The effects of forest cover change and polydomous colony organisation on the wood ant *Formica lugubris*

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Abstract

Anthropogenic land use changes, such as deforestation, generally have negative effects on ecosystems. However, in Europe recently, historic trends in deforestation are being reversed due to increases in planted forests, and it is becoming much rarer to replace native forests with plantations. British forests have undergone centuries of degradation and fragmentation, and increases in forest cover due to plantations represent a potential positive for forest specialist species struggling in isolated fragments. In this thesis, I assess forest cover change and the demographic and genetic health of populations of the wood ant *Formica lugubris*, a forest specialist, in the North York Moors National Park, UK. I show that, contrary to expectations, non-native conifer plantations have had incredibly beneficial effects on this forest specialist species. Populations of *F. lugubris* have expanded from historically isolated fragments, and show no evidence of this expansion ceasing. Furthermore expanded populations are genetically diverse in both nuclear and mitochondrial DNA, and show evidence of commercial forests connecting previously isolated population fragments. There is strong divergence within mitochondrial DNA across the landscape in *F. lugubris*, which suggests either a cryptic species within the study population, or an historic hybridisation event. *Formica lugubris* exhibits polydomous colony organisation, whereby multiple spatially separate nests display social and cooperative connections, and are therefore one colony. I show that socially connected nests are socially and cooperatively distinct from their neighbouring colony, but show no equivalent genetic distinction. The findings within this thesis support growing evidence that non-native conifer plantations can have positive effects on forest biodiversity, and that some wood ant populations within the UK are healthy and under no threat of extinction. Furthermore polydomous colonies are cooperative but not genetic units, and division of colonies in this species may be ecologically, rather than genetically determined.

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Author Declaration

I hereby declare that this submission is entirely my own work, except where acknowledgement is given

Chapter 1A is part of a collaborative review in review in Insectes Sociaux. It is the section I wrote.

Chapter 2 has been published in Forest Ecology and Management (Procter et al. 2015). It is presented as published

Chapter 3 is in preparation for submission to PLoS ONE

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Chapter 1: Introduction

1.1 General Introduction

Anthropogenic land use change generally has detrimental effects on ecosystems. The utilisation of large parts of natural ecosystems for agriculture and industry has decimated organisms that rely upon that habitat. However, anthropogenic land use change does not have to have negative consequences. There are large areas of forest in Britain that were created by man on land which was not forest beforehand (Forestry Commission 2013a). These forests are not structured like natural forest, they have lower variation in species, trees tend to be of even age, many of the species are not native, and the forests are interspersed with large tracks to allow access of forestry vehicles (Ratcliffe & Petty 1986). However, despite this non-natural situation, this is a forest habitat, which could potentially allow forest specialist species to utilise it, if the quality of the habitat is sufficient.

In order to properly understand the modern situation, both current and historical trends must be taken into account, along with the life history of species in question. Furthermore explaining of current trends is not sufficient, prediction of future patterns is essential in a changing world, where many species are faced with extinction. In this thesis, I present a series of studies that assess the quality of this man-made forest for a forest specialist, and poor disperser, the wood ant *Formica lugubris*. I assess whether this artificial forest can make a positive contribution to the persistence of forest specialists in Britain, using *F. lugubris* as a case study. I then go on to assess social organisation in *F. lugubris*, and whether it can be explained by genetic distinctions.

Figure 1.1 The surface of a *F. lugubris* nest taken in spring, when workers mass on the nest surface, then move into the nest core once warm, a form of behavioural thermoregulation

1.2 Habitat Fragmentation

The development of human societies and the exploitation of natural resources generally has negative effects on the maintenance and survival of natural systems. The degradation of habitats and their separation into isolated fragments is one of the most well-known examples of such negative impacts. Loss of habitat has obvious negative effects on an organism; without a habitat in which to forage and reproduce a species is doomed to extinction. However, separation of habitats into smaller fragments has less obvious effects, for example fragmented habitat causes reduced population per patch and reduced dispersal between patches (Wilcox & Murphy 1985). Lower populations are more susceptible to both stochastic demographic events and rare natural catastrophes, which can lead to extinction of that patch. Reduced dispersal between patches means that extinction of single patches can lead to permanent loss of that patch within the range of the species (Lima & Zollner 1996). Furthermore, reduced dispersal between patches also means reduced gene flow between patches, which leads to inbreeding within patches (Templeton *et al.* 1990). For any organism that feeds on another i.e. virtually all animals, there is also the fact that the organisms being fed upon are undergoing the negative effects of habitat fragmentation, therefore feeding resources more than likely diminish as fragmentation increases. There are also less obvious negative effects to habitat fragmentation. Separation of large areas of one habitat type into a network of fragments can vastly increase the edge to interior ratio without large differences in total area (Wilcox & Murphy 1985). For a great many organisms there are negative effects of living near habitat edges, for example increased predation due to exposure to predators from other habitats (Saunders, Hobbs & Margules 1991). As a result the overall quality of the habitat can be reduced by fragmentation far more than would be suggested by the reduction in area.

In reality, to say that fragmentation of habitats has negative effects is too simple. There are a vast number of species that have been negatively affected by habitat fragmentation, for example red squirrels, *Sciurus vulgaris* (Verboom & Apeldoorn 1990), the common frog, *Rana temporaria* (Hitchings & Beebee 1997) and the alpine butterly *Parnassius smintheus* (Roland, Keyghobadi & Fownes 2000) to name but a few. However, the effect of habitat fragmentation on a species will depend on the ecology and behaviour of that species. For example an interior specialist needs large areas of contiguous habitat in order to thrive, and separation of that habitat will have negative effects on the species in question. Similarly large bodied species, with large home ranges, are more susceptible to the loss of habitat due to fragmentation than smaller bodied species (Bennett 1990). Many species perform very well along the edges of habitats, particularly forests (Buckley, Howell & Anderson 1997; Calladine, Bielinski & Shaw 2013). A degree of fragmentation can therefore be a positive for many species, allowing increases in populations. The ability of a species to disperse between separate fragments will also affect their response to fragmentation. Long distance dispersers can maintain populations across multiple

spatially separate fragments, whereas short distance dispersers are much more prone to isolation (Hanski 1999). This leads to different landscape configurations having different effects on the ability of organisms to disperse between patches and persist in a landscape (With 2015). The realisation of the interaction between the properties of an individual organism, its habitat and the configuration of the habitat across the landscape has spawned entire areas of research such as metapopulation ecology and landscape ecology. The literature for these disciplines is far too large to discuss here, and both have a selection of full books in their own right (e.g. Hanski 1999; Turner & Gardner 2015).

Figure 1.2. An example of current forest habitat in the UK. Small fragments of broadleaved trees are surrounded by vast coniferous plantations. This photograph is taken from a clear-felled area approximately 500m x 500m, highlighting the difference in disturbance to natural forest

Alongside the ecological effects of habitat fragmentation, there are genetic effects that must be considered. Reduction of population sizes increases inbreeding within the population and also the negative effects of genetic drift (Höglund 2009). Inbreeding is the mating of related individuals, which, in itself, is not a problem. However, repeated inbreeding can lead to the expression of recessive negative alleles; the reduction in fitness due to the expression of alleles caused by inbreeding is termed inbreeding depression (Templeton *et al.* 1990). Genetic drift is the change of allele frequency within a population due to random mating (Hamilton 2009). With

a smaller number of individuals alleles can go to fixation by chance much more easily, therefore deleterious alleles can become fixed in small populations. The combination of a reduced starting genepool within the fragment, and then the action of inbreeding and genetic drift means that populations within fragments can suffer from low genetic diversity. Threatened species show reduced genetic diversity in comparison to non-threatened species (Spielman, Brook & Frankham 2004), which can correlate with reduced fitness (Westemeier *et al.* 1998). Furthermore, in populations with low genetic diversity, fitness of members of the population increases with increased genetic diversity (Ingvarsson 2002). Conservation of species therefore requires the consideration of not just ecological effects, but genetic effects too.

Inbreeding should be particularly costly for the haplodiploid hymenoptera, because it leads to the production of diploid males, as well as the negative effects of inbreeding depression (Hölldobler & Wilson 1990; Harper *et al.* 2016). Diploid males are both sterile and cannot function as workers within the colony, therefore represent a total waste of resources. Despite this apparent high cost of inbreeding within the eusocial hymenoptera, inbreeding is often found within ecologically successful species (Sundström, Keller & Chapuisat 2003; Hannonen, Helanterä & Sundström 2004; Kureck *et al.* 2012) , including some of the most damaging invasive species worldwide (Keller & Fournier 2002; Fournier *et al.* 2012). The parasitoid *Cotesia flavipes* also shows no negative effects after 10 generations of lab inbreeding (Trevisan *et al.* 2016), therefore there may be methods by which members of the hymenoptera can offset the negative effects of inbreeding.

1.3 Forests and forest cover change

Human activities worldwide have led to a continuous decline in forest cover (FAO 2010). Forests support the majority of terrestrial biomass, and declines in forest cover can have hugely detrimental effects on the communities those forests support (Aerts & Honnay 2011). In Europe, forest decline is slowly being reversed, with recent increases in forest cover resulting from a combination of natural regeneration and increases in planted forests (FAO 2010). Britain is a prime example of this trend; historical deforestation meant that a minimum forest cover of 5% was reached at around 1900 (Mason 2007), which has since recovered to the current figure of 13% (Forestry Commission 2013a). Increases in British forest cover were triggered by a shortage of wood in the First World War (Forestry Commission 2016). As a response, the Forestry Commission was established, and large areas were planted with trees, as a strategic reserve of timber (Forestry Commission 2016). The increase in forest cover Britain has seen, has primarily consisted of fast growing conifer species for commercial forestry (Fig. 1.2). The legacy of this planting can be seen today, because the non-native conifer Sitka spruce, *Picea sitchensis*, is currently the most common tree in British forests (Forestry Commission 2013a).

Afforestation, the planting of forests on previously non-forest land, primarily occurred in the upland areas of Britain. Planting of large non-native conifer forests on upland areas slowly results in a flora and fauna more characteristic of a forest ecosystem (Ratcliffe 1986). Loss of species characteristic of upland ecosystems is a serious negative of the afforestation program in Britain, and the effects have been well documented (Thompson et al. 1988 and references therein; Moore and Allen 1999). Increases in forest have the potential to have positive effects on organisms dependent on forest habitat. However, commercial forests do not represent the same habitat as native broadleaved woodland (Fig. 1.3). Commercial forests are characterised by high planting densities, low tree species diversity, and forests interspersed with wide tracks and openings to allow access of forestry vehicles (Ratcliffe & Petty 1986). It is therefore vital to assess whether forest specialist species are able to utilise the non-natural situation that now dominates the British landscape, if conservation of forest species is to be achieved.

