens

In June and July 2012, 50 *G. maculosus* specimens were collected from eleven different locations within Ireland (Fig. 12). Slugs were sampled from tree trunks or rocks from seven different habitats (determined using Fossitt, 2000): blanket bog, heath, exposed siliceous rock, wet grassland, deciduous woodland, mixed woodland and coniferous plantations. They were transferred into sterile petri dishes and observed until they defecated. Freshly collected faeces

were transferred into sterile Eppendorf tubes which were initially stored in a mobile freezer compartment at -6 °C before being moved to a -80 °C freezer in the laboratory two days later.

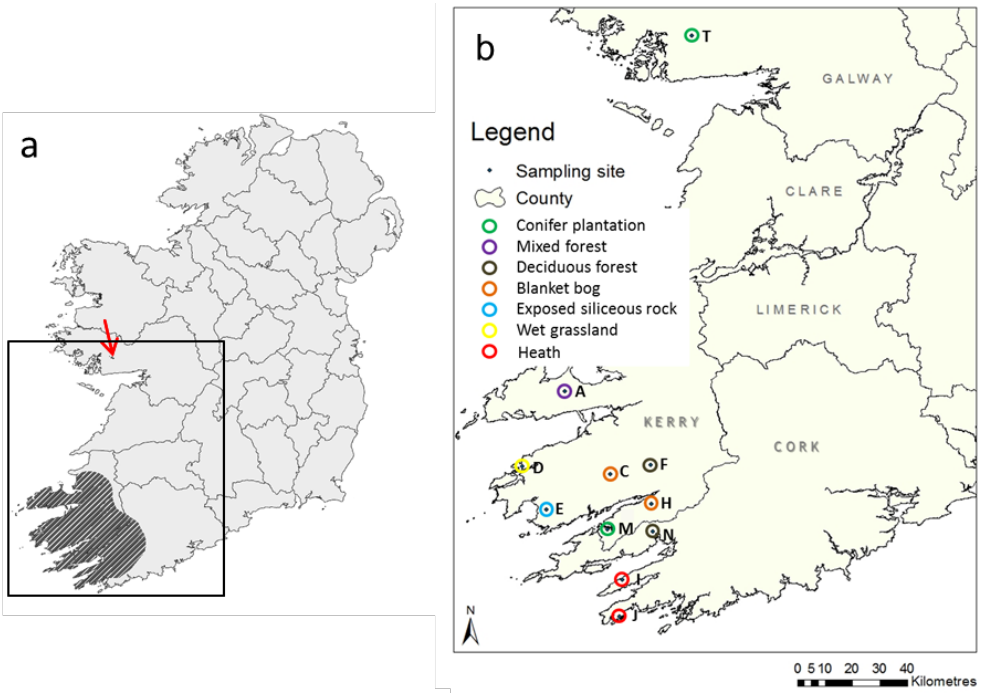

Figure 12. (**a**) The Irish distribution area of *G. maculosus* (shaded), the arrow indicates the localised population of the species in County Galway (modified from G. Kindermann); (**b**) Sites sampled during this study, different habitats are encircled in a different colour. The number of faeces samples used in the following analyses from each site is given in brackets. **A** Glanteenassig Forest (3), **C** Ballaghbeama Gap (3), **D** Ballycarbery (3), **E** Lough Currane (3), **F** Derrycunnihy Woods (3), **H** Barraduff (3), **I** Crookhaven (2), **J** Raferigeen (1), **M** Derreen Forest (3), **N** Glengarriff Woods (3), **T** Cloosh Forest (3).

Laboratory reared specimens

In June 2014, a clutch of ten eggs was laid by a slug captured two weeks beforehand from the mixed woodland site in Glanteenassig (site A, Fig. 12). The eggs were removed from the parent slug and put into a petri dish containing moist tissue paper and kept at room temperature. After hatching, each slug was transferred into a single

petri dish where four slugs were fed with porridge oats, while three slugs were fed with different lichens collected from Cloosh Forest (Fig. 12). Three eggs did not hatch. As faecal amounts of the juveniles were small, they were collected after three weeks in the same manner as described above and immediately stored at -80 °C.

4.3.2. DNA extraction, PCR and sequencing

DNA was extracted from the faeces samples collected from the fifty sampled and six reared slugs (faeces weight ranging from 0.03 to 0.17g) using the PowerSoil DNA Isolation Kit (MoBIO, Carlsbad, CA, USA). The V3 region of the 16S rRNA was amplified with the universal bacterial primers 341F (5'-CTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') using the following conditions: two minutes initial denaturation at 98 °C followed by 30 cycles of 20 seconds at 98 °C, 30 seconds at 58 °C and 30 seconds at 72 °C. The final extension step was for five minutes at 72 °C. One µl of purified DNA was added to a 24 µl PCR mixture containing one unit of Q5 High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA) and 0.25 µM of each primer. Each sample was amplified three times and the combined PCR products were run on a 2 % agarose gel and subsequently excised and gel extracted using the MinElute Gel Extraction Kit (QIAGEN, Hilden, Germany). As not all 50 samples amplified satisfactorily, the purified PCR products of 36 samples (30 from wild specimens, six from reared specimens) were sent to Research and Testing Laboratory, Texas, USA for sequencing on Illumina MiSeq.

The contamination of samples with foreign DNA can pose a problem (Salter *et al*., 2014), especially when working with low microbial biomass samples as in this study. Therefore, a negative control using only PCR water (Bioline, London, UK), which was extracted and amplified following the same protocol as that of the faeces samples,

was also included for sequencing. Additionally, the risk of skewing our results was prevented by using the same extraction and PCR kit for all samples (Salter *et al*., 2014).

4.3.3. Sequence analyses

The majority of analyses were carried out with QIIME 1.9.1 (Caporaso *et al*., 2010a). Overlapping paired-end reads were stitched together and primer sequences trimmed. Sequences were multiplexed and quality-filtered, removing reads which were shorter than 75 % of the input read length after they had been truncated due to three or more consecutive low quality base calls. Chimeric sequences were identified using USEARCH 6.1 (Edgar *et al*., 2011) with reference based detection suppressed and then filtered from the samples. Operational taxonomic units (OTUs) were clustered at 97 % similarity against the latest Greengenes database (August 2013; DeSantis *et al*., 2006) using the USEARCH 6.1 (Edgar, 2010) method and open-reference OTU picking. Singleton sequences were removed to reduce noise by specifying the minimum OTU size to two. Taxonomy was assigned with the RDP classifier (Wang *et al*., 2007) based on the Greengenes reference database (McDonald *et al*., 2012). Sequences were aligned with PyNAST (Caporaso *et al*., 2010b) and a maximum-likelihood tree was produced using only one unique sequence for each OTU with FASTTREE2 (Price *et al*., 2010). Chloroplast sequences were filtered from the OTU table, leaving reads per sample from 7,939 to 18,264 with a mean sequence depth of 12,719 reads per sample.

Alpha diversity (observed OTUs plus amount of singletons and doubletons, Phylogenetic Diversity (PD) and Simpson Index) and beta diversity (weighted and unweighted UniFrac (Lozupone & Knight, 2005)) were calculated for the data rarefied to a common depth of 7,939 reads. Principle coordinates analyses (PCoA) were

conducted on both, weighted and unweighted UniFrac distances to examine how changes in relative taxon abundance as well as presence or absence of taxa influence the clustering of individual faecal samples. The samples from the reared specimens were not included in this and the following analysis due to the time lag in faeces collection. To test if the centroids among the groups (sample sites) were significantly different, we used the method *adonis*, a PERMANOVA implemented in the R *vegan* package v.2.0-10 (Oksanen *et al*., 2013) followed by a PERMDISP analysis (implemented in the same package) to test for homogeny of within-group dispersions. As only two and one specimen(s) respectively were collected from sample sites I and J, they were not included in these analyses to ensure a balanced design. Both tests were run with 10,000 permutations on the unweighted and weighted UniFrac distances. To investigate whether potential contaminants, which could have been introduced into the samples during the DNA extraction and PCR steps, had an effect on our results, the OTUs found in the negative control sample were filtered from all other samples and the resulting data were analysed with *adonis* and PERMDISP in the same way as described above.

The core microbiome found in all samples and the shared OTUs occurring in 100 % of the samples grouped by habitat and by sample site were calculated from the non-rarefied data.

4.4. Results

4.4.1. Microbial diversity

Excluding those which occurred exclusively in the negative control, a total of 4,211 OTUs which belonged to 27 phyla and 68 associated classes of bacteria were observed within our samples. The most

frequently observed group which was dominant in nearly all samples were the Proteobacteria, with Gammaproteobacteria accounting for 46 % of all sequences, followed by Alphaproteobacteria (21 %) and Betaproteobacteria (5 %) (Fig. 13).

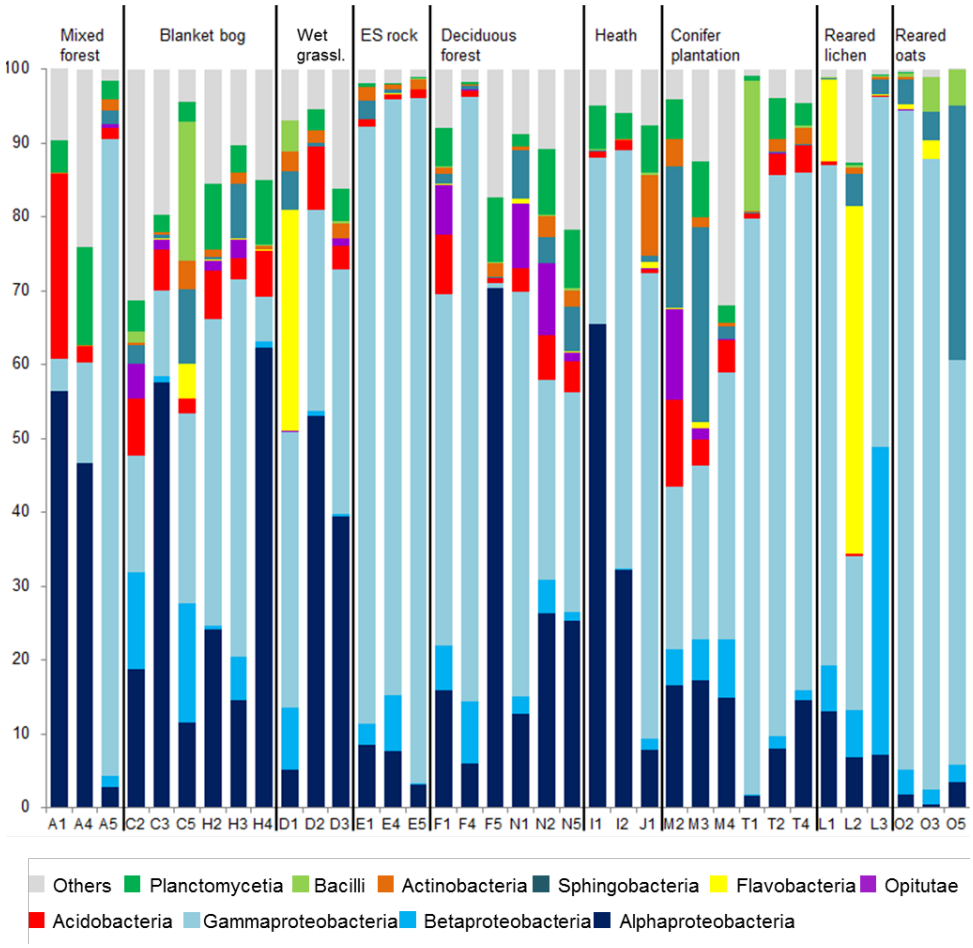

Figure 13. The most abundant classes of bacteria (> 1 % of all sequences) which were found in all samples. The letters refer to the sample sites (Fig. 12) with L and O indicating the reared specimens fed on lichens and oats respectively.