Figure 1.3. An example of the difference in structure between ancient broadleaved woodland (left) and coniferous plantation (right). Coniferous plantations have much higher trunk density and low levels of ground flora, broadleaved woodland is characterised by much denser undergrowth and lower tree density.

How much of a positive impact commercial forests, comprised primarily of non-native conifer plantations, can have on biodiversity is unclear. Commercial forests can display lower species richness or diversity than native broadleaved woodland (Fahy & Gormally 1998; Pedley *et al.* 2014), or the opposite can be true (Day, Marshall & Heaney 1993), or there can be no difference between the two habitats (Bibby, Phillips & Seddon 1985; Fuller, Oliver & Leather 2008; Pedley *et al.* 2014). Each of the studies mentioned above were conducted on too small a scale to give an overall picture of the country-wide effects; the only study on a sufficiently large scale to quantify country-level patterns is the Forestry Commission's Biodiversity Assessment Project, which assessed plant, fungal, microbial, vertebrate and bird communities in plantation forests

across Britain. Overall the study found no difference in the species richness supported by native or non-native stands (Quine & Humphrey 2010), and concluded that plantations made a significant contribution to the maintenance of woodland biodiversity (Humphrey, Ferris & Quine 2003). However, there were no control plots in natural/semi-natural woodland, therefore we cannot be sure how positive this effect is compared to a more natural scenario.

The forest cover of Britain is highly fragmented (Peterken 1993), with 74% of forest within 100m of an edge (Riutta *et al.* 2014). Many of the non-native conifer plantations that have been planted in the recent past connect previously isolated patches of natural or semi-natural woodland (Vanhala *et al.* 2014; Procter *et al.* 2015). If non-native conifer plantations can support populations of forest specialists or facilitate dispersal between semi-natural fragments then the connectivity of woodland populations may be massively increased by afforestation with non-native conifers. Only assessment of effects on forest specialists in the wild will inform us whether this is the case. Increases in forest cover in this country have not come to an end; there are currently plans to increase the forest cover in England by a further 2% by 2060 (Forestry Policy Team 2013). If we are to maximise the positive effects of both current forests and further forests yet to be planted, understanding the effects of non-native conifer plantations on forest-dependent organisms is essential.

Ideally in order to predict effects across species, the effects of forest cover change on each individual species would be known and then collated together to give a holistic, and overarching strategy. However such knowledge would be prohibitively expensive in both time and money to collect. Collecting data on species that should represent a range of species with similar habitat requirements is a more sensible way to assess habitat level effects. Furthermore if species have beneficial effects on both ecosystem function and the presence of the promotion of biodiversity then data on them is of more value.

1.4 The study species

The mound building red wood ants of the *Formica rufa* group are common across the forests of Eurasia and are generally accepted to comprise the following species: *F. rufa, F. polyctena, F. lugubris, F. aquilonia, F. paralugubris, F pratensis, F. frontalis and F. truncorum* (Goropashnaya *et al.* 2012; Stockan *et al.* 2016). The ants are dependent on forest cover due to the majority of their calorific intake during the active summer months coming from honeydew collected from aphids feeding on trees (Rosengren & Sundström 1991). Wood ants do not depend on a single aphid species, instead they can feed on a number of aphid species, across multiple tree hosts (Domisch, Risch & Robinson 2016). Wood ants are keystone organisms in woodland systems and have strong effects on invertebrate community structure, as well as being a potential food source for predators (Hughes & Broome 2007). Nests can be over 1m high and consist of various plant matter dependent on the forest in which they are found. The

construction of nests results in modification of the soil structure, increasing porosity (Frouz & Jilková 2008), accumulation of food and detritus makes nests hotspots of nutrient exchange in the forest system (Domisch *et al.* 2009) and wood ant nests are sources of carbon dioxide and methane production (Jílková *et al.* 2016). Wood ant nests themselves are supporters of biodiversity, with many species dependent on wood ant nests as habitat. Wood ant dependent species include: annelid worms, pseudoscorpions, spiders, centipedes and millipedes, beetles, bugs (Hemiptera), crustaceans and even other hymenoptera including species of ants, such as *Formicoxenus nitidulus,* which nests within sticks in the wood ant nest mound (Härkönen & Sorvari 2014; Parmentier, Dekoninck & Wenseleers 2014; Robinson, Stockan & Iason 2016). When present, wood ants are a positive influence in forest ecosystems, therefore they are excellent species to assess when assessing forest cover change.

Figure 1.4. The wood ant *Formica lugubris.*

The *F. rufa* group are very similar in morphology, with differences between species usually judged by hair patterns (Collingwood 1979; Seifert 1996; Stockan *et al.* 2016). As a result species identification within the group can be problematic, but a recent phylogeny based on mitochondrial DNA clearly separated the different Palearctic species (*Formica* s. str. in Fig. 1.5, Goropashnaya et al. 2012), in agreement with the morphological evidence. Recently there has been the confirmation of one cryptic species (Seifert 1996) and the suggestion of a second (Bernasconi *et al.* 2011) from the Alps, therefore the final arrangement of the *F. rufa* group is not fixed. Sequencing of mitochondrial DNA can be a useful method for identification of

morphologically close species (Bernasconi, Pamilo & Cherix 2010). However, the *F. rufa* group also exhibits extensive hybridisation (Seifert & Goropashnaya 2004; Czechowski & Radchenko 2006; Seifert, Kulmuni & Pamilo 2010).This means that at times sequencing mitochondrial DNA can lead to misidentification (Seifert & Goropashnaya 2004), due to the presence of mitochondrial haplotypes from a species with which hybridisation has occurred.

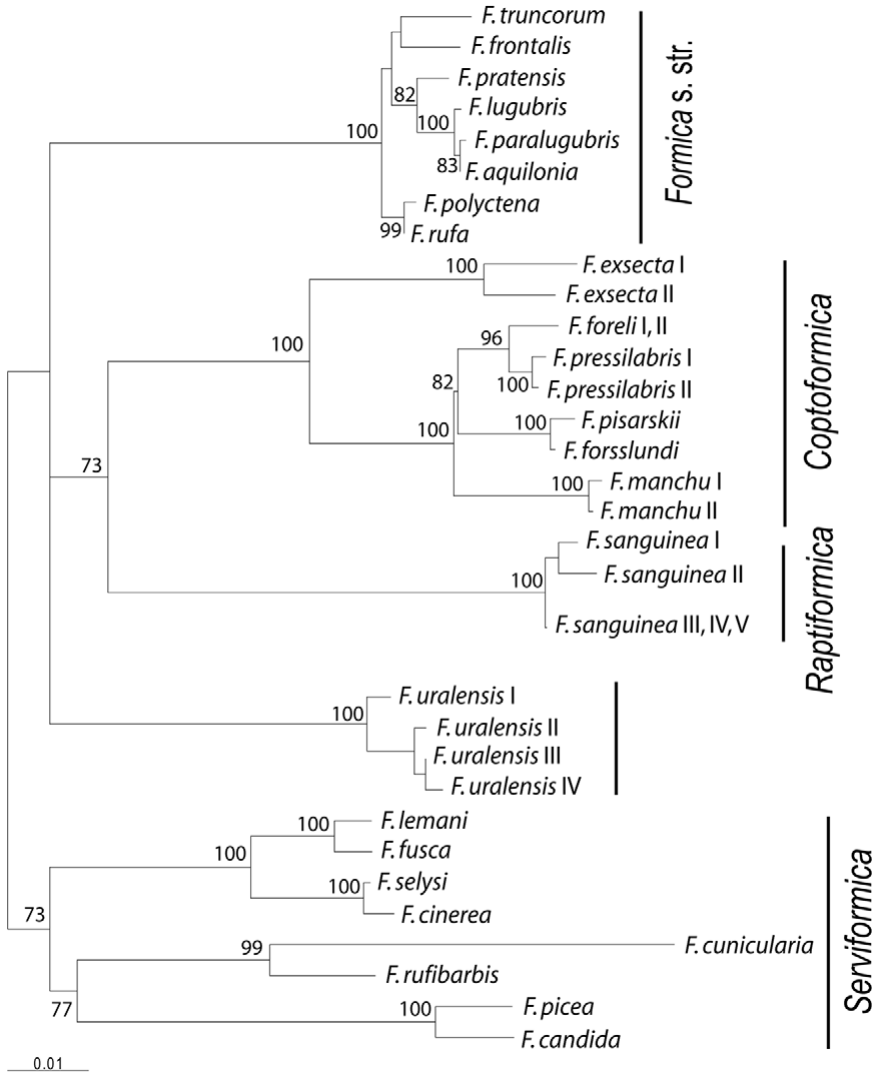

Figure 1.5. A phylogeny of 32 mitochondrial haplotypes within the genus *Formica*, taken from Goropashnaya *et al.* (2012). Values per node are percentage bootstrap values.

Within the *F. rufa* group there is variation in life history strategies. Some species, for example *F. rufa*, are mainly monogynous (one queen per nest), monodomous (one nest per colony) and exhibit long range flighted dispersal and independent colony founding, whereas other species, such as *F. aquilonia*, are highly polygynous (multiple queens per nest), highly polydomous (multiple nests per colony) and exhibit dependent colony founding (Maeder *et al.* 2016). Generally a species used to be classed as monogynous or polygynous, however more recently this has been revised as variation within species has been revealed (Heinze 2008). Individual

species sit somewhere on a continuum between these two extremes, for example *F. truncorum* can be monogynous and monodomous or polygynous and polydomous dependent on the population (Sundström 1993).

We chose *F. lugubris* (Fig 1.1, 1.4) as a study organism for this thesis for a number of reasons. As a member of the *F. rufa* group, it is dependent on forest cover, and a positive influence when found within forests. Therefore it is an excellent organism to study when assessing the effects of forest change on native populations. *Formica lugubris* varies in dispersal ability across its range, but in the UK, it is a short range disperser due to founding new colonies by budding as opposed to flighted dispersal (Gyllenstrand & Seppä 2003; Bernasconi *et al.* 2005; Mäki-Petäys & Breen 2007). Therefore expansion of populations should happen in a stepwise fashion, which we can predict, and it should be representative of the part of the forest ecosystem which responds slowest to changes in forest structure. The populations of *F. lugubris* that the studies within this thesis are based on are not bordered by any other species within the *F. rufa* group. Therefore, there was no need for time consuming morphological identification of samples for every study that must be conducted. There are several practical advantages to the study of *F. lugubris* as well. Nests are conspicuous and, once you have become used to spotting them, can be identified from a long distance. It is therefore unlikely that nest abundance will be under-estimated with a rigorous mapping technique. Nests are also long lived, therefore sampling and re-sampling of nests over time is an option (Rosengren 1971). Nests have worker populations in the tens to hundreds of thousands (Chen & Robinson 2013), therefore it is easy to sample workers for genetic work without disturbing the functioning of the colony. *F. lugubris* exhibits polydomous colony organisation in the UK (see section below), which allows us to ask questions relating to how this fascinating form of social organisation functions. Finally, there were known populations of *F. lugubris* in the North York Moors, which allowed easy access for fieldwork from the University of York. The other English species of wood ant, *F. rufa*, has a more sparse distribution and, though many populations are known, there is not such a landscape with multiple substantial populations as we find in *F. lugubris*.

1.5 Polydomous colony organisation

The classical view of an ant colony is a single queen heading a single nest, with the workforce comprising her sterile daughters. However, this view of an ant colony is increasingly being shown to be a gross oversimplification (Heinze 2008). Ant nests can contain a single breeding queen (monogyny), or multiple breeding queens (polygyny). Furthermore, ant colonies do not have to be restricted to a single nest, they can comprise multiple spatially separate but socially connected nests (Fig. 1.6), a phenomenon termed polydomy (Debout *et al.* 2007).Species can be entirely monodomous e.g. the common black ant *Lasius niger*. Some species, such as *F. lugubris*, exhibit variation in social organisation across their range, with populations in Ireland,

Finland and parts of Switzerland being monogynous and monodomous, whereas in the UK and other parts of Switzerland populations are polygynous and polydomous (Gyllenstrand & Seppä 2003; Bernasconi *et al.* 2005; Hughes 2006; Mäki-Petäys & Breen 2007). Other species, such as the invasive Argentine ant, *Linepithema humile*, can vary the level of polydomy seasonally, retreating to single winter nests and then expanding into polydomous networks in the summer (Gordon & Heller 2014). In the most extreme form of polydomy, certain species, for example *Formica paralugubris*, are unicolonial, whereby the entire population functions as a single colony (Holzer *et al.* 2006).The level of polydomy should therefore be seen as on a continuum, from exclusively monodomous species at one extreme to highly polydomous species at the other extreme. Interestingly, although there is a tendency for highly polygynous species to exhibit polydomy (Debout *et al.* 2007), polydomy is not restricted to polygynous species, and entirely monogynous species can exhibit polydomous colony organisation (e.g. *Cataulacus mckeyi*, Debout et al., 2003).

Figure 1.6. Polydomous nest organisation in *F. lugubris*, the nests are connected by trails of workers. Photo by Sam Ellis at Longshaw Estate in the Peak District, the North York Moors field sites are not quite so lacking in undergrowth.

Polydomy is present in a wide range of species (Debout *et al.* 2007), including widespread and ecologically dominant species (Ellis & Robinson 2014) and some of the world's most damaging

invasive species (Fournier *et al.* 2012; Gordon & Heller 2014; Hoffmann 2014). It is therefore likely that there are strong benefits associated with polydomy, and many have been suggested: polydomy may allow the spreading of the risk of damage between multiple nests (van Wilgenburg & Elgar 2007a), it may allow efficient resource exploitation and acquisition (Schmolke 2009; Cook, Franks & Robinson 2013), release from inefficiency associated with a particularly large nest (Robinson 2014; Kramer, Scharf & Foitzik 2014), or escape from the limitations of a single nest site (Cao 2013). Due to the wide diversity of ant species that exhibit polydomous colony organisation, it is unlikely that only one benefit is universal, and it is entirely possible than any one polydomous species is benefitting from more than one advantage simultaneously.

Polydomous colonies are defined as spatially-separate nests that exhibit social connections (Debout *et al.* 2007). Social connections can be incredibly obvious, such as those in *F. lugubris*, where trails of workers continually move back and forth between nests within the same colony (Fig. 1.6, Ellis et al. 2014). However, not all species exhibit such strong and obvious social connections, therefore there are a variety of methods by which polydomous colony boundaries can be measured, such as resource movement, aggression, spatial clustering and genetic distinctions (for an overview of genetic delineation of colony boundaries see Chapter 1A). Both workers and food can be marked, in order to track resource movement between nests to assign colony boundaries (McIver 1991; Buczkowski & Bennett 2006; van Wilgenburg & Elgar 2007a). Tracking resource movement not only allows a study to track social connections, but ensure those connections are cooperative.

Aggression bioassays are often used to assign colony identity, based on the assumption that lack of aggression between nests is representative of colony identity (Pirk *et al.* 2001; Debout *et al.* 2003; Holzer *et al.* 2006; Buczkowski 2011). However there is evidence of workers being able to recognise non-nest-mates or non-colony-mates without aggression (Holzer *et al.* 2006; Björkman-Chiswell *et al.* 2008), therefore a lack of aggression does not mean a lack of recognition. Different aggression bioassays differ in their repeatability (Roulston, Buczkowski & Silverman 2003) and all suffer badly from observer bias (van Wilgenburg & Elgar 2013), therefore aggression bioassays should be carefully designed if they are used at all.

Polydomous boundaries have often been inferred from spatial clustering of nests, based on the assumption that nests in competition should be equally spread (overdispersed), whereas clustering represents a shared territory (Sudd *et al.* 1977; Levings & Traniello 1981; Dillier & Wehner 2004; Santini *et al.* 2011). Spatial clustering must be used with care though, because there are a great many reasons why nests may cluster that are not to do with social organisation. The *F. rufa* group of wood ants, for example, are dependent on trees for food; therefore their spatial organisation should be affected by the location of trees to some degree. Failure to assess

ecological variables that may affect the spatial distribution of nests could easily confound methods based on spatial clustering.

Polydomy is present in phylogenetically diverse species (Debout *et al.* 2007), and is probably severely under-reported. Therefore, there is not simply a subset of species in which polydomy must be assessed. Irrespective of the method by which polydomous colony boundaries are defined, assessment of the scale of colony organisation in a species is essential if research is to be put in proper context. Nests within a polydomous colony are not independent data points, therefore sampling from a population without assessing the scale of colonies can invalidate conclusions. Furthermore, ignoring the scale of colony boundaries can mask effects of interest. For example the sex ratios present within ant nests can be explained by inclusive fitness (Sundström, Chapuisat & Keller 1996). However if the population is highly polydomous, then different nests may adopt different sex ratios as part of an overarching colony strategy. Failure to assess variation within the colony would mask the true patterns within the population.

An ant colony is expected to be a cooperative, selective and reproductive unit, and this applies whether referring to a monodomous or polydomous colony. It is therefore expected that nests within polydomous colonies have some form of resource exchange. In *F. lugubris* there is considerable evidence that there is resource exchange between nests (Ellis *et al.* 2014; Ellis & Robinson 2015a; b), therefore the logic that the colony is cooperative certainly stands. The presence of workers, which do not reproduce, within social insect colonies can be explained by inclusive fitness (Hamilton 1963, 1964). Workers contribute to the reproduction of a queen, or many queens, to whom they are related, and a proportion of their genes are passed on. This should continue to apply to polydomous colonies. In this thesis I assess whether there is sufficient relatedness within polydomous colonies to maintain altruistic behaviour, and assess whether it is lack of relatedness between neighbours which determines why there are not connections between polydomous networks.

1.6 The North York Moors National Park

All of the studies in this thesis are based on the North York Moors National Park, in the North-East of the UK (Fig. 1.7, Long/Lat: 54,289, -1.059). This study site was chosen for a number of reasons. Firstly there are historic records of wood ant presence within the park (Yarrow 1955), which were checked for persistence in 2011 (EJH Robinson unpublished data). Therefore we knew that there were extant wood ant populations that had persisted for some time. Secondly the landscape exhibits a high level of forest cover, compared to much of Britain (21.5% compared to 13% for Britain overall: Forestry Commission 2013), the majority of which consists of non-native conifer plantations. Therefore, this landscape makes an excellent study site for the effects of non-native conifer plantations on native forest specialist species. Thirdly the primary manager of forest across the landscape is the Forestry Commission, which gave us

access to the Forestry Commission sub-compartment database. This database contains data describing the characteristics of each compartment (management unit) of forest across Forestry Commission managed land. Data is extensive, containing primary, secondary and tertiary tree species, date of planting, age structure of the trees, and a variety of other data. This wealth of data allows us to put the presence of wood ants into ecological context.

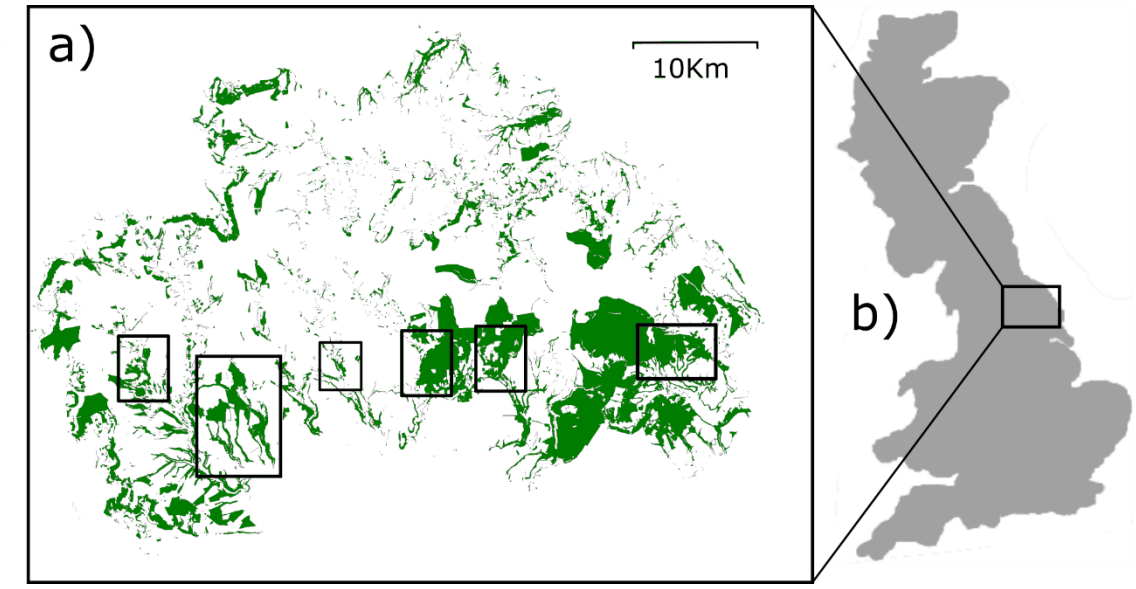

Figure 1.7. a) The study landscape, the North York Moors National Park. Green polygons show forest cover, the location of known wood ant populations are outlined by boxes. **b**) The position of the landscape within Britain.