The negative control contained 379 OTUs, the majority of which belonged to the Proteobacteria and Actinobacteria, both accounting for more than 40 % of the negative sample. The most abundant OTU was a member of the Intrasporangiaceae (Actinobacteria: Actinomycetales) (20.2 %), while Rubrobacter (Actinobacteria:

Rubrobacterales) was the most abundant OTU classified to genus level (9.6 %). The dominant OTUs of the negative control were found only in low abundances in all other samples (≤ 0.1 % of sequences per sample), and 103 of the 379 OTUs of the negative control were not found in any other samples.

Not including the negative control, an average of 391 OTUs (± 150 standard deviation (SD)) were observed per sample, ranging from 160 (O5) to 709 OTUs (F1 and N5) (Table 1). Many of these were low abundance OTUs: between 34.5 % (D1) and 59.2 % (E5) were observed as singletons within a sample (mean: 46 % ± 4.8 SD) and between 12.7 % (T2) and 21.3 % (O3) were doubletons within a sample (mean: 16 % ± 2.3 SD). Simpson's Index of Diversity ranged from 0.56 (E5) to 0.97 (C2) (mean: 0.85 ± 0.11 SD) and phylogenetic diversity ranged from 9.7 (O5) to 26.6 (F1) (mean: 18 ± 4.1 SD). The three specimens which were reared in the laboratory and fed with oats had the lowest number of observed OTUs and the lowest phylogenetic diversity compared to all of the other specimens (Table 9).

Table 9. Alpha diversity measures. The letters refer to the sample sites (Fig. 12), L and O indicate the reared specimens fed on lichens and oats.

Sample	Observed OTUs	Singletons (%)	Doubletons (%)	Simpson's 1-D	Phylogenetic diversity
A1	353	44.19	15.01	0.96	16.49
A4	367	46.32	14.44	0.92	17.05
A5	347	48.41	17.29	0.65	15.06
C2	481	40.54	16.01	0.97	18.38
C3	407	49.88	17.44	0.93	20.02
C5	511	44.03	15.07	0.95	20.45
D1	362	34.53	19.89	0.87	17.21
D2	436	49.08	19.04	0.91	22.38
D3	477	48.01	16.98	0.95	22.11
E1	349	39.54	17.19	0.76	17.08
E4	322	53.11	14.29	0.57	17.29
E5	260	59.23	18.46	0.56	17.26
F1	709	49.65	15.09	0.95	26.64
F4	322	52.80	14.29	0.60	18.03
F5	197	42.64	20.81	0.74	13.67
H2	493	49.09	13.18	0.93	20.91
H3	509	50.29	14.73	0.93	18.96
H4	327	44.34	14.07	0.89	13.65
I1	261	44.44	14.56	0.92	15.32
I2	310	48.06	15.16	0.80	12.01
J1	704	42.90	20.88	0.78	24.74
M2	532	44.36	16.73	0.96	20.44
M3	505	45.35	13.27	0.94	21.76
M4	433	49.65	13.63	0.90	19.65
N1	470	47.87	16.81	0.86	22.07
N2	656	48.48	17.07	0.94	23.98
N5	709	48.94	14.67	0.95	25.32
T1	237	47.26	17.72	0.84	14.60
T2	362	48.62	12.71	0.79	18.66
T4	373	38.07	14.75	0.82	17.64
L1	229	45.85	13.97	0.85	12.99
L2	322	39.44	15.22	0.87	15.93
L3	223	44.84	13.90	0.89	18.05
O2	203	45.81	14.78	0.76	12.09
O3	183	38.80	21.31	0.86	10.51
O5	160	43.13	16.25	0.84	9.74

4.4.2. The drivers of the gut microbiome composition

Core microbiome

Only two OTUs were found in all faeces samples of the wild and reared slugs, both belonged to the Enterobacteriaceae (Gammaproteobacteria: Enterobacteriales) one of which was only classified to family level while the other one was assigned to *Citrobacter freundii* (100 % sequence identity, BLAST). They accounted for approximately 14 % and 10 % (*C. freundii*) of all sequences respectively and were the most abundant OTUs alongside two other not further classified Enterobacteriaceae (7.5 % and 3.5 %), one member of the Methylocystaceae (4.5 %) and one member of the Acetobacteraceae (3.5 %) (Fig. 14), which occurred in 89, 50, 92 and 83 % of samples respectively.

Gut microbiome of *G. maculosus*

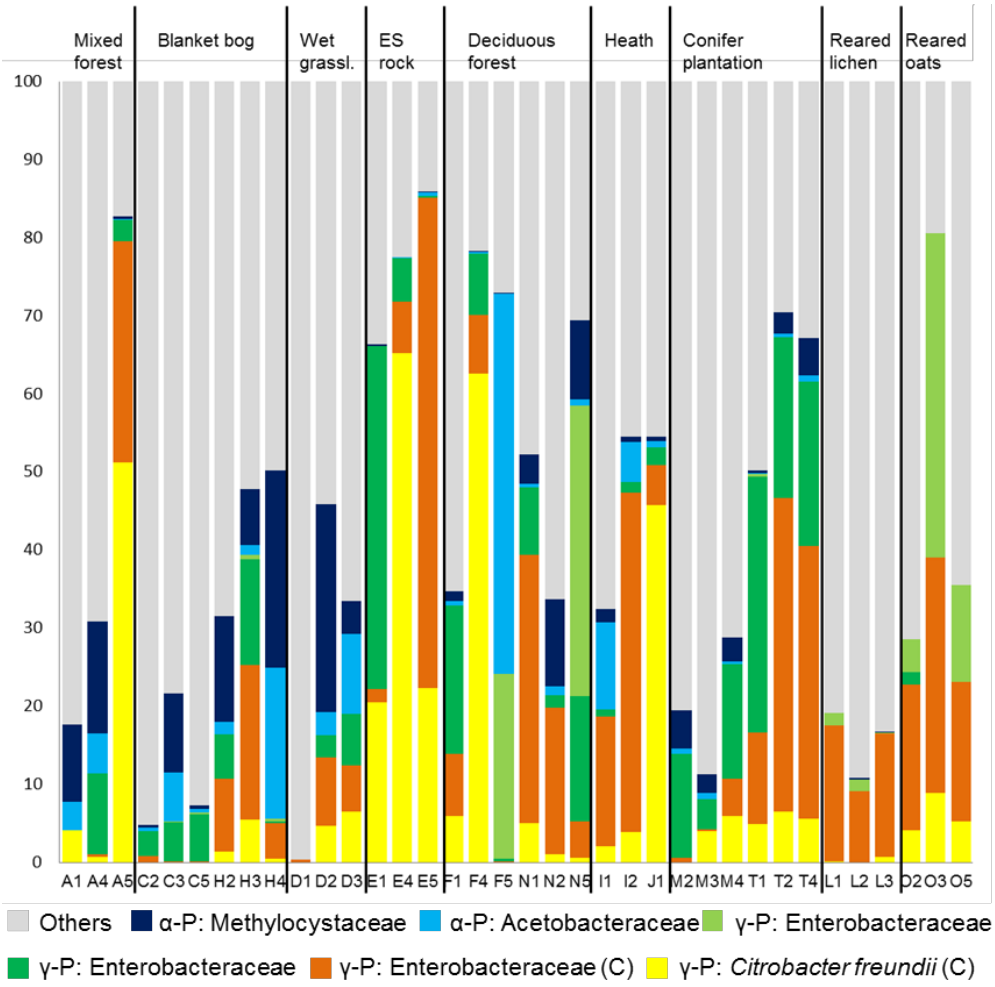

Figure 14. The six most abundant operational taxonomic units (> 10,000 sequences per OTU) including the core OTUs (marked '**C**') and their abundance within the individual samples. The letters refer to the sample sites (Fig. 12) with L and O indicating the reared specimens fed on lichens and oats respectively.

Influence of sample site and habitat on gut microbiome

No clear separation of samples by either site (Fig. 15a, c) or habitat (Fig. 15b, d) could be observed in the PCoA plots. However, certain samples from the same site and/or habitat were found in close proximity to each other, including those from Lough Currane or those from the conifer plantations at Derreen and Cloosh Forest, which are also clustered relatively closely in the habitat plot when using the

unweighted UniFrac distances (Fig. 15b). While the specimens collected from the heath habitat did not share a high level of similarity using the unweighted distances, they were clustering together using the weighted UniFrac (Fig. 15d).