1.7 Thesis outline

In this thesis I present a series of studies, which assess various aspects of the biology of *F. lugubris* and its interaction with habitat and conspecifics. In chapter 2, I use historic forest cover, ant population mapping, and habitat suitability modelling to assess whether the expansion of commercial forests, comprised of non-native conifers, has benefitted or harmed *F. lugubris*. The expectation is that non-native conifer monocultures do not support native diversity; I test whether that is the case. In chapter 3 I extend the habitat suitability modelling from the previous chapter to include a variety of spatial scales. I test whether our findings in chapter 2 stand up to a more thorough approach, and assess landscape level factors relevant for conservation. In chapter 4 I bring in genetic data, to assess the genetic diversity of *F. lugubris*, and assess whether there is evidence of the connection of different, previously isolated, populations by increases in commercial forests. In chapter 5 I assess mitochondrial DNA, again assessing diversity, but also investigating possible evidence of ancient hybridisation events within this landscape. Finally in chapter 6 I move on to social organisation, and I ask whether

polydomous colonies defined by social connections are genetically distinct from their neighbours.

Chapter 1A. Genetic delineation of polydomous colony boundaries

Genetic tools allow inference of both evolutionary and historic patterns within and between populations of polydomous colonies. An evolutionary example is the divergence between Argentine ant, *Linepithema humile*, supercolonies (Giraud, Pedersen & Keller 2002), and an example of historic patterns is the identification of source populations of the invasive garden ant (e.g. *Lasius neglectus* Ugelvig et al. 2008). Historical and evolutionary perspectives can complement more functional resource based and spatial methods, possibly explaining why neighbouring nests are cooperating or clustered together.

1A.1 Theoretical basis

When a colony is described as a genetic unit, that means a nest or grouping of nests which contains workers with an allele frequency distribution or collection of DNA sequences that is more similar to other workers within the colony than it is to workers in the population at large. Genetically similar nests should both show evidence of shared descent, and represent a reproductive unit. Genetic divisions can be drawn on a number of measures, such as genetic relatedness, genetic differentiation or distinct matrilines, all of which will be discussed here.

Genetic delineation of colony boundaries offers a fundamentally different perspective on the colony as a unit than other methods discussed within this review. Throughout this review we have adhered to the polydomous colony definition of Debout *et al* (2007), whereby spatially separate but socially connected nests are considered part of the same colony. Assuming the same definition of a colony but using genetic measures as the method to draw boundaries, implicitly assumes that social connections form along genetic lines. Social connections appear to represent cooperative interactions (Buczkowski 2012; Gordon & Heller 2014; Ellis *et al.* 2014), and cooperation is more likely when the organisms in question are more related to one another (Hamilton 1964; Bourke 2011). It is therefore a reasonable assumption that social connections correlate with genetic links. However, while social connections can form along genetic lines (Banschbach & Herbers 1996), genetic differentiation can be found within socially connected nest networks (Chapuisat, Goudet & Keller 1997; Holzer, Keller & Chapuisat 2009). Socially unconnected nests can also display no genetic distinction from unconnected nests (Chapter 6). Therefore, genetic methods for colony delineation potentially do not correlate with social connections. It may be useful to use other, more functional methods of colony delineation alongside genetic methods, in order to better understand the study system.

1A.2 Limitations

The level of sampling needed to distinguish between colonies is determined by the genetic variability of those colonies and the level of difference attempting to be distinguished. Workers within monogynous colonies , whether monodomous or polydomous, are highly related, and within nest genetic diversity is fairly low, making distinguishing colony boundaries simple using genetic tools (e.g. Foitzik and Heinze 2001; Debout et al. 2003). However, in polygynous colonies, as the number of queens per colony increases so does the amount of genetic diversity contained within that colony, and worker relatedness decreases (Ross 2001), frequently approaching zero (Pedersen & Boomsma 1999; Tsutsui & Case 2001; Pamilo *et al.* 2005). As genetic diversity increases and worker relatedness decreases the level of sampling must increase in order to detect genetic differences. Increasing the level of sampling can be done by sampling more workers per nest, assaying more loci per worker, or utilising more variable loci, or a mixture of all three (Pedersen & Boomsma 1999). Practically, a high sampling effort for highly polygynous systems means that genetic determination of colony structure can be expensive both in time and money compared to more ecological methods. Whether this investment is worthwhile will depend on the goals of the study in question.

Polydomous populations often exhibit short distance dispersal, leading to strong spatial genetic structuring (Sundström, Seppä & Pamilo 2005). This means that nests closer to one another are more genetically similar than to the rest of the population. Spatial genetic structuring needs to be accounted for in analyses before trying to distinguish between neighbouring colonies. The stronger the spatial structuring, the more of the variation in allele frequencies is explained by space, and not colony membership. In practice this means that in a population with strong spatial genetic structuring, more loci or more variable loci are required to distinguish between neighbouring colonies.

Genetic differences build up over long timescales, often allowing inference of past patterns within or between populations e.g. *Formica aquilonia* in response to forest cover change (Vanhala *et al.* 2014), sources of invasive populations of *Linepithema humile* (Tsutsui *et al.* 2001) and *Lasius neglectus* (Ugelvig *et al.* 2008). Long timescales for differentiation can also cause a problem though, because recently separated colonies may not yet have begun to diverge. As a result neighbouring colonies may display clear ecological separation, but be indistinguishable in genetic terms (Chapter 6). A combination of genetic methods with ecological or behavioural methods may allow clearer inference of colony boundaries.

Whereas resources can flow in one direction but not the other between a pair of nests, genetic measures do not have a direction to them. There is only a single measure of genetic differentiation or inter-nest genetic relatedness for a pair of nests; therefore it is not possible for directionality in relatedness or differentiation. A colony, defined along social connections, must

be expected to contain genetic variation, and not have identical allele frequencies in each nest. Therefore, it is possible that there will be situations where, in a group of three nests, nest A is not significantly differentiated from nest B or C, yet nests B and C show significant differentiation from one another. In this situation it would be very difficult to know where to draw a colony boundary. We are unaware of any examples of this yet discovered, but a similar situation has been observed with aggression assays (Ugelvig *et al.* 2008).

In polygynous populations, polydomy has often been inferred from the presence of associated features of polydomy such as low relatedness of nest-mates, the presence of budding dispersal and strong spatial genetic structuring of populations (Pamilo *et al.* 2005; Zinck *et al.* 2007). However, associated features do not inform about the scale of polydomous colonies i.e. are the polydomous colonies two connected nests over 5m or 30 connected nests over 200m? Furthermore, features associated with polydomy do not inform about the frequency of polydomy within the population i.e. are all colonies polydomous or is there a mix of monodomous and polydomous colonies? Inferences of polydomy from correlated traits are usually unexpected side effects of studies looking at other questions. However, the presence of polydomy can lead to false inference from studies assessing other questions. For example, if the sampling of multiple nests has taken place, and they are assumed to be independent, the presence of polydomy within the population may mean that some of those sampled nests are not independent data points. Analyses that do not take polydomous population structure into account may risk drawing incorrect conclusions (Seppä & Walin 1996).

1A.3 Methods

Individuals within a colony are more genetically related to one another than they are to individuals from other colonies within the population. To determine whether two nests are within the same polydomous colony, pairwise inter-nest relatedness estimates between workers of the nests in question can be examined. Expected inter-nest relatedness within the polydomous colony will depend on the level of relatedness found within each nest. Pairwise inter-nest relatedness estimates can then be adjusted to account for within nest relatedness (Pedersen & Boomsma 1999), or the distribution of pairwise relatedness estimates can be compared to both within nest relatedness and relatedness between distant unrelated nest pairs (Pamminger *et al.* 2014). Neither method has been widely applied, possibly because variation in pairwise relatedness estimates is high within samples. Therefore, discrimination would be difficult in situations with low within-nest relatedness, as is common in ants.

Instead of using relatedness to determine how similar workers within separate nests are, measures of genetic differentiation such as F_{ST} can be used to determine how different they are. Under this methodology, two nests that do not display statistically significant differentiation are said to be from the same colony, and nests that do display significant differentiation are said to

be from different colonies (Elias, Rosengren & Sundström 2005; Dronnet *et al.* 2005; Steinmeyer, Pennings & Foitzik 2012). An alternative approach to F-statistics is G-distance (Pedersen & Boomsma 1999). This adapted measure of standard G-statistics (Sokal & Rohlf 1981) compares the heterogeneity of genotypes of workers sampled from different nests. The application of G-distance will produce a statistic whose magnitude correlates with genetic distance. The values for G-distance will be influenced by the number and variability of loci used, and therefore cannot be compared between studies. Furthermore, G-distance should be used to reinforce conclusions based on other genetic methods, not as a stand-alone method (Pedersen & Boomsma 1999). Conclusions about colony structure based on genetic differentiation should be made with care. This is especially true in polygynous species or populations where within-nest genetic diversity is high, and in species with local dispersal where strong spatial genetic structuring is present. A lack of significant genetic differentiation is only evidence of two nests being part of the same colony if the study involved sufficiently numerous and variable loci to enable discrimination between neighbouring colonies. Statistical power analyses before embarking on studies dependent on genetic differentiation are advised, and reinforcing conclusions based on genetic differentiation with other measures is recommended (Pedersen & Boomsma 1999; Dronnet *et al.* 2005).

Groupings of genetic data can be determined by Bayesian clustering algorithms such as Structure (Pritchard, Stephens & Donnelly 2000), BAPS (Corander, Waldmann & Sillanpää 2003) or Geneland (Guillot *et al.* 2012), which are used widely in population level studies. These methods assess the number of clusters that best explain variation present in genetic data and the likelihood that each sampled individual belongs to each cluster. To our knowledge these have not yet been applied to colony boundaries but colonies determined by genetic methods are genetic units and so should be just as detectable as any level of genetic division. There should be some caution in the spatial scale of data analysed by these methods, however, because large populations may contain genetic subdivisions above the level of the colony which the clustering algorithms will identify, masking smaller scale colony boundaries. The necessary spatial scale for application of these analyses will have to be determined for each study.

When dealing with highly variable markers and trying to assign nests to groups, it can be most informative to look at rare genotypes within the population and the nests which share them. Common genotypes can often be found within neighbouring nests by chance. However, alleles rare within the population, but present in two neighbouring nests, are unlikely to be shared by chance (Pedersen & Boomsma 1999). Ants within neighbouring nests sharing alleles rare enough in the population that they should only be found in a single nest can be termed a ‘rare genotype sisterhood’ (Pedersen & Boomsma 1999). If neighbouring nests contain ‘rare genotype sisterhoods’, then it is likely that they share common descent and so it is more likely that they are from the same colony. However, the lack of a rare genotype sisterhood does not

prove that two nests are not within the same colony; they just may not have a genotype rare-enough to fulfil the necessary criteria. As mentioned earlier in this section, genetic differentiation works on a longer timescale than ecological or behavioural processes. Neighbouring colonies in a population may share common descent and so contain rare-genotype sisterhoods without currently functioning as single colonies. This could make inferences from rare allele methods such as 'rare genotype sisterhoods' unreliable, and therefore we would only recommend their use for this purpose in conjunction with other methods if at all.

Most studies that attempt to determine colony boundaries have done so using either allozymes or micro-satellite markers. Though perfectly valid, these techniques have been restricted to nuclear DNA. Many ant species are known to display sex-biased dispersal, with males usually dispersing further than females (Doums, Cabrera & Peeters 2002; Clémencet, Viginier & Doums 2005; Soare *et al.* 2014). The sequencing of mitochondrial DNA (mtDNA) may help to reveal distinctions between nests that nuclear DNA does not. If there is strong sex biased dispersal within the population, then neighbouring nests may exchange nuclear DNA via males but no mitochondrial DNA because females do not disperse. This would could lead to different mitochondrial haplotypes present in neighbouring nests that show no nuclear genetic distinction. The utility of mtDNA will depend on how variable it is within the study population: in a population containing very few mitochondrial haplotypes, mtDNA sequence is unlikely to further inform colony structure.

We are not aware of any examples of next generation sequence data having been applied to this question of colony boundaries. With ever decreasing costs we hope this will be an option in the near future, and the massively increased power available using those techniques may help to deal with some of the problems that currently exist in distinguishing colony boundaries. For an overview of the potential of next generation sequencing see Nygard and Wurm (2015).

1A.4 Conclusion

As with any form of experimental design, the appropriate genetic methods used to determine colony boundaries will depend on the system in question. With species or populations where queen numbers are low, genetic tools can put colony boundaries in an evolutionary perspective with relative ease. However, in polygynous species or populations we would recommend the application of functional measures of colony boundaries in addition to multiple genetic measures, in order to put the genetic patterns into ecological context. We would also recommend the use of statistical power analyses before embarking on a project, to be sure that there is enough power to distinguish any boundaries that may be present. Genetic tools offer the potential to elucidate evolutionary and historic patterns that are not available to other methods, and are therefore potentially very useful, but not without weaknesses.

Chapter 2: Do non-native conifer plantations provide benefits for a native forest specialist, the wood ant *Formica lugubris*?

2.1 Abstract

Recent increases in plantation forestry are starting to reverse the global decline in forest cover, in some areas of the world. Britain has practiced afforestation, primarily with non-native conifers, for over a century. It is unclear whether these new plantations have the potential to support native forest species.

We quantify afforestation across the North York Moors National Park, UK, deriving a chronology of afforestation from historic maps at six time points from 1854 to 2013. We map the location of current wood ant (*Formica lugubris*) nests and set their distribution in the context of historic forest cover. We use these nest locations and the features of the habitat in which they occur to model the suitability of recently established conifer plantations for wood ants using MaxEnt. We determine whether non-native conifers offer suitable habitat for a forest specialist species, and assess the lag between establishment of conifer plantations and colonisation by wood ants from historic woodland fragments.

Forest cover increased by 229% over 160 years and is now dominated by non-native conifer plantations. Our survey data show that current wood ant populations extend hundreds of metres from where forest was in the past, demonstrating geographical population expansions into newly formed forest, comprised of non-native conifer plantations. Both our data and model reveal that the recently planted non-native conifer plantations are a suitable habitat for this forest specialist species. Our model reveals that *Formica lugubris* has not yet spread through all available suitable habitat due to very poor dispersal ability, displaying a severe lag behind the availability of habitat.

Managers should not assume that unoccupied habitat is unsuitable nor should they expect to see immediate colonisation of plantations. Future forest creation should be targeted close to existing forests to facilitate colonisation of forest specialists.

2.2 Introduction

Forest cover worldwide has undergone massive decreases in the past 300 years due to conversion of forested land into cropland (Ramankutty & Foley 1999). In South America, Africa and Oceania this trend is still ongoing: all showed further decreases in forest area between 2000 and 2010 (FAO 2010). In contrast, historical deforestation in Europe is in the process of being reversed and forest cover is now increasing, with a combination of natural expansion of forests and afforestation, the planting of forests on previously un-forested land (FAO 2010). Afforestation in Great Britain provides a prime example of this trend, because forest cover was at a minimum of 5% in 1900 (Mason 2007) and has since recovered to the current figure of 13% (Forestry Commission 2013a). During the first half of the twentieth century, British forestry policy was focussed on the creation of large plantations of fast-growing non-native conifer species for commercial objectives (Quine, Bailey & Watts 2013). These plantations account for the major increase in forest cover within Britain. In the latter half of the twentieth century, forest policy gradually shifted to encompass a broader range of objectives for forests and to emphasize the importance of native species (Forestry Commisson 2011; Quine *et al.* 2013). However, the legacy of afforestation with non-native conifers is still evident in Britain, for example, the non-native Sitka spruce, *Picea sitchensis*, is now the most common tree species in British forests (Forestry Commission 2013a).

Creation of non-native conifer plantations on previously unforested land gradually results in a flora and fauna more representative of a forest ecosystem (Ratcliffe 1986). The loss of species specific to the land prior to afforestation has been extensively documented as a negative effect of afforestation (Moore and Allen, 1999; Thompson et al., 1988 and references therein). However, on the plus side, the progression towards a forest ecosystem offers potential benefits to forest-dependent species, if the conifer plantations offer similar habitats to native woodland. Although planted forests exhibit lower biodiversity than natural forests in South East Asia (Kanowski, Catterall & Wardell-Johnson 2005; Fitzherbert *et al.* 2008), the situation in Britain is less straightforward; there can be lower species richness or diversity in conifer plantations than mixed or broadleaved woodland (Fahy & Gormally 1998; Pedley *et al.* 2014), whereas the reverse can also be observed (Day *et al.* 1993), or there may be no difference between the habitats (Bibby *et al.* 1985; Fuller *et al.* 2008; Pedley *et al.* 2014). However, the scales over which these studies were conducted were too narrow to determine whether there is a general direction of change. The only study on a sufficiently large scale to quantify country-wide patterns was the Forestry Commission's Biodiversity Assessment Project, which found no difference in species richness between native and non-native stands (Quine & Humphrey 2010) and concluded that plantations made a significant contribution to the maintenance of woodland biodiversity (Humphrey *et al.* 2003). General studies measuring biodiversity or species richness, though of great value, do not inform about the status of individual populations within non-native

conifer plantations; the presence of a species within a plantation does not necessarily mean there is a healthy breeding population utilising that habitat, and this must be confirmed with more in depth studies.

Historic deforestation has left the forest cover of the UK highly fragmented (Peterken 1993). Fragmentation of a landscape has detrimental effects on populations dependent on those fragments, increasing local extinctions and inbreeding (Wilcox & Murphy 1985; Templeton *et al.* 1990). Connection of fragments of native woodland by conifer plantations has the potential to defragment the landscape, if forest specialists can utilise this new plantation habitat. Non-native conifer plantations have been shown to increase the connectivity of previously isolated populations in the red squirrel, *Sciurus vulgaris* (Hale *et al.* 2001) and the wood ant *Formica aquilonia* (Vanhala *et al.* 2014). While this is a welcome and positive effect of non-native conifers it is not clear from these studies whether such plantations provide a valuable habitat in their own right or if they merely represent a matrix that facilitates dispersal of forest specialists.

Species' responses to ecological change are known to be slow. It can take over a century for fragmentation and isolation of a population to result in extinction (Vellend *et al.* 2006), a phenomenon known as extinction debt (Tilman *et al.* 1994). The current distribution of a species in a recently changed landscape is therefore not expected to be in equilibrium. Species composition of plantations change throughout their development cycle, with the oldest stands being the most species rich (Moss, Taylor & Easterbee 1979; Brunet *et al.* 2011) and with a community structure more similar to natural woodland than earlier stages (Humphrey *et al.* 2000). As plantations progress beyond their first rotation, there are also opportunities for management to enhance plantation forest, in terms of its conservation potential (Nature Conservancy Council 1986). Opportunities have been taken to improve management in Britain, with emphasis now on benefitting biodiversity as well as a range of other considerations (Forestry Commisson 2011). Presence of a given species in a section of habitat depends both on the suitability of the habitat for that species and the species' ability to disperse to that habitat (Saunders *et al.* 1991). We may therefore expect that plantations which are a long way from historic fragments of forest will have fewer of the species that are characteristic of forest habitat (Wallace & Good 1995). This mismatch between the numbers of species a newly formed habitat is capable of supporting and the number currently found there can be termed colonisation lag. If the effect of creating large areas of conifer plantations is to be properly understood, the speed at which organisms colonise this new habitat must be assessed.

We chose the wood ant *Formica lugubris* as our study species. It is a member of the mound-building red wood ants of the *Formica rufa* group, common across the temperate and boreal forests of Europe and Asia (Goropashnaya *et al.* 2004). Nests can be as high as 1m and consist of various components of dead vegetation, depending on the type of forest in which they occur.