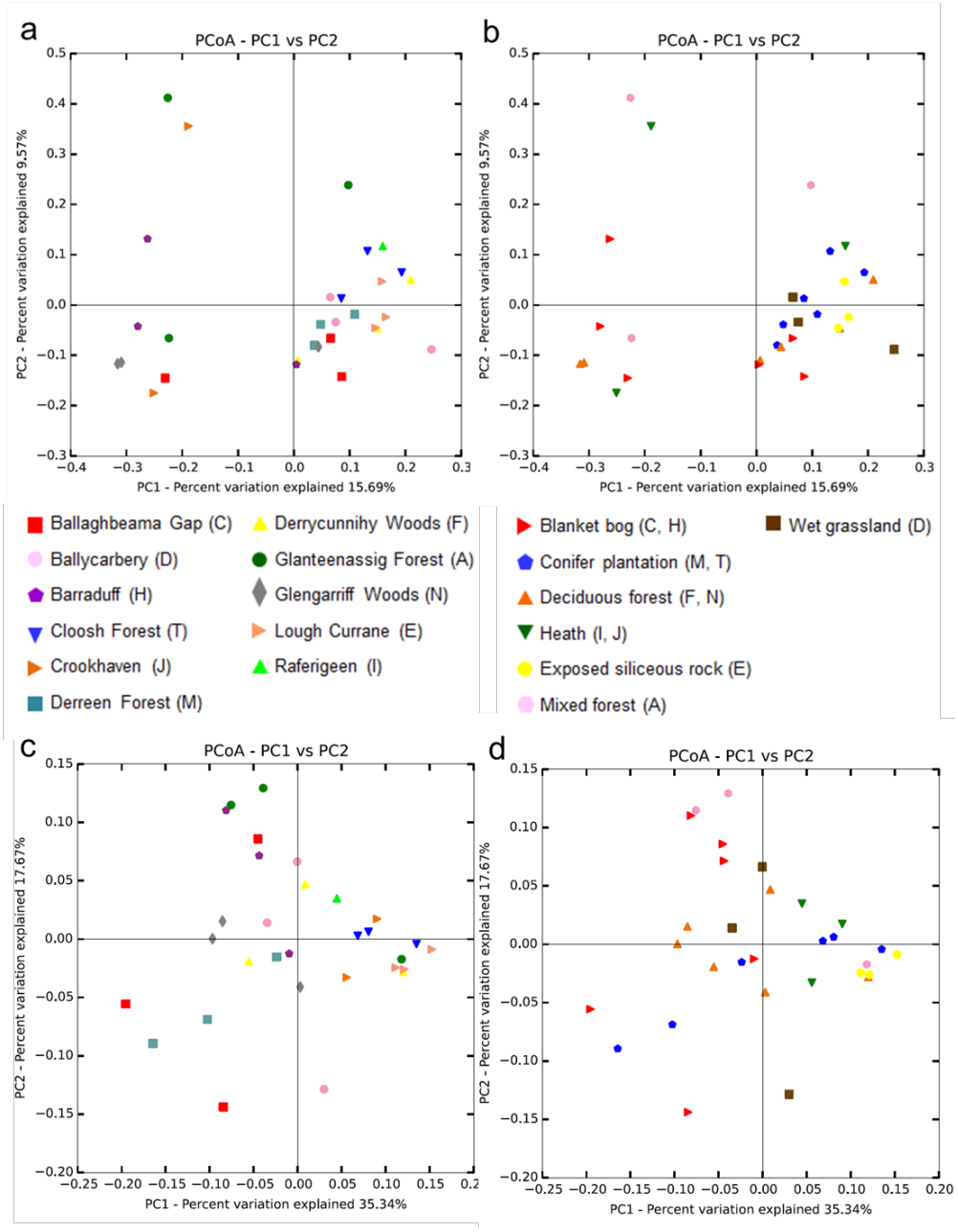

Figure 15. Principal coordinate analysis plots based on the unweighted (**a**, **b**) and weighted (**c**, **d**) UniFrac distance matrix. Coloured by 'sample site' (**a**, **c**) and by 'habitat' (**b**, **d**). The letters refer to the sample sites (Fig. 12).

No separation of clusters was observed by either the substrate from which the slugs were collected (rock or tree) or by the environment type (forest or open habitat) (Appendix 7). The samples are roughly spread along the x-axis by the amount of observed OTUs (unweighted UniFrac) or by the Simpson's Index of Diversity (weighted UniFrac; Appendix 7).

While the PERMANOVA found the centroids of the groups to be significantly different when the faecal samples were grouped by sample site (N = 9; R^2 = 0.48, P < 0.001 (unweighted UniFrac) and R^2 = 0.48, P = 0.002 (weighted UniFrac)), the multivariate spread between these groups was also significantly different (PERMDISP, F = 3.97, P = 0.007 (unweighted UniFrac), F = 3.86, P = 0.006 (weighted UniFrac)). This indicates that unequal variances rather than actual differences between the centroids of the groups were the reason for the significant PERMANOVA which is supported by the absence of clearly separated groups in the PCoA plots (Fig. 15).

4.4.3. Vertical transmission of microbes

As the reared slugs were all form the same parent, we determined whether they shared a higher amount of OTUs between them than the slugs collected from the wild which were of unknown kinship. Only a marginally higher percentage of OTUs was shared between all three individuals from the reared slugs fed on lichens or on oats (12.3 and 13.7 % respectively, mean: 13 % ± 1 SD) compared to the wild specimens which were collected from the same site (mean: 10.5 % ± 4.5 SD). Those from Cloosh Forest and Derreen Forest even had a higher percentage of shared OTUs than the samples from the reared specimens (15.6 and 14.5 % respectively) (Fig. 16, Appendix 8). When pooling the specimens reared on lichens and those reared on oats, they shared only 2.3 % of their OTUs among all six individuals, whereas an average of 3.1 % ± 1.3 SD was shared

between the six wild specimens collected from the same habitat where two sites per habitat were sampled (conifer plantation, deciduous forest and blanket bog) (Appendix 8).

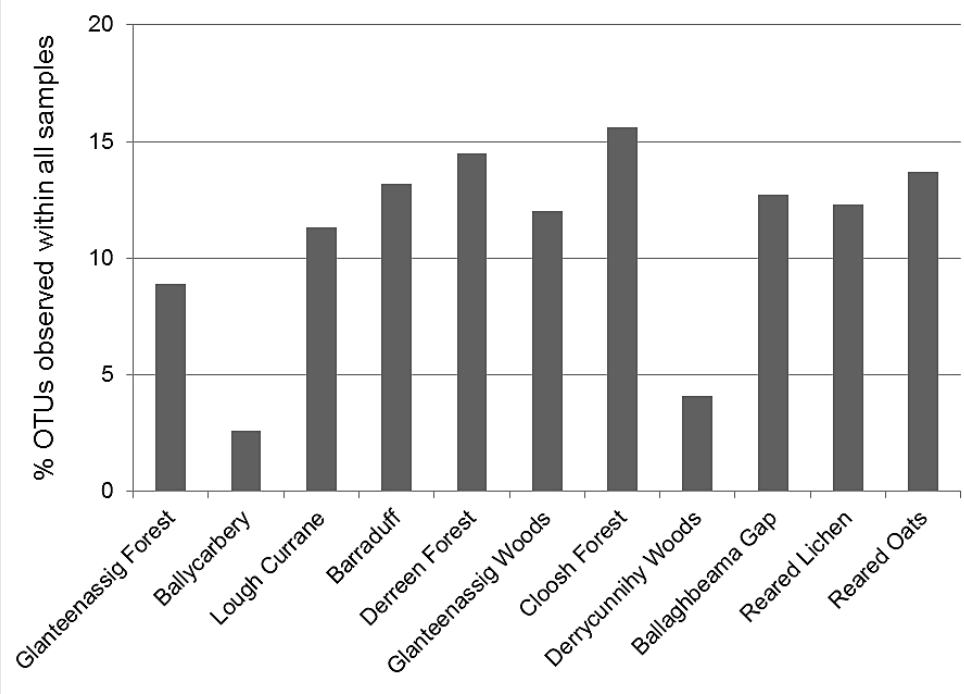

Figure 16. Percentage of OTUs shared between all individuals at each site/fed on the same diet (N = 3). Samples from Raferigeen and Crookhaven are not included as only one and two samples respectively were taken at these locations.

4.5. Discussion

The faeces samples of *G. maculosus* were found to harbour a very diverse microbial community which differed greatly between individuals. In fact, 27 % (1,119) of OTUs were found exclusively in single specimens while only two OTUs were found in all slugs. More than 40 % of sequences that were found in the faeces samples belonged to the Enterobacteriaceae, including the most abundant OTUs (Fig. 14). Members of this family have been reported to be abundant in the guts of insects (Engel & Moran, 2013) and

gastropods, including slugs (e.g. Wilkinson, 2010; Joynson *et al*., 2014) and snails (e.g. Watkins & Simkiss, 1990; Charrier *et al*., 2006; Cardoso *et al*., 2012). Enterobacteriaceae are part of the gut flora of many animals as well as humans and are also frequently found in water and soil (O'Toole, 2003) and their dominance within the faeces samples suggests that they might be of some importance for *G. maculosus*. In fact, several species within this family including *Citrobacter freundii*, which was one of the observed core OTUs, possess cellulolytic and xylanolytic activity (Anand *et al*., 2010) and might serve a key role in the digestion for the slug which primarily feeds on lichens and bryophytes (e.g. Platts & Speight, 1988; Reich *et al*., 2012). Interestingly, when eliminating the reared slugs from the analysis, two more core OTUs were found including a member of the Acetobacteraceae (Alphaproteobacteria: Rhodospirillales) and a member of the Isospheraceae (Planctomycetia: Gemmatales), both of which were very abundant in the samples with nearly 4,000 sequences each. So while surprisingly few core OTUs were found within all samples, it is likely that several members of the above mentioned families have similar functions within the host but that the bacterial phylotypes vary according to the habitat the slugs were found.

An investigation of lichen associated bacteria found members of Acidobacteria, Alphaproteobacteria (Rhizobiales and Rhodospirillales), and Gammaproteobacteria (Hodkinson & Lutzoni, 2009), all of which were usually abundant throughout our samples (Fig. 13). Interestingly, Acidobacteria, Acetobacteraceae (Alphaproteobacteria: Rhodospirillales) and Methylocystaceae (Alphaproteobacteria: Rhizobiales) were not found in the faeces of the oat-fed slugs but were present in all other specimens indicating that these microbes could have been introduced through the consumption of lichens.

Gut microbiome of *G. maculosus*

The percentage of OTUs shared between all three individuals collected from the same site was highest at Cloosh Forest with 15.6 % and lowest at Ballycarbery (2.6 %) (Fig. 16). Where six individuals were sampled per habitat, the highest proportion of shared OTUs was observed within conifer plantations (4.4 %) and the lowest in deciduous forests (Appendix 8). This result highlights the large variability of microbes observed within the faeces collected from each site and habitat and is reflected by the absence of any clear clustering by either habitat or sample site which was observed in the PCoA plots. While the PERMANOVA found the centroids of the sample sites to be significantly different, the significant result obtained from the PERMDISP analysis indicates that this might be due to unequal dispersions rather than significant differences in the centres (Anderson, 2001). As the lichen and bryophyte species which *G. maculosus* feeds on are likely to differ between habitats and even between the trees and rocks within one site, so are the associated microbes which have been ingested from the environment through feeding. Taking into account that the species does not cover large distances within its habitat (Mc Donnell & Gormally, 2011; Reich *et al*, in prep.), the big differences that were observed even between the faecal bacterial communities of specimens collected from the same site can likely be attributed to the micro-structuring within their habitat. Interestingly, conifer plantations was the only habitat which was clustering closely together in the PCoA plot using the unweighted UniFrac distances and the highest proportion of shared OTUs was observed in conifer plantations and in particular Cloosh Forest. This could be tied to the observed lower species richness of lichens and bryophytes found in British and Irish plantations compared to semi-natural woodlands (Humphrey *et al*., 2002; Coote *et al*., 2007) resulting in less diverse food sources for *G. maculosus* and hence a less variable microbial community.

Gut microbiome of *G. maculosus*

Only 2.3 % of all OTUs observed within the reared specimens were shared between all six slugs (Appendix 8). This is surprising, as these six slugs were from the same parent and were reared on identical substrate before the samples were taken and indicates that diet has a major influence on the composition of their gut microbiome. However, the two OTUs which were observed in all of the wild specimens were also found in the faeces of the reared specimens which suggests that there is at least a limited vertical transfer of gut microbes from the eggs to the slugs. The only other study on gastropods which researched the aspect of vertical transfer is from Ducklow *et al*. (1981) who found that there was no transmission of the internal microbiome from parents to their young in freshwater snails, albeit their study was based solely on cultured isolates representing only a small fraction of the total microbial community. Another possible route for vertical transmission in *G. maculosus,* which was not tested in this study, is through coprophagy: slugs of this species have frequently been observed to eat their own as well as other slug's faeces (pers. obs.) which could facilitate the transfer of microbes between specimens.

Due to the very small size of the juvenile's faecal pellets, we were forced to wait three weeks before we could collect sufficient faecal material for DNA extraction. This may have biased the end results, as the DNA extraction was from a composite sample. While the samples were not subjected to changing environmental conditions or shifts in temperatures, a recent study (Menke *et al*., 2015) describes that facultatively aerobic and aerobic bacteria increase while anaerobic bacteria decrease within faecal samples over time, thus affecting the final proportions of taxa. A more precise way of describing the microbial communities found within hatchlings, would be the dissection of the juvenile slugs and the examination of the bacteria associated with their gut rather than their faeces.

4.5.1. Conclusions and future work

In conclusion we showed that the microbial communities found within the faeces samples of *G. maculosus* are highly variable even between slugs collected from the same site. We hypothesize that this reflects the significant influence of diet and patchiness of microhabitats on the composition of the microbial gut community of *G. maculosus*. Only two core OTUs were found within the faeces samples of all of the wild and the reared slugs and, as these were also likely of benefit to the slug, we suggest that they could have been vertically transmitted from the egg to the slug during hatching.

Future work should examine the bacterial communities found in the habitats and on the food-plants of *G. maculosus*. Additionally, a functional analysis of the microbes found within the gut of *G. maculosus* could give indications to the role these organisms might have within their host.

Chapter 5:

General Discussion

5. General Discussion

This doctoral thesis aimed at furthering the knowledge of *G. maculosus* by employing both, ecological and molecular methods. The particular aims were:

1. To investigate the genetic variability within the Irish and Iberian populations of *G. maculosus* with a view to determining the origin of the Irish population

2. To assess the suitability of the mark-recapture method for estimating population sizes of *G. maculosus* and the sympatric *L. marginata* using visible implant elastomers and to determine the optimal time for a mark-recapture survey. The impacts of forestry management practices on both species were also explored.

3. To determine the influence of sample site, diet and vertical transfer on the microbial signature of the faeces of the Irish *G. maculosus* and to identify the range of bacterial phylotypes found within the faeces of *G. maculosus* with view to identifying habitat specific and core microbiome members.

5.1. Discussion

Geomalacus maculosus is a Lusitanian slug species and, with a view to identifying the origin of the Irish population, we determined the genetic diversity throughout its range using the two mitochondrial markers 16S rRNA and COI as well as one nuclear marker ITS-1. We observed that the genetic variability of the Irish *G. maculosus* specimens was greatly reduced in comparison to the Iberian specimens to an extent that the sampled Irish populations could not be distinguished using mitochondrial markers. Theoretically, the observed low genetic diversity of the Irish *G. maculosus* could have

arisen from a population bottleneck due to a near extinction event within Ireland occurring during the last glacial maximum (LGM). However, none of the observed Irish ITS-1 haplotypes were unique and the genetic distance between Irish *G. maculosus* and slugs from the north coast of Spain was significantly less compared to differences found between some Iberian populations. This highlights a strong link between Irish and Iberian specimens and a post-glacial introduction rather than survival within a refugium in Ireland seems more likely based on our findings. This is also supported by Clark *et al*. (2012) who suggest that no ice-free corner existed in Ireland during the LGM.

While we could not pinpoint the exact origin of the Irish population, the monophyletic grouping of the Irish *G. maculosus* with specimens from Northern Asturias and Cantabria make it reasonable to assume that it originated from the North of Spain. This is corroborated by the genetic similarities of people from Ireland and the Basque country (Hill *et al*., 2000) which indicates an early connection between these two countries as well as by trade links between Ireland and Spain that have been shown to reach back as far as the Mesolithic (Corbet, 1961; Cunliffe, 2001). The time of introduction is uncertain and even when an accelerated rate of 10 % divergence of the 16S rRNA is assumed, the fragment obtained with our primer set was too short (255bp) to detect any mutation more recent than about 30,000 years ago. As a post-glacial colonisation would have happened less than 20,000 years ago, the whole 16S rRNA gene (~1,500bp) or a faster evolving marker such as microsatellites (in particular simple single repeats) could have provided a better background for the exact timing of separation and related lineages. However, due to financial constraints, this was not possible in this study. Sampling of further *G. maculosus* populations along the north coast of Spain might yield the source population and more insights into the timing and mode of

introduction could be gained by investigating possible connections between this place and Ireland. Another approach could be to employ Approximate Bayesian Computation (ABC) to unravel the origins of the Irish population but this methodology also necessitates a faster evolving marker.

Our results also found that the Iberian *G. maculosus* populations were highly structured with low intra- and high inter-population genetic diversity. This indicates that these populations diverged allopatrically and survived the ice ages in different Iberian refugia during the Pleistocene (Gómez & Lunt, 2007) and in the case of the populations located south of the river Douro even possibly during the Pliocene. Further analyses such as a nested clade analysis could give insights into the population history of *G. maculosus* and the timing of population expansions and contractions. In combination with geological events these data can also help to estimate an approximate divergence rate for the 16S rRNA of *G. maculosus* (see Pinceel *et al*., 2005b). However, more than one locus should be investigated when inferring historical demographic events and as the ITS-1 region we sequenced showed only little variability, a faster evolving marker should be sequenced before employing these methodologies. Future research should also investigate whether the highly divergent Iberian populations are reproductively isolated using an array of different genetic markers as well as morphological and behavioural studies.

The findings of this study have two major implications for *G. maculosus*:

1. The low genetic diversity of the Irish population

   This is critical due to the relative rarity of *G. maculosus* and its association with mainly undisturbed habitats which are under threat from land modification or pollution (NPWS, 2010). While

the conservation status of the Irish population is favourable, that of the Spanish populations is not and the conservation status of the Portuguese populations is unknown (Eionet, 2014). Additionally, future climate change (Moreno *et al*., 2005) or habitat modifications (NPWS, 2010) could render the habitat conditions unsuitable for *G. maculosus* in Iberia. However, several introduced slug species are successfully colonising new areas despite originating from a small founding population (e.g. Allendorf & Lundquist, 2003; Pinceel *et al*., 2005a) and as *G. maculosus* populations in Ireland are expanding, indications are that the limited genetic variability is not posing any problems for this species.

2. The (most likely) human mediated introduction of the Irish *G. maculosus*

   Despite it's possibly 'unnatural' arrival in Ireland, in the light of its restricted distribution and especially in the context of climate change where predicted temperature rises (Moreno *et al*., 2005) could threaten Iberian *G. maculosus* populations while the range of Irish *G. maculosus* is likely to expand with milder and wetter winters (Coll *et al*., 2012), its protection status within Ireland should be upheld. Additionally, *G. maculosus* is not an agricultural pest but nevertheless its presence within Ireland may affect native species occupying similar niches, such as *L. marginata* which is widespread in Ireland. Possible impacts of the presence of *G. maculosus* on this and other species need to be investigated.

Despite of its protected status, there is no standardised method for the estimation of population sizes of *G. maculosus* in its associated habitats. Consequently, a mark-recapture experiment was carried out in six different sites within Cloosh Forest, the first conifer plantation

from which the species was reported in 2010 (Kearney, 2010). It was the first long-term survey for estimating the population densities of both *G. maculosus* and sympatric *L. marginata* using visual implant elastomers as markers which were found to be long-lasting and did not affect the survival of the slugs. While no difference in capturability between adults and sub-adults was observed, the assumption that the species had no trap response did not hold: the goodness-of-fit test that was carried out for the Jolly-Seber method found that previously captured *G. maculosus* specimens had a higher capture probability than those which were not previously captured. The trap response of an animal can lead to biased population size estimates when applying the mark-recapture approach as the percentage of marked individuals found below the traps might not reflect that of marked individuals in the actual population (Greenwood, 1996). As the slugs were observed to be trap-happy, this results in a subsequent underestimation of their population size. The repeated finding of the same individuals below the traps, an observation which had been mentioned previously for *G. maculosus* by McDonnell and Gormally (2011a), also probably indicates a homing behaviour in both species which would lead the slugs to return to the same traps after foraging. The result of this study supports this in that an average of 9 % *G. maculosus* and 15 % of *L. marginata* which were marked in the first period were still being found below the traps in the final period. Additionally, slugs captured in the final period were marked three (*G. maculosus*) and four (*L. marginata*) times on average and the maximum amount of different colour tags within one single specimen was nine (*G. maculosus*) and eight (*L. marginata*) respectively indicating that most specimens were present within the same plot for several months. To further test this hypothesis, a small mobility experiment was carried out between December 2013 and July 2014 where 33 *G. maculosus* were injected with pet microchips for individual marking and released beneath a metric refuge trap

wrapped around a tree. This tree was surrounded by 23 further trees all of which had metric band traps installed. The experiment found no tagged *G. maculosus* behind any of the surrounding trees until nearly four months after the start of the survey when six specimens were located on five different trees between one and seven meters away from the release tree, while three specimens were recaptured six times in a row beneath the traps on the release tree during the same period (Appendix 9). These results indicate that, at least when traps are employed, *G. maculosus* is faithful to its habitat and does not move much between trees. This obviously has implications for the use of mark-recapture experiments as marked slugs may remain beneath the traps and consequently the resulting population size estimates are underestimated. Possible counter actions could be to move the traps between the different primary periods or to not release the slugs directly behind the traps from which they were removed but rather place them on the ground in the middle of the plot. Something that will have to be investigated is whether the decay of the epiphytes which grow beneath the metric refuge traps affects the capture success of the slugs. While a previous study (Reich *et al*., in prep.) found no effect using baited refuge traps this does not necessarily apply when using unbaited traps. A possible method could be to compare the capture success using unbaited metric refuge traps which had been installed for different periods of time. These traps should be installed in one site on neighbouring trees with similar epiphyte cover to eliminate the effect of habitat variability.

Our results show that *G. maculosus* was consistently found in greater numbers than *L. marginata*. While this indicates that *G. maculosus* is more suited to the conditions found in conifer plantations and is the dominant slug species on tree trunks in this habitat, many more replicates are needed from other plantations and habitats to draw any definite conclusions about the general relationship between

these two species. It is also possible that the trapping method is biased towards *G. maculosus*. This could be tested by setting up metric refuge traps in an experimental plot where both slugs are present in equal (known) numbers and by comparing the amount of captured specimens of each species.