The ants are dependent on forest cover because the majority of the food coming in to the nest is honeydew from aphids, tended by ants on the trees (Rosengren & Sundström 1991). Wood ants are keystone species in woodland ecosystems, with effects on the community structure of local invertebrates as well as providing a food source for predators (Hughes & Broome 2007). Nest construction results in modification of soil structure, increasing porosity (Frouz & Jilková 2008) and accumulation of food and detritus makes nests hotspots of nutrient exchange (Domisch *et al.* 2009). Nests support high levels of biodiversity, including many species that are dependent on the nests as habitat (Härkönen & Sorvari 2014; Parmentier *et al.* 2014). In the UK, *F. lugubris* exhibits budding dispersal (Hughes 2006), whereby a newly mated queen moves a short distance from her natal nest to form a new nest with a subset of the workers from the natal nest. Short distance dispersers are particularly susceptible to the negative effects of habitat fragmentation, such as local extinctions and inbreeding (Wilcox & Murphy 1985; Templeton *et al.* 1990). Potential connection of historic fragments by afforestation, effectively defragmenting the landscape, would mean that *F. lugubris* might benefit greatly if it can make use of planted forests and overcome historic fragmentation. Due to its role as a keystone woodland species and promoter of biodiversity through nest building, *F. lugubris* has a positive role in the woodlands in which it is found.

Here we combine mapped populations of the wood ant *Formica lugubris*, historic forest cover data and habitat suitability modelling over the landscape of the North York Moors National Park to answer the following questions:

1. How has recent afforestation impacted the forest cover of our study landscape?
2. Do non-native conifer plantations offer suitable habitat for *F. lugubris*?
3. What degree of lag is there between establishment of non-native conifer plantations and their colonisation by this forest specialist species?

This information will help us to understand the role that non-native conifer plantations currently have in providing suitable habitat for a woodland specialist species. It will provide new insights into the time taken to occupy these plantations and will clarify whether habitat suitability and/or ability to disperse limit occupancy of these new forest habitats.

2.3 Materials and Methods

The study area comprises the southern half of the North York Moors National Park, in the north east of England, UK (Fig. 2.1). We assess forest cover in all 1:10,000 Ordnance Survey grid squares that cover some area of the National Park in an area cornered by the Lat/Long coordinates 54.3916, -1.3073 (North West) and 54.2110, -0.4695 (South East). The area of the study area within the National Park is 934km^2. This landscape, as with many upland areas in Britain, has been extensively planted with non-native conifer plantations over the last century. It

is also home to wood ant populations, each based around ancient woodland fragments next to which plantation forest has been established. Historic records exist on the presence of ant nests within each of the mapped ant populations (Yarrow 1955), pre-dating the establishment of conifer plantations, though without any detail on the area those populations cover or numbers of nests within those populations. This change in the forested landscape occupied by wood ants allows us to examine the potential benefit of non-native conifer plantations on the expansion of this forest specialist species. The Forestry Commission manages 60% of the forest area across this landscape, enabling us to access data from the extensive Forestry Commission sub-compartment database for use in modelling the suitability of non-native conifer forest as wood ant habitat. The sub-compartment database contains the current distribution of Forestry Commission forests, as well as data on the age and species composition of each plantation block as well as a number of other variables.

The plantations throughout the study landscape contain over 40 tree species as well as mixed stands, but the most common species are Sitka spruce, *Picea sitchensis* (22.2% of land area), Scots pine, *Pinus sylvestris* (15.5% of land area), Japanese larch, *Larix kaempferi* (8.6% of land area) and Hybrid larch, *Larix x leptolepis* (4.8% of land area). In terms of age, approximately one third of plantations are 30 years old or younger (28.4% of land area), a further third are 31-60 years old (32.1% of land area), and the remainder are either older than 60 years (15.7% of land area) or undefined (23.8% of land area).

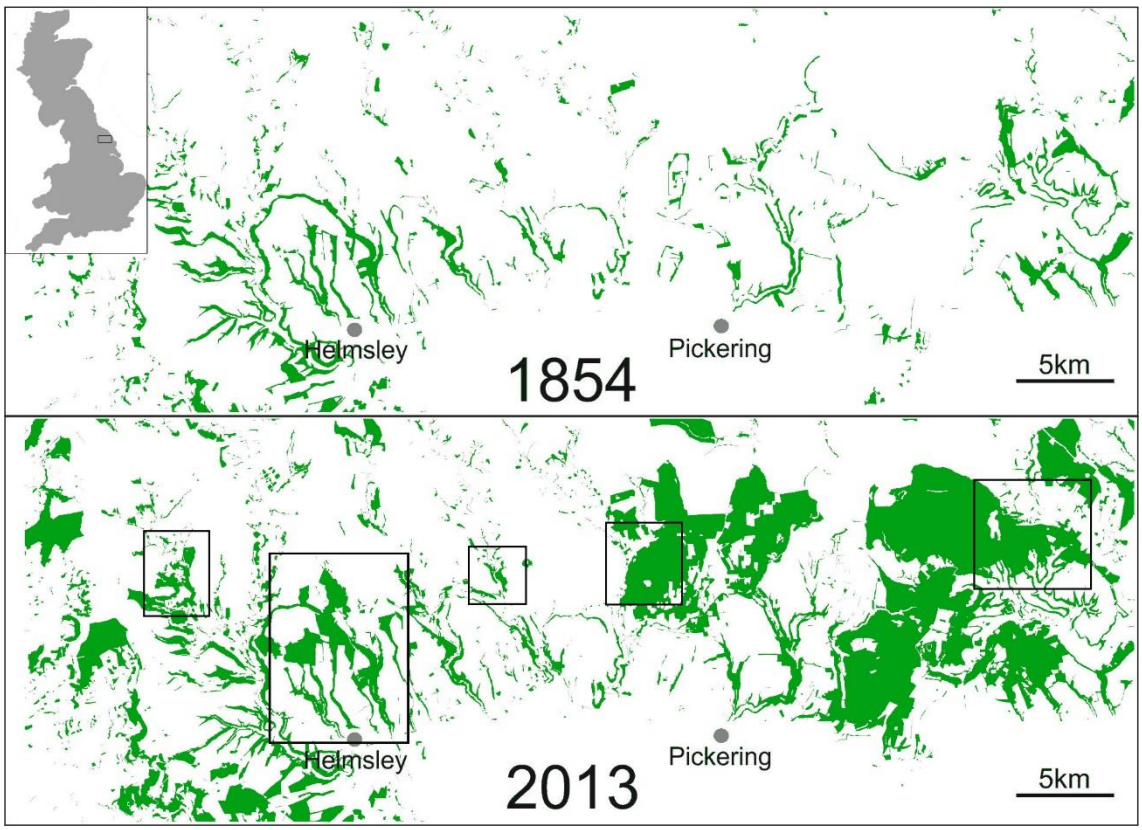

Figure 2.1. Forest cover changes between 1854 and 2013; the green polygons show forest cover at the time points stated on each map. The insert shows the location of the study landscape within Britain. Boxes in the lower panel show the location of wood ant populations displayed in Fig. 2.2 and enclose the land for which the estimates of area around current ant populations are provided in Table 2.1.

Creating a chronology of forest cover change

We manually produced forest cover data by creating polygons around forests depicted on historic maps in ArcMap 10.1. We obtained four maps from the county series 1:10,560 (© Crown Copyright 2014. An Ordnance Survey/EDINA supplied service) dated for the study area in question to 1854, 1894-5, 1914 and 1952. We also obtained two maps from the National Grid 1:10,000 series (© Crown Copyright 2014. An Ordnance Survey/EDINA supplied service) from 1976-81 and 2013. Changes in forest cover were assessed both across the whole study area and in a restricted area incorporating all land within 1km of current ant population edges (Table 2.1, Figs 2.1 and 2.2).

Table 2.1. Forest area and percentage increase in forest cover since 1854 at different time points for both the landscape as a whole (Fig. 2.1) and a reduced area extending 1km in each compass direction from the edges of each ant population (Fig. 2.2)

	Whole Study Site		Area around current ant populations	
Time of Map	**Area of forest (km^2)**	**Percentage increase since 1854**	**Area of forest (km^2)**	**Percentage increase since 1854**
1854	73.08	-	17.84	-
1894	93.78	28.3	20.70	16.0
1914	95.12	30.2	20.82	16.7
1952	130.24	78.2	29.44	65.1
1976	230.60	215.6	46.78	162.2
2013	240.75	229.5	49.05	175.0

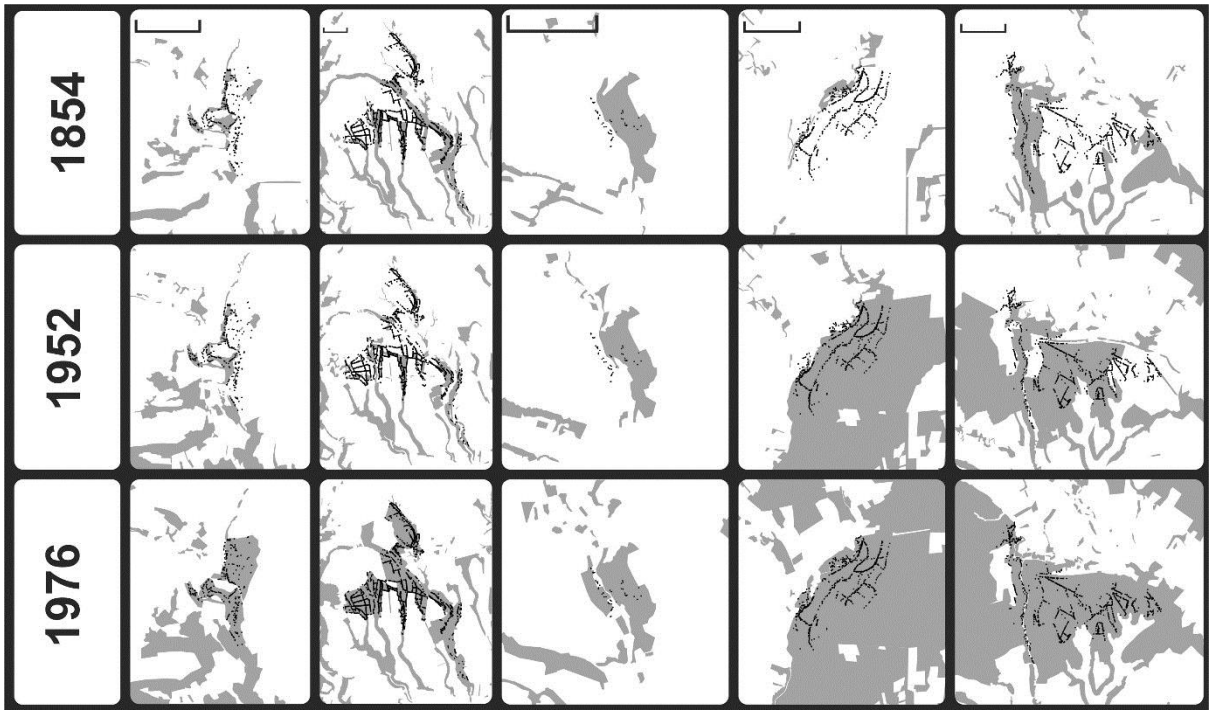

Figure 2.2. The changes in forest cover over time for areas within 1km of the current *F. lugubris* population boundaries. Each column represents a different population, with their relative location depicted in Fig. 2.1. Grey polygons are forest cover at the dates on the left hand side. Points are the locations of *F. lugubris* nests in 2013. The scale bar in the upper left of each column is 1km wide. The dates chosen represent the start point of forest cover for the study in 1854 and the two major periods of afforestation, in 1952 and 1976. Numbers of nests per population, mapped in 2013, left to right: 400, 2938, 48, 856, and 1264.