The distribution of *G. maculosus* and *L. marginata* was found to be highly variable throughout the surveyed plantation and significantly less slugs of both species were found in a plantation site where epiphyte cover and thickness of the epiphyte layer were significantly lower than in the other plantation sites indicating that these are important factors. This is hardly surprising as a thick epiphyte layer retains moisture and provides food and shelter for both species and our observation is corroborated by findings from a previous study (Reich *et al*., 2012) which also found epiphyte cover to be the main predictor of *G. maculosus* abundance. A future study should determine if epiphyte species richness also has an impact on the abundance of both slugs and while a small set of choice experiments have indicated that *G. maculosus* prefers foliose and fruticose lichen over crustose ones (Reich *et al*., 2012), the identification of its favoured food plants could help determine further habitat requirements of the species.

The presence of *G. maculosus* in Irish conifer plantations has also implications for forestry services which need to ensure that management practices do not negatively impact on the slug. A potential mitigation measure of the traditional clear-felling in which trees are cut down to the base was tested in this study. This modification i.e. partial clear-felling, involves the retention of three meter high tree trunks and eliminates the steps of stripping side branches and taking the trees off-site. Population densities of both *G. maculosus* and *L. marginata* were found to be significantly greater in this site compared to the traditional clear-fell, indicating that this

method seems to offer, at least in the short term, favourable conditions for *G. maculosus*. However, many more replicates are needed especially since our survey also showed how patchy the populations of both of these species are with population sizes varying dramatically between plantation sites that were only a few hundred meters apart. A survey undertaken pre- and post-felling would have also given better estimations of the direct impact of traditional and partial clear-felling on the population sizes of the slugs but no felling operations took place in relevant sites during the time of the study.

In conclusion, the mark-recapture method using unbaited metric refuge traps and visible implant elastomers is a useful tool for estimating population densities of *G. maculosus* and *L. marginata* and, in contrast to a simple count, populations can be monitored for a set time frame using this approach. This would be especially appropriate for forestry managers who want to investigate the impact of management operations on *G. maculosus*: by comparing population densities before and after management practices such as felling take place, informed decisions can be made on which methods to employ in the future. It can also be monitored whether marked animals reside within a site during operations such as felling or whether the slug populations found post-felling consist largely of unmarked individuals which re-populated the area. The trap-happiness and the subsequent underestimation of the actual population size should be of little concern as in the case of forestry operations where the objective is likely to be the selection of a certain site over another depending on how many slugs are observed there, the precision of these estimates is not relevant, but rather the values in comparison. When setting up a survey we recommend using at least 30 trapping trees and to conduct the survey between May and October to ensure sufficient captures for the estimation of the population sizes. Using a single census is not recommended as we

have shown that capture success of both *G. maculosus* and *L. marginata* is strongly influenced by weather conditions and therefore, data obtained in this way could simply reflect the sampling conditions rather than providing a realistic picture of the actual number of slugs present. However, if no estimation of population size is required and time is restricted, multiple capture-removal censuses, where animals are not marked but removed from the site to avoid counting individuals more than once, could prove useful to compare abundances between sites (as in Reich *et al*., 2012). In this case it is especially important to sample only during conditions where capture probabilities are high and which have been identified in this study.

The environment which a species inhabits is one of the main factors which influences the microbial community within its gut (e.g. Engel & Moran, 2013; Newton *et al*., 2013). To examine the effect of sample site and habitat as well as diet and vertical transfer on the faecal microbiome of *G. maculosus*, bacterial 16S rRNA sequences were obtained with next generation sequencing. We identified more than 4,000 bacterial phylotypes within the faeces samples which were extremely variable even within the faeces of specimens collected from the same site. In the absence of a clear separation of the faecal samples by sample site or habitat in the PCoA plots, we concluded that the microbial signature is instead reflective of the microbial community of the microhabitat such as the individual tree or rock from which the slug was collected. This is corroborated by the fact that many of the bacterial families we observed were also found on lichen (Hodkinson & Lutzoni, 2009), one of the main food sources of *G. maculosus*. Additionally, the highest proportion of shared OTUs was observed in conifer plantations which could be due to the lower species richness of epiphytes in this habitat, at least compared to semi-natural woodlands (Humphrey *et al*., 2002; Coote *et al*., 2007).

To support this theory, sequences from the microhabitats in which the slugs were collected could be obtained. Alternatively, slug faeces sampled from the same rock or tree could be compared to those collected from different trees within the same site to test if they would be more similar. More samples obtained from each site and a balanced sampling design would have increased the statistical power and would have allowed to calculate a two-way nested PERMANOVA which could have tested whether habitat or sample site had a larger effect on the similarity of microbial communities.

There is still some uncertainty about which food plants *G. maculosus* consumes in the wild and observational experiments in the laboratory are time consuming and therefore only focus on a few selected species. As we suspect a high association between the microbial communities within the faeces of *G. maculosus* and those in its microhabitat, a future study could investigate whether the diet of the slug can be inferred from the microbial signature found in its faeces. The first step would be to establish if the microbial communities found on the food plants are species-unique. If this is true, a number of slugs should be fed a particular species of lichen and the faecal microbiome alongside that of the lichen species should be compared to see to what extent the lichen associated microbes are present in the faeces. Additionally, comparisons of the gut microbial communities of *G. maculosus* with that of *L. marginata* or even more generalist slugs such as *Arion vulgaris* or *Deroceras reticulatum* could aid to give insight into why the distribution of *G. maculosus* is restricted within Europe. While factors such as climate and habitat availability are likely to be the main determinants for the presence of the species, food might also be of importance. Its absence from localities with underlying limestone geology in Ireland even when these are located within areas inhabited by the slug (Platts & Speight, 1988) could indicate that *G. maculosus* has an inability to

metabolise secondary compounds found in food plants growing in limestone habitats. This in turn might be reflected in their gut microbial community when compared to slug species thriving in limestone areas.

Only two core OTUs were observed in the faeces samples including those of the six juvenile slugs. This finding indicates that there is only a very limited vertical transfer of gut microbes via the egg in *G. maculosus* or that many microbes which were taken up during the hatching process do not permanently colonise the gut. As at least one of these core OTUs has a proven xylanolytic and cellulolytic ability it can be assumed that it might be selected by the host as it aids its digestion. A future study should focus on the autochthonous bacteria within the gut of *G. maculosus* which involves starving the slugs for at least 72 hours prior to sampling to purge them from transient bacteria (Wilkinson, 2010; Cardoso *et al*., 2012). The microbial community obtained in this way should then consist of more persistent members which may indicate their proceedings to the fitness of *G. maculosus*. A functional analysis of the most abundant phylotypes could then give insight into their role within the gut microbiome of *G. maculosus*.

A problem that could have occurred with our sampling design concerns the contamination of the faeces with slug mucus and the subsequent presence of non-gut-associated microbes within the samples. However, every care was taken to remove the faeces as quickly as possible from the petri dish after defecation and hence the contamination should have been minimal. An alternative to faeces sampling is the removal and subsequent sequencing of the slug's gut (e.g. Joynson *et al*., 2014) but also in this procedure contamination can occur during the dissection. A distinct disadvantage of the dissection method is that it involves the killing of the organism and a

continuous assessment of the same animal over time is not possible. This would be necessary if one wants to investigate shifts in microbial communities due to dietary or other changes in the same animal. However, in the case of our juvenile slugs where sampling of sufficient faecal matter was only possible after three weeks, this method would provide a good alternative. When selecting either method it has to be noted that the microbial communities change depending on which part of intestinal tract is sampled: Cardoso *et al*. (2012) found that the bacterial community structure in the faeces of *A. furcata* was different to that in the crop fluid of the species. This highlights the importance of only comparing samples obtained in the exact same way.

5.2. Key findings and conclusions

- The population genetic study shows that the Irish *G. maculosus* population has a low genetic variability and while its exact origin could not be determined, a great genetic similarity to specimens from Northern Asturias and Cantabria was observed. The Iberian *G. maculosus* populations are highly structured and the large mitochondrial sequence divergences of often more than 10 % between populations indicate long-term allopatric divergence.

- The mark-recapture study shows that population densities of *G. maculosus* and *L. marginata* are highly variable even within a single forest and a thick epiphyte cover of the trunk is an important factor for both species. In the surveyed plantation, *G. maculosus* was the dominant slug species in every site where the species co-occurred and it was found that the partial clear-fell was, at least in the short term, a suitable

mitigation method where populations of *G. maculosus* and *L. marginata* populations could be maintained post-felling. As capture success was found to be positively correlated with temperature, future surveys should ideally take place between May and October. Surveying within the forest should be avoided during heavy rainfall as capture success was negatively correlated with rainfall in the plantation sites. To ensure sufficient capture numbers for the calculation of reliable population density estimates, 30 trapping trees should be installed in commercial plantation sites. The trap-happiness of *G. maculosus* and the subsequent underestimation of its population sizes should be kept in mind.

- The study of the microbial diversity within the faeces of *G. maculosus* found that the microbial communities are highly variable even between slugs collected from the same site. The two core OTUs which were found within all slugs are likely beneficial symbionts of *G. maculosus* and we suggest that they could have been vertically transmitted from the egg to the slug during hatching.

It was shown that the Irish *G. maculosus* was most likely post-glacially introduced from the North of Spain and that the Irish population has a greatly reduced genetic variability, which could impact negatively on its ability to cope with environmental changes, however, the current expansion of the species within Ireland suggests otherwise. Here the range of the species has spread from largely undisturbed habitats to commercial conifer plantations and it was established that a deep epiphyte cover is an important factor for *G. maculosus* in this habitat as well as for the sympatric species *L. marginata* which was found in fewer numbers than *G. maculosus* in

the surveyed plantation Cloosh Forest. As *G. maculosus* is a protected species, forestry operations have to take its vulnerability into account and consequently a methodology which can be used to investigate population sizes of the species before and after management operations has been established in this study. The gut microbiome of *G. maculosus* was found to be very variable and the majority of bacteria found within their faeces are likely associated with the diet and microhabitat of the species. However, two bacterial phylotypes were identified that are suspected to be vertically transferred via the egg and which might fulfil important roles within the gut of the slug. This indicates that there might be symbionts that *G. maculosus* relies on for its survival and further research on slug gut microbiomes might yield information as to why the distribution of this species is so limited.

5.3. Recommendations for further study

- Further studies should be undertaken to determine the exact origin of the Irish *G. maculosus* as this could give further insight into the mode and timing of its introduction. These should involve further sampling of slug populations from additional locations within Iberia as well as the sequencing of additional genetic markers which could then also allow for analyses into the phylogeographic history of the Iberian populations. In combination with morphological studies, it should be established whether the highly diverged Iberian populations are already reproductively isolated.

- The response of *G. maculosus* to forestry management operations such as different types of clear-felling should be investigated at several more sites using the mark-recapture

method to obtain population sizes before and after felling. The results can inform Coillte (Ireland's leading forestry company) and other management operatives as to which procedures would allow the maintenance of the species in conifer plantations.

- Ecophysiological factors that limit the distribution of *G. maculosus* should be identified to inform effective habitat management for its conservation. Food plants could be determined with DNA metabarcoding of its faeces samples. Comparing the gut symbionts of *G. maculosus* with those of more widespread species might reveal why it is not found in certain habitats such as limestone areas.

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Appendices

Appendix 1. Mean p-distances within (diagonal, bold) and between (down) all sampled *Geomalacus maculosus* populations for 16S rRNA (**a**), COI (**b**) and ITS-1 (**c**) datasets.

a **16S rRNA**	S1	S2	S3	S4	S5	S6	S7	P1	P2	P3	IRE
S1	**0.052**										
S2	0.067	**0.007**									
S3	0.059	0.067	**0.003**								
S4	0.058	0.067	0.013	**0.008**							
S5	0.07	0.082	0.037	0.029	**0.005**						
S6	0.079	0.080	0.048	0.044	0.050	**0.014**					
S7	0.128	0.137	0.112	0.104	0.107	0.106	**0.007**				
P1	0.075	0.050	0.062	0.059	0.067	0.075	0.133	**0.000**			
P2	0.121	0.125	0.101	0.093	0.101	0.087	0.022	0.122	**0.000**		
P3	0.125	0.129	0.104	0.097	0.105	0.091	0.025	0.122	0.012	**0.000**	
IRE	0.074	0.082	0.037	0.038	0.044	0.048	0.106	0.079	0.091	0.099	**0.000**

b COI	S1	S2	S3	S4	S5	S6	S7	P1	P2	P3	IRE
S1	**0.037**										
S2	0.072	**0.006**									
S3	0.088	0.086	**0.004**								
S4	0.086	0.083	0.010	**0.002**							
S5	0.071	0.069	0.028	0.019	**0.005**						
S6	0.114	0.114	0.083	0.076	0.082	**0.001**					
S7	0.104	0.100	0.084	0.082	0.077	0.113	**0.047**				
P1	0.080	0.058	0.067	0.065	0.057	0.098	0.090	**0.004**			
P2	0.101	0.106	0.097	0.095	0.090	0.120	0.041	0.098	**0.001**		
P3	0.101	0.103	0.102	0.099	0.092	0.124	0.041	0.105	0.008	**0.000**	
IRE	0.082	0.086	0.030	0.025	0.027	0.081	0.087	0.065	0.103	0.107	**0.000***

* this value is not zero but due to rounding is shown as 0.000.

c ITS-1	S1	S2	S3	S4	S5	S6	S7	P1	P2	P3	IRE
S1	**0.001**										
S2	0.003	**0.003**									
S3	0.003	0.003	**0.000**								
S4	0.003	0.003	0.000	**0.000**							
S5	0.004	0.004	0.002	0.002	**0.002**						
S6	0.009	0.009	0.008	0.003	0.009	**0.004**					
S7	0.002	0.003	0.002	0.002	0.003	0.008	**0.002**				
P1	0.004	0.005	0.004	0.004	0.005	0.007	0.004	**0.004**			
P2	0.016	0.017	0.016	0.016	0.017	0.022	0.018	0.018	**0.001**		
P3	0.003	0.004	0.002	0.002	0.003	0.009	0.002	0.002	0.016	**0.000**	
IRE	0.006	0.005	0.004	0.004	0.005	0.011	0.007	0.007	0.019	0.006	**0.002**

Appendix 2. Node ages in millions of years (Myr) and 95 % highest posterior density intervals (HPDI) for the 16S rRNA dataset and three different divergence rates obtained for gastropods from the literature in ascending order of age.

Divergence rate	2 %		5.4 %		10 %	
Splits	Node age (Myr)	95 % HPDI	Node age (Myr)	95 % HPDI	Node age (Myr)	95 % HPDI
1 P2 -- P3*	0.72*	0.19-1.43	0.27*	0.08-0.53	0.14*	0.04-0.29
2 S3 -- S4	0.9	0.31-1.73	0.33	0.11-0.63	0.17	0.06-0.34
3 P2/P3 -- S7	1.52	0.72-2.58	0.56	0.26-0.95	0.3	0.14-0.51
4 S3/S4 -- S5*	2*	0.93-3.27	0.75*	0.38-1.26	0.4*	0.2-0.68
5 IRE -- S1-4**	2.16**	1.1-3.6	0.81**	0.41-1.35	0.43**	0.21-0.7
6 S2/S1-3 -- P1	3	1.46-4.86	1.04	0.58-1.83	0.6	0.32-1
7A IRE/S1-4 -- S3/S4/S5**	3.03**	1.76-4.5			0.61**	0.35-0.91
8A IRE/S1-4/S3/S4/S5 -- S6	3.75	2.24-5.71			0.75	0.45-1.15
7B IRE/S1-4 -- S6**			1.11**	0.6-1.6		
8B IRE/S1-4/S6 -- S3/S4/S5			1.4	0.82-2.12		
9A IRE/S1-4/S3/S4/S5/S6 -- S2/S1-3/P1**	6.23**	3.69-8.95				
10A IRE/S1-4/S3/S4/S5/S6/S2/S1-3/P1 -- S1	7.38	4.72-10.87				
9B S2/S1-3/P1 -- S1*			2.08*	1.26-3.16	1.13*	0.69-1.69
10B IRE/S1-4/S3/S4/S5/S6 -- S2/S1-3/P1/S1			2.73	1.71-4	1.47	0.94-2.16
11 IRE/S1-4/S3/S4/S5/S6/S2/S1-3/P1/S1 -- P2/P3/S7	13.63	7.81-22.48	5.08	2.84-8.23	2.73	1.57-4.47
Age of Irish cluster	0.43	0.14-0.86	0.16	0.05-0.32	0.09	0.03-0.18

* posterior probability < 0.95, ** posterior probability < 0.5. A and B indicate alternative splits observed in the data.

Appendix 3. Environmental measurements at sites P1, P3 and P4.

Site	CBH [cm]	Bark structure	Epiphyte cover	Epiphyte thickness (bottom trunk)	Epiphyte thickness (trunk above trap)	Epiphyte thickness (average)	Light intensity at the centre of the plot [lux]
P1	98	2	3	2	1	1.5	500
	57	1	3	3	2	2.5	
	78	1	3	3	2	2.5	
	25	1	3	2	1	1.5	
	64	1	3	3	2	2.5	
	54	1	3	2	2	2	
	43	1	3	2	2	2	
	86	1	3	2	3	2.5	
	65	1	3	2	2	2	
P3	170	3	2	2	2	2	300
	61	2	3	3	1	2	
	103	1	3	3	1	2	
	69	1	2	3	1	2	
	84	1	3	3	2	2.5	
	116	1	3	3	3	3	
	122	1	2	3	2	2.5	
	52	1	3	3	1	2	
	123	1	3	3	3	3	
P4	66	1	1	1	1	1	10,000
	27	1	2	2	1	1.5	
	80	1	2	2	1	1.5	
	37	2	2	1	1	1	
	33	1	3	1	1	1	
	72	1	3	1	1	1	
	62	2	2	1	1	1	
	60	3	1	1	1	1	
	39	1	3	1	1	1	

CBH: circumference at breast height; Bark structure: 1: smooth, 2: some cracks/flaking bark, 3: many cracks/flaking bark; Epiphyte cover: 1: < 30%, 2: 30-60%, 3: > 60%; Epiphyte thickness: 1: < 0.5cm, 2: 0.5cm - 1cm, 3: > 1cm.

Appendix 4. Formulae of the Schnabel (**a**) and Jolly-Seber (**b**) methods.

a Schnabel method

1. Population size estimate

$n_i =$ number of animals in the ith sample

$m_i =$ number of animals in the ith sample that are already marked

$u_i = n_i - m_i =$ number of unmarked animals in the ith sample

$M_i = \sum_{j=1}^{i-1} u_j =$ number of animals marked prior to the ith sample

$\hat{N} =$ population size estimate

$A = \sum n_i M_i^2$

$B = \sum m_i M_i$

$C = \sum m^2{}_i / n_i$

$\hat{N} = A/B$

2. Confidence intervals

$t =$ Student's t for $S-2$ degrees of freedom at the 5% significance level

$A / [B \pm t\sqrt{(AC - B^2) / (S-2)}$

3. Goodness-of-fit test

If the resulting G-value is smaller than the critical χ^2 value at 5 % significance for S-2 degrees of freedom, the regression line is a satisfactory fit for the data and the estimate is unbiased. If any observed or expected value was < 1, consecutive samples were added together (Greenwood, 1996).

$O_j =$ the jth value of the observed m_i and u_i

$E_j =$ the jth value of the expected m_i and u_i

expected $m_i = M_i n_i / \hat{N}$

expected $u_i = n_i -$ expected m

$G = 2\sum O_j \log_e(O_j / E_j)$

b Jolly Seber method

1. Population size estimate

$n_i =$ total number of animals caught in the *i*th sample
$R_i =$ number of animals that are released after *i*the th sample
$m_i =$ number of animals in the *i*th sample that were previously marked
$m_{hi} =$ number of animals in the *i*th sample that were last caught in the *h*th sample
$r_h =$ number of animals released from the *h*th sample and were subsequently recaptured
$z_i =$ number of animals caught before and after the *i*th sample but not in the *i*th sample
$\hat{M}_i = m_i + (R_i + 1)z_i / (r_i + 1) =$ number of marked animals in the population when the *i*th sample is taken
$\hat{N}_i = \hat{M}_i(n_i + 1) / (m_i + 1) =$ population size estimate at the time of the *i*th sample

2. Confidence intervals

$$T_i = \log_e \hat{N}_i + 0.5\log_e[0.5 - 3n_i / 8\hat{N}]$$

$$s_{Ti} = \sqrt{(\frac{\hat{M}_i - m_i + R_i + 1}{\hat{M}_i + 1})(\frac{1}{r_i + 1} - \frac{1}{R_i + 1}) + \frac{1}{m_i + 1} - \frac{1}{n_i + 1}}$$

$$T_{iL} = T_i - 1.65s_{Ti}$$

$$T_{iU} = T_i + 2.45s_{Ti}$$

$$W_{iL} = e^T iL$$

$$W_{iU} = e^T iU$$

$(4W_{iL} + n_i)^2 / 16W_{iL} =$ Lower confidence limit

$(4W_{iU} + n_i)^2 / 16W_{iU} =$ Upper confidence limit

3. Goodness-of-fit test

If the resulting G-value is lower than the critical χ^2 value at 5 % significance for S degrees of freedom, there is no difference between the capture probabilities of previously marked and unmarked animals.