Repeatability of the method to obtain forest cover data from historical maps was assessed by repetition of the manual creation of forest cover estimates in 10 randomly assigned 1kmx1km squares across the landscape. This was repeated by the first author then again by an independent

assessor who had not previously been involved in the work. The forest cover of the 10 areas was re-mapped for the 1894, 1952 and 2013 maps, giving a total of 30 re-mapped areas. Estimates of forest area per square showed a strong correlation between the original and when remapped by the first author (Pearson's correlation, r=0.995, Appendix 1, Fig A1.1). Estimates of forest area per square were also strongly correlated between the original data and when remapped by an independent assessor (Pearson's correlation, r=0.986, Appendix 1, Fig A1.1).

Mapping populations of the study species and their spread

Sites within the study area with historic population records (Yarrow 1955) were surveyed for *F. lugubris* population persistence in 2011. These populations, along with large areas of forest currently unoccupied by wood ants, were re-surveyed during January and February 2013 to assess the geographic area colonised by the wood ant nests. During initial surveys we noted that most nests appeared to be close to the forest edge in plantations. In order to establish whether there was a relationship between distance to the forest edge and the location of wood ant nests, fourteen blocks of plantation forest were mapped during April 2013. These fourteen initial transect blocks were mature plantation at least 150m wide and with a slope of less than 30° above horizontal, spread throughout four populations of *F. lugubris* across the study landscape. Each transect block consisted of 15 transect lines, each extending 75m into the forest from an edge, separated by 5m intervals. Transect lines of this length were chosen as this ensures sufficient penetration into plantation woodland to be under very dense canopy. Blocks of woodland are rarely greater than 150m in width without some form of break, so transect lines longer than 75m would merely result in being closer to another track or path than the point from which the transect began. Nest locations were recorded using a Garmin eTrex H handheld GPS device and their distance to the path measured. Initial transect results revealed that 78.5% of nests are found within 10m of forest edges adjacent to paths (total 121 nests, Appendix 1, Fig. A1.2).

To assess the accuracy of detection of wood ant nests using the methodology above, a subsample of six transect blocks was repeated by an independent assessor not involved in the original mapping work. There was 96% agreement between original and repeated surveys (55 vs 53 nests total). The difference between surveys was due to two small nests being overlooked by the second survey; no additional nest locations were found.

Our initial transect blocks confirmed that most *F. lugubris* are found in the first 10m from a plantation edge (see above), therefore we only mapped the first 10m from each edge into plantations. We conducted 10m long transects into the plantations, spaced by 5m, along every edge of the forest in which ants were found. Edges were defined as tracks or rides through the forest which were wide enough to cause a gap in the canopy, including all external edges of the forest and the perimeter of felled areas. Internal edges between different plantation blocks

without any form of track in between them were not included in the survey. Due to the importance of sunlight in the thermoregulation of wood ant nests (Kadochová & Frouz 2014; Chen & Robinson 2014), the higher solar radiation available at the margins of plantations makes *F. lugubris* an edge specialist. In contrast, natural or naturalised woodland has a much lower density of trees, which allows greater penetration of sunlight at ground level. Consequently, in natural/naturalised woodland there is no reason to expect such a strong relationship with the forest edge. We therefore decided to map natural/naturalised areas using transect lines that extended all the way through the woodland, spaced by 5m. Five populations were mapped using these transect based methods between April and July 2013 (Fig. 2.2).

Our null hypothesis was that there has been no expansion of wood ant nests into non-native conifer plantations and our prediction therefore is that there should be no difference in the distance of current nest locations to the nearest forest cover at various points in the past; because wood ants are forest specialists they will always have been within forest. The Kruskall-Wallis test with multiple comparisons was used to test the difference between distances from current wood ant nest locations to the nearest forest cover was at various times in the past (Fig 2.4), using the kruskall function in the agricolae package of R (de Mendiburu 2009; R Core Team 2015).

Habitat Suitability Modelling

Suitability of the forest habitat across the landscape was modelled using the maximum entropy modelling software MaxEnt version 3.3.3k (Phillips, Anderson & Schapire 2006). MaxEnt uses spatial habitat data and the presence of the ant nest locations to assess the characteristics of the habitat in which nests are found. The habitat characteristics of ant nest locations are compared with the habitat characteristics at pseudo-absence points i.e. locations in the habitat in which there are not ant nests. Habitat suitability was modelled in all of the areas on the landscape managed by the Forestry Commission, as that allowed us to use the extensive data of the Sub-compartment Database (http://www.forestry.gov.uk/datadownload) to include more relevant variables than would have otherwise been possible. As only 60% of the forested land in the study area is owned by the Forestry Commission, this approach led to a reduction in the number of nest locations that could be included from 5506 to 3811 before further data preparation (see below), a number which is nevertheless more substantial than many datasets used in such models. To create the modelled area, a layer of all non-forest areas around each of the five ant populations, such as tracks, roads and open ground, was manually created from published maps of the area and our survey data in ArcGIS 10.1, including the edges of forest from which transects were started in order to map ant populations. A buffer of 25m into forests was then applied to the layer of non-forest areas and edges to allow for the 10m transect distance plus some inaccuracy of the GPS device used to map nests. The Forestry Commission land within

this buffered layer was the modelled area used. Sampling bias is known to be a problem in MaxEnt modelling (Elith *et al.* 2011), however for our data, sampling effort was even across the modelled area, therefore bias files are not required.

The variables included in the model, all rasters at 10m resolution, were as follows: distance to forest cover in 1854, primary tree genus, slope of the ground, hillshade (a measure of the shadiness of the landscape that essentially takes into account aspect and the height of the sun at a given position on the globe), mean percentage of conifers within 50m, mean percentage of broadleaves within 50m, mean percentage of open land within 50m and four variables for the mean percentage of different age classes of forest within 50m (Appendix 1, Table A1.1). The age classes were: under 20 years, 20-30 years, 31-80 years and over 80 years, based on the summary of age classes of woodland in Franklin et al. (2002). All genera used in the 'primary tree genus' variable were represented by at least five sub-compartments within the modelled area. Genera occurring in fewer than 5 sub-compartments were binned as 'other broadleaves' or 'other conifers'. The mean percentage of open ground within 50m has a minimum value of 15% because all sub-compartments are assumed by the Forestry Commission to have at least 15% open ground incorporated into them, to allow for rides and tracks between plantation blocks. Slope and hillshade were calculated from a digital elevation model (© Crown Copyright 2014. An Ordnance Survey/EDINA supplied service) using the 'Slope' and 'Hillshade' tools in the Spatial Analyst toolbox of ArcGIS 10.1. Distance to forest cover in 1854 and all variables of percentages within 50m were calculated using the Multiscale MaxEnt ArGIS toolbox (Bellamy, Scott & Altringham 2013). A biologically relevant scale was chosen, as wood ants will forage extensively within that 50m circle around the nest (Ellis *et al.* 2014) and though they are known to forage further, this occurs relatively rarely. Variables were checked for multicolinearity in ENMTools 1.43 (Warren, Glor & Turelli 2010), but as there were no correlations greater than 0.46 (Appendix 1,Table A1.2) this was not deemed to be a problem.

In order for habitat suitability models to be fitted reliably, spatial independence of points is a prerequisite. Clustering of points within homogenous areas leads to over-fitting towards environmental biases and false inflation of model performance values (Veloz 2009; Boria *et al.* 2014). To deal with this problem, heterogeneity of spatial covariates was assessed using the 'Calculate climate heterogeneity' step 1 and 2 tools in SDMToolbox v1.1 (Brown 2014). Repeat points within areas of spatial homogeneity were then removed using the 'Spatially Rarefy Occurrence Data' tool in SDMToolbox 1.1. The modelled area was separated into five categories of heterogeneity based on natural breaks in the data, implemented in ArcMap by Jenks' optimisation algorithm, and duplicate points were removed within 10m radius for the highest heterogeneity category then at 70m, 130m, 190m and 250m for the categories of reducing heterogeneity. The 10m radius was chosen as that is the resolution of the spatial covariates so it is not possible to have spatial heterogeneity within that scale. The maximum

value of 250m was chosen after visual inspection of test output values as it led to very small numbers of occurrence points being within the areas of low heterogeneity. The numbers of points removed at the different levels were 1169 at 10m, 650 at 70m, 131 at 130m, 51 at 190m and 16 at 250m. After removing points within each level of heterogeneity, 1734 unique occurrence points remained upon which to build the model.

Models were initially tested for feature combinations and values of regularisation multiplier by running 5-fold cross-validated models with raw output and each combination of: linear features only, linear and quadratic features, linear, quadratic and hinge features, hinge features only and all features together and regularisation multiplier set at 1,5,10 and 20. The regularisation multiplier affects the smoothness of the modelled relationships between variables, with higher values giving smoother results (Elith *et al.* 2011). These models were compared in ENMTools 1.43 (Warren & Seifert 2011) using Akaike Information Criteria (AIC) to select the best performing model. Models containing all feature combinations together consistently performed best in terms of AIC irrespective of regularisation multiplier (Appendix 1, Table A1.3). A regularisation multiplier of one was found to perform best in terms of AIC so that was used for the remaining analyses (Appendix 1,Table A1.3).

Models were run with raw output, 5000 maximum iterations and five-fold cross validation, in which the study site data were randomly partitioned into five approximately equal subsets, four of which were used to train the model and one to test the model. Five repeats of the model were run with averages across models reported. Model selection was done in ENMTools 1.43 (Warren & Seifert 2011) using AIC. Model pruning consisted of removing each variable and comparing the difference in AIC between each pruned model and the full model, the best performing of which was then used and pruned further if possible. Models were considered equivalent if the difference in AIC was within two of the minimum AIC. The minimum model was then re-run with logistic output which can be interpreted as probability of occupancy relative to a given level of sampling effort (Elith *et al.* 2011). This scaling of probability of occupancy with sampling effort can lead to problems when comparing between species; however, this is not a problem for our analysis because we are comparing different variations of the model within one species and using the same dataset.