$f_{1i} =$ first captured before this sample, subsequently recaptured

$f_{2i} =$ first captured before this sample, not subsequently recaptured

$f_{3i} =$ first captured in this sample, subsequently recaptured

$f_{4i} =$ first captured in this sample, not subsequently recaptured

$$n_i = f_{1i} + f_{2i} + f_{3i} + f_{4i}$$

$$a_{1i} = f_{1i} + f_{2i}$$

$$a_{2i} = f_{3i} + f_{4i}$$

$$a_{3i} = f_{1i} + f_{3i}$$

$$a_{4i} = f_{2i} + f_{4i}$$

$$g_{1i} = \sum_{i=1} f_{xi} \log f_{xi}$$

$$g_{2i} = \sum_{i=1} a_{xi} \log a_{xi}$$

$$G = \sum_{i=1} 2(g_{1i} - g_{2i} + n_i \log_e n_i)$$

Appendix 5. Results from the goodness-of-fit tests for the Schnabel (**a**) and Jolly-Seber (**b**) methods.

Bold values indicate that the G-value is smaller than the critical χ^2 value at 5 % significance and that the population size estimate is unbiased and n/a indicates that no value could be obtained for these months due to low capture numbers.

a **2012/2013**	Site	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Mar**	**May**	**Jun**	**Jul**
G. maculosus	P1	**4.42***	34.09*	**3.25***	4.06**	**0.19****	n/a	**0.23****	**2.7***	**0.02****	**0.33***
L. marginata	P1	**1.08****	n/a	n/a	n/a	**0.2****	n/a	n/a	**0.65****	n/a	n/a
	P2	**0.7***	**0.14****	n/a	6.17*	n/a	n/a	n/a	n/a	**0.36****	n/a
2013/2014		**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Mar**	**May**	**Jun**	**Jul**	**Aug**
G. maculosus	P3	**6.44**	**0.52***	n/a	n/a	n/a	**0.25****	**0.79****	n/a	9.31*	**0.76***
	P4	n/a	n/a	n/a	n/a	n/a	n/a	**0.07****	**0.25****	n/a	n/a
	C1	**4.22**	**0.96**	12.9	n/a	n/a	**0.85****	**2****	**0.05****	**2.82**	**0.6**
	C2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
L. marginata	P3	**2.33***	**0.13****	**1.57****	n/a	n/a	n/a	**2.13****	n/a	**1.96****	n/a
	P4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	C1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	**0.2****	n/a
	C2	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
G. maculosus	overall								92.08^{+}		
L. marginata	overall								**17.62^{++}**		

* 2 degrees of freedom, ** 1 degree of freedom, all other samples 3 degrees of freedom; $^{+}$ 45 degrees of freedom, $^{++}$16 degrees of freedom.

b	**2012/2013**	Site	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Mar**	**May**	**Jun**	**Jul**
	G. maculosus	P1	***I***	6.75	**2.37**	9.50	5.24	**2.22**	**2.07**	**1.03**	8.77	/
	L. marginata	P1	***I***	n/a	n/a	n/a	**1.19**	n/a	n/a	n/a	n/a	/
		P2	***I***	**0**	**0.01**	**0.57**	**0.15**	n/a	n/a	n/a	**1.08**	/
	2013/2014		**Sep**	**Oct**	**Nov**	**Dec**	**Jan**	**Mar**	**May**	**Jun**	**Jul**	**Aug**
	G. maculosus	P3	***I***	n/a	n/a	n/a	n/a	n/a	**3.79**	**0.62**	10.97	/
		P4	/	45.34	n/a	n/a	n/a	n/a	n/a	**0.18**	5.8	/
		C1	***I***	n/a	3.84	n/a	n/a	n/a	**2.13**	**2.01**	**1.06**	***I***
		C2	/	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	/
	L. marginata	P3	***I***	5.21	n/a	n/a	n/a	n/a	**0**	n/a	n/a	/
		P4	/	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	/
		C1	/	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	/
		C2	/	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	/
	G. maculosus	overall										113.7^{+}
	L. marginata	overall										$\mathbf{8.22^{++}}$

$^{+}$ 18 degrees of freedom, $^{++}$ 8 degrees of freedom.

Appendix 6. Schnabel (**a**) and Jolly-Seber (**b**) population size estimates (PSE) with 95 % confidence intervals (CI).

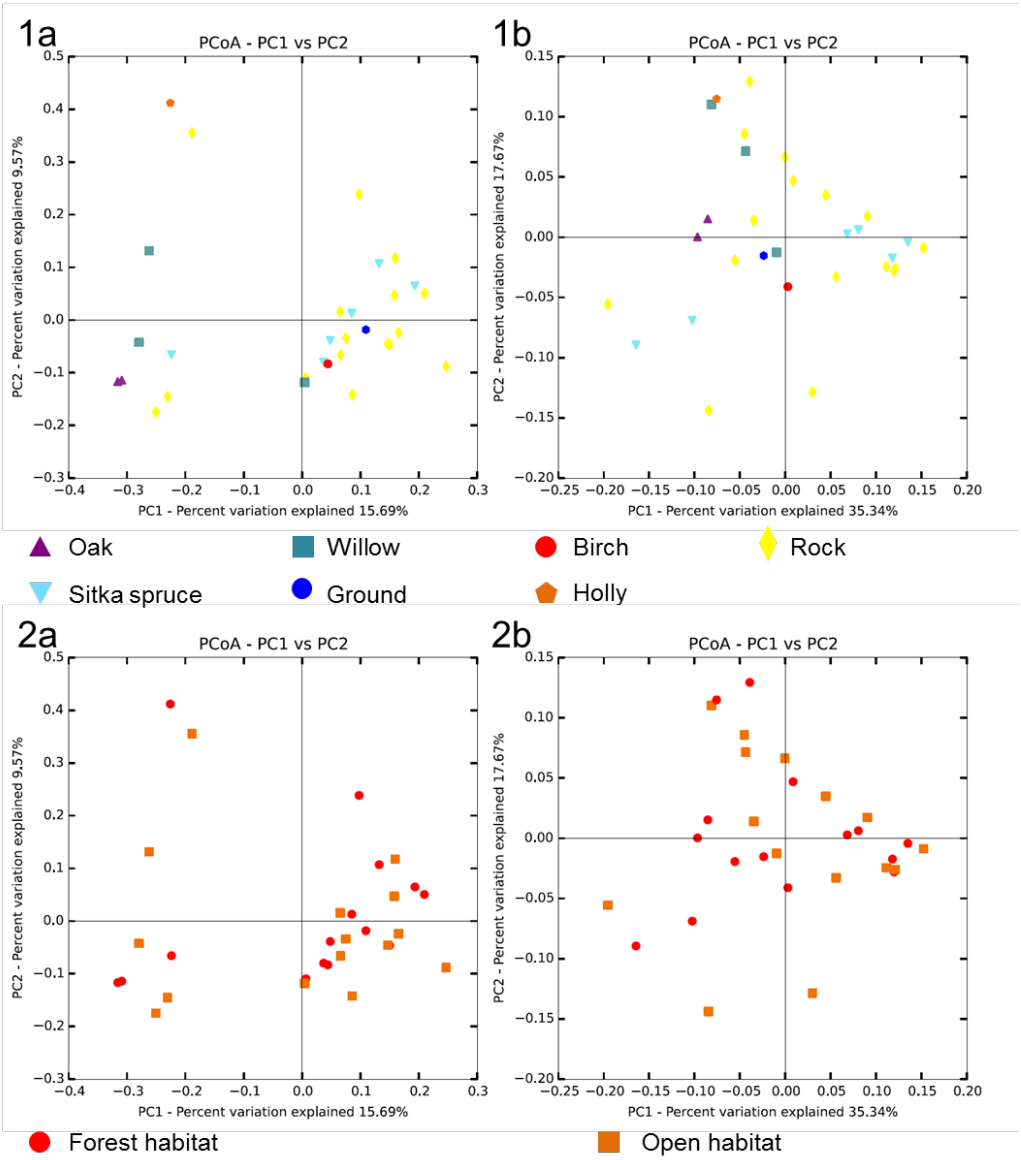
a		Site P1		Site P2		Site P3		Site P4	Site C1		Site C2
		G. maculosus	*L. marginata*	*L. marginata*		*G. maculosus*	*L. marginata*	*G. maculosus*	*G. maculosus*	*L. marginata*	*G. maculosus*
PSE	Aug-12	65.75	14.88	16	Sep-13	44.59	9.63	14.78	50.79	5	8
95 % CI		47.91 - 104.77	10.27-23.08	10.5-27.2		30.39-71.27	0.07-16.37	9.59-23.79	29.92-87.76	0.38-98.04	1.5-156.86
PSE	Sep-12	56.27	7.76	15.96	Oct-13	17.14	9.46	38	28.89	5	2
95 % CI		41.61-86.92	5.02-13.75	11.07-23.79		11.68-27.39	5.76-18.4	11.08-214.08	22.74-38.02	0.25-18.34	2
PSE	Oct-12	46.39	13.08	18.14	Nov-13	6.89	3	5	19.56	0	2.33
95 % CI		36.05-65.06	9.08-19.5	14-24.67		4.47-11.09	0.3-16.9	0.94-98.04	12.32-32.61	0	0.86-8.56
PSE	Nov-12	16.85	3	10	Dec-13	41	3	0	8	0	0
95 % CI		12.28-24.24	0.47-2.85	5.77-20.67		12.26-230.99	0.3-16.9	0	1.5-156.86	0	0
PSE	Dec-12	23.33	5.4	7	Jan-14	10.85	2.67	0	1.75	0	2.5
95 % CI		17.23-33.07	2.42-13.71	0-2.13		7.23-16.93	0.25-9.78	0	0.73-5.12	0	0.75-14.08
PSE	Jan-13	13.66	6.2	3	Mar-14	27.78	10.06	2	15.67	1	0
95 % CI		9.67-20.79	3.52-11.65	1.25-8.78		21.23-36.37	6.34-16.77	0.74-7.33	9.25-28.12	0.19-19.61	0
PSE	Mar-13	10.67	2	0	May-14	31.45	15.65	17.2	24.67	0	2

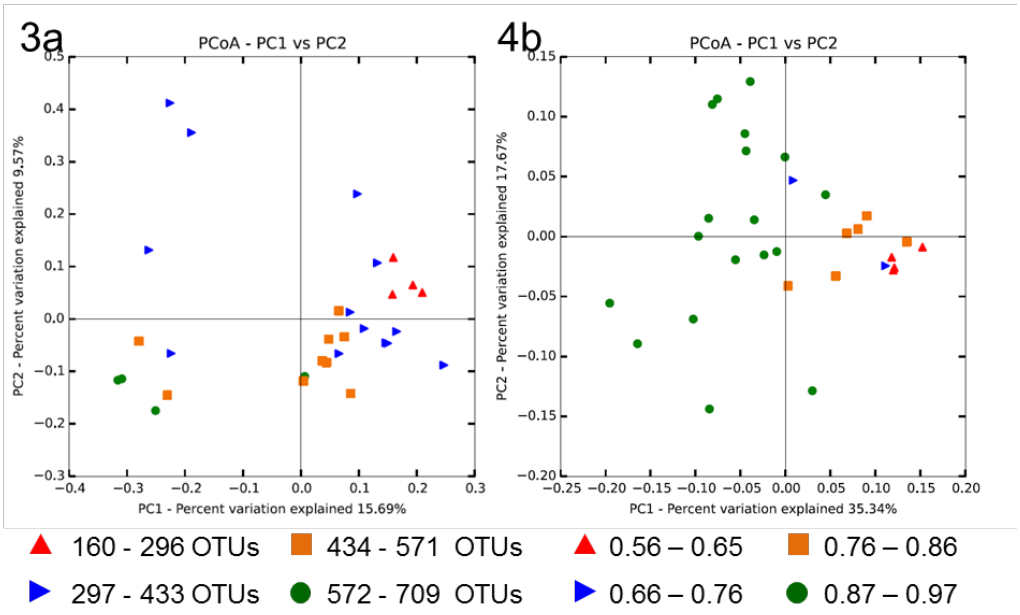
95 % CI		5.73-21.52	0.32-1.9	0		23.57-43.7	10.8-24.27	9.75-32.31	13.24-49.78	0	0.3-5.63
PSE	May-13	20.06	6.22	7.18	Jun-14	10.67	3.33	10.33	46.11	0	0
95 % CI		15.33-27.78	3.34-12.56	4.15-14.84		3.95-39.12	1.23-12.22	4.84-23.73	24.75-93.05	0	0
PSE	Jun-13	32.73	14	9.83	Jul-14	20.67	10	16.33	27.96	8.67	3
95 % CI		18.9-67.63	4.19-78.87	4.6-22.58		13.51-38.26	0.16-22.98	7.65-37.5	18.89-43.09	0.25-31.78	0.56-58.82
PSE	Jul-13	33.81	2	3.75	Aug-14	16.71	12.14	10	26.67	6	0
95 % CI		24.39-47.55	0.94-4.59	1.56-10.89		8.5-35.62	0.15-25.88	6.59-11	14.31-35.81	0.15-12.79	0

b		Site P1		Site P2		Site P3		Site P4	Site C1		Site C2
		G. maculosus	*L. marginata*	*L. marginata*		*G. maculosus*	*L. marginata*	*G. maculosus*	*G. maculosus*	*L. marginata*	*G. maculosus*
PSE	Aug-12	n/a	n/a	n/a	Sep-13	n/a	n/a	n/a	n/a	n/a	n/a
95 % CI		n/a	n/a	n/a		n/a	n/a	n/a	n/a	n/a	n/a
PSE	Sep-12	49.77	10	20.95	Oct-13	24.55	10.42	15.5	63.31	4.5	4.5
95 % CI		43.82-70.38	8.09-20.48	16.38-44.39		18.78-46.14	9.03-23.16	13.01-35.87	44.53-131.72	7.93-10.68	2.11-31.66
PSE	Oct-12	59.51	15.05	20.9	Nov-13	39.31	13.33	10	147.88	2	6.22
95 % CI		51.00-80.71	13-29.99	19.01-34.66		17.68-149.8	4.85-86.08	5.06-41.41	63.57-584.98	0.21-59.58	3.19-34.87

PSE	Nov-12	48.88	12	16.01	Dec-13	40	10.67	9	25.5	4	28
95 % CI		32.21-101.53	4.56-93.02	14-29.59		24.35-96.88	4.39-58.44	0.94-268.09	11.91-139.08	11.48-15.98	6.52-483.4
PSE	Dec-12	51.35	7	19.5	Jan-14	38.4	12	9	38	2	6
95 % CI		35.84-98.57	5-25.06	7.68-112.22		22.43-96.87	4.76-66.83	0.94-268.09	10.19-277.1	1.01-28.05	2.03-107.33
PSE	Jan-13	33.62	5.25	11.56	Mar-14	44.66	20.95	13.5	38.61	2	1
95 % CI		22.12-72.11	8.38-9.74	4.39-73.37		34.36-76.13	12.21-66.92	3.98-112.61	23.27-96.53	3.27-5.53	0.1-29.79
PSE	Mar-13	63.75	10.5	7	May-14	72.89	18.03	54	52.64	1	2
95 % CI		36.5-154.41	4.72-40.53	7		51.75-133.45	16.02-33.82	27.84-171.11	38.16-90.14	1	6.12-5.63
PSE	May-13	34.98	5.83	8.44	Jun-14	27.96	4	15	43.09	1	0
95 % CI		26.66-61.33	7.56-8.95	7-20.94		13.06-113.65	3-13.83	9.87-41.99	31.79-86.03	1.79-3.28	0.03-7.45
PSE	Jun-13	51.52	6.86	10.03	Jul-14	44.7	17.08	12.6	68.94	3.5	2
95 % CI		35.11-105.6	9.1-11.86	8-26.56		25.8-138.32	10.56-49.99	17.09-22.17	42.2-168.56	16.09-13.08	13.95-19.83
PSE	Jul-13	n/a	n/a	n/a	Aug-14	n/a	n/a	n/a	n/a	n/a	n/a
95 % CI		n/a	n/a	n/a		n/a	n/a	n/a	n/a	n/a	n/a

Appendix 7. Principal coordinate analysis plots based on the unweighted (**a**) and weighted (**b**) UniFrac distance matrices. Coloured by 'substrate' (**1**), 'environment type' (**2**), 'OTU number' (**3**) and 'Simpson's Index of Diversity' (**4**).




3a
PCoA - PC1 vs PC2
PC2 - Percent variation explained 9.57%
PC1 - Percent variation explained 15.69%
4b
PCoA - PC1 vs PC2
PC2 - Percent variation explained 17.67%
PC1 - Percent variation explained 35.34%
160 - 296 OTUs
297 - 433 OTUs
434 - 571 OTUs
572 - 709 OTUs
0.56 – 0.65
0.66 – 0.76
0.76 – 0.86
0.87 – 0.97

Appendix 8. The total amount of operational taxonomic units (OTUs) and the number and percentage of OTUs present in all individuals at each site except Raferigeen (**a**), in all individuals at each habitat for which more than one site was sampled (**b**) and in all individuals of reared slugs (**c**).

a	Site name	GF	BC	LC	BD	DF	GW	CF	DW	BG	CH
	Number of samples	3	3	3	3	3	3	3	3	3	2
	Total number of OTUs within group	876	1236	796	1101	1124	1489	808	1259	1228	550
	Number of OTUs observed in all samples within group	78	32	90	145	163	178	126	52	156	110
	OTUs observed in all samples within group [%]	8.9	2.6	11.3	13.2	14.5	12.0	15.6	4.1	12.7	20.0

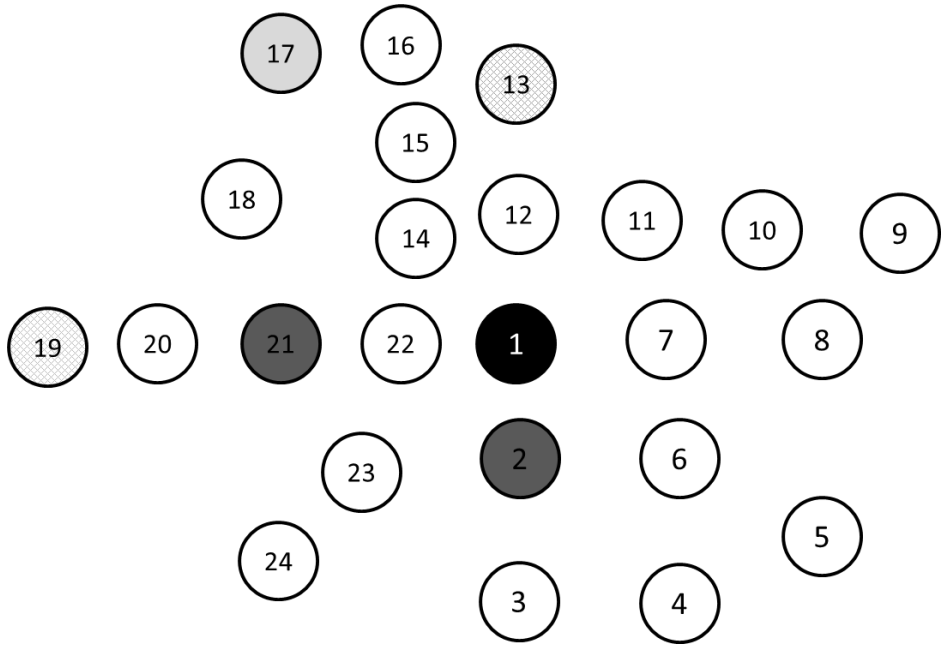
GF: Glanteenassig Forest, BC: Ballycarbery, LC: Lough Currane, BD: Barraduff, DF: Derreen Forest, GW: Glengarriff woods, CF: Cloosh forest, DW: Derrycunnihy woods, BG: Ballaghbeama Gap, CH: Crookhaven.

b	Habitat name	Blanket bog	Conifer plantation	Deciduous forest	Heath
	Number of samples	6	6	6	3
	Total number of OTUs within group	1806	1511	2122	1253
	Number of OTUs observed in all samples within group	57	66	39	80
	OTUs observed in all samples within group [%]	3.2	4.4	1.8	6.4

c	Reared slugs (lichen-fed)	Reared slugs (oat-fed)	All reared slugs
Number of samples	3	3	6
Total OTUs within group	653	401	859
Number of OTUs observed in all samples within the group	80	55	20
Percentage of OTUs observed in all samples within the group	12.3	13.7	2.3

Appendix 9. Schematic of the trees at the experimental site in relation to each other (**a**) and number of the tree behind which each micro-chipped slug was captured between 6.12.2013 and 26.06.2014 (**b**).

a



Black indicates the central release tree while the grey shades indicate the trees at which *Geomalacus maculosus* specimens were found during March 2014 (dark grey), April 2014 (patterned) and May 2014 (light grey).

b	Microchip ID	6.12.2013	11.12.2013	17.12.2013	14.1.2014	30.1.2014	28.2.2014	28.03.2014	29.04.2014	30.05.2014	26.06.2014
	48030	1		1							
	48092	1									
	48017		1								
	48023			1		1	1		1		
	48013	1									
	48011										
	48496	1									
	48493	1	1								
	48505	1									
	48028		1								
	48022	1		1							
	48506		1						13		
	48025		1	1	1	1	1	1			
	48497		1		1						
	48021		1	1				2		1	
	48015	1									
	48018	1									
	48014							1			
	48024										
	48012	1	1	1	1	1	1				
	48946										
	48016				1	1	1				
	48027										
	48020				1		1		19		
	48948				1						
	48950			1							
	48019	1								19	
	48935										
	48947	1	1	1	1	1	1			17	
	48944	1									
	48498										
	48029	1	1	1			1	21			
	48945								1		