In order to test the predictive power of the model, it was then projected across all of the study landscape for which data were available. There are two other wood ant populations within the study landscape, which were identified in the initial survey work and are of known geographical extent. These populations were not mapped accurately and so their data are not included in the model. If the model predicts that these areas containing other populations have a high probability of occupancy of wood ant nests then that constitutes a test of the predictive power of the model.

2.4 Results

Forest cover change

Between 1854 and 2013, forest cover across the whole of the study area increased from 73.1 km^2 to 240.8 km^2, an increase of 229.5% (Table 2.1, Fig.2.1), the majority of which occurred between 1952 and 1976-81 (Table 2.1). In the area within 1km of existing wood ant populations the percentage increase was slightly lower at 175% (Table 2.1, Fig. 2.2) but shows the same general pattern. The majority of current forests across the study landscape consist of conifers and non-native species (Table 2.2).

Table 2.2. Current percentages of conifer and broadleaved forest and non-native and native species both with the area in which habitat suitability was modelled and across all Forestry Commission (FC) land across the landscape; other land did not have the data available.

Within area used in habitat suitability model				**Within all FC land across landscape**			
Forest type	**Percentage of forest**	**Species origin**	**Percentage of forest**	**Forest type**	**Percentage of forest**	**Species origin**	**Percentage of forest**
Conifer	61.0	*Non-native*	43.2	*Conifer*	65.0	*Non-native*	50.6
Broadleaf	16.1	*Native*	34.0	*Broadleaf*	11.3	*Native*	25.6
Not specified	22.9	*Not specified*	22.9	*Not specified*	23.7	*Not specified*	23.8

Mapping populations of the study species and their spread

In total, we discovered 5506 nests of *F. lugubris* distributed across five geographically discrete populations (Fig. 2.2). There is a minimum distance of 6km between two areas we define as different populations. Nests were unevenly distributed among populations with nests per population numbering 48, 400, 856, 1264 and 2938 (Fig. 2.2). Due to the mapping methods (see Methods section) this should represent approximately 80% of the true number of nests per population.

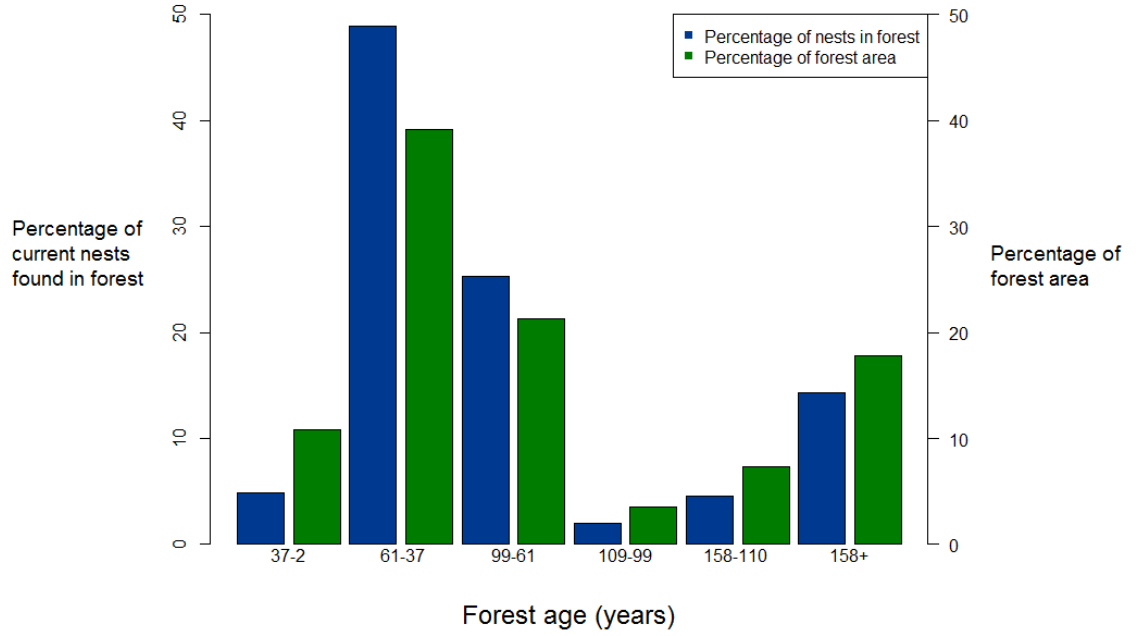

Figure 2.3. Percentage of current *F. lugubris* nests found in forest (light bars) and the percentage area of that forest in the study landscape (dark bars) against the age of the forest.

The majority of current nest locations (87.9%) are in areas that have been planted with forest since 1854 (Fig. 2.3). Almost half the current nests (49.7%) occur in areas that were planted with forest only between 61 and 37 years ago (Fig. 2.3). Current nest locations were significantly further away from historic forest cover than current nest locations were from more recent forest cover (Kruskall-Wallis, χ_5=9530.6, P<0.001, Fig. 2.4). Current nest locations were significantly further from the nearest forest cover in 1854 than from the nearest forest cover at all other time points (Fig. 2.4, K-W multiple comparisons, P<0.001). Current nest locations were not significantly further away from forest cover in 1894 than 1914 but distances at both these dates were significantly greater than to those at all subsequent time points (Fig. 2.4, K-W multiple comparisons, P<0.001). Therefore, there was no detectable expansion of populations into forests planted between 1894 and 1914 but expansion into forest planted after 1914 clearly occurred. Current nests were significantly further away from the nearest forest cover in 1952 than in 1976 and 2013 (Fig. 2.4, K-W multiple comparisons, P<0.001) but no significant difference in this distance between the 1976 and 2013 time points (Fig 2.4, K-W multiple comparisons, P=0.13). Therefore, during the intervening periods between those time points that differ least in forest cover (1894 and 1914 and 1976-81 and 2013) there were no significant geographic population expansions. In contrast, the time periods during which there were substantial changes in forest cover were accompanied by population expansions of ant nests. Although we did detect evidence of ant population expansions into plantations, the total expansion distance is low given the long time period, with the furthest a current nest is found

from the nearest forest cover in 1854 being 773m (Fig 2.4). This equates at most to a mean rate of population expansion of only 5m yr^{-1}. In comparison, referring to the forest contiguously connected to current ant populations, the maximum distance of current forest from forest cover in 1854 is 4500m. Therefore, there is a large amount of accessible forest into which wood ants have not yet spread.

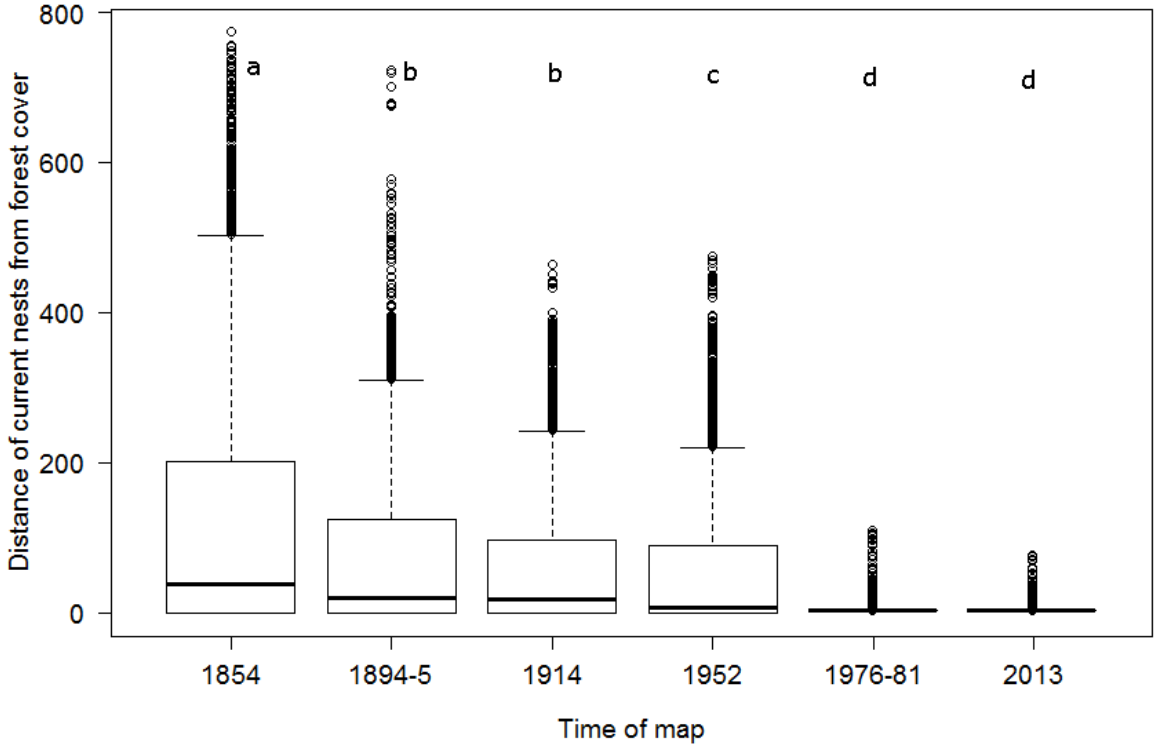

Figure 2.4. The distance of current nest locations from where forest cover was at the times of different maps; an estimate of the expansion of the population. Letters denote significant differences (Kruskal-Wallace with multiple comparisons, all $P<0.001$)

Habitat Suitability modelling

The previous section describes how *F. lugubris* expanded in the past; in order to allow us to predict whether this expansion is likely to continue and whether a lag in colonisation of suitable habitat is present, we modelled habitat suitability across our study site. We found that the most important variable in determining where wood ant nests are currently found is the distance to the nearest forest cover in 1854 (Table 2.3). If we remove this effect, large areas of currently unoccupied forest are predicted to have a high probability of occupancy for *F. lugubris.*

Table 2.3. Relative importance of variables to the model. Percentage contribution is determined by summing the increase in regularised training gain due to that variable per iteration of the model. Permutation importance is a measure of how much worse the model performs if that variable is randomised.

Variable	Percent contribution	Permutation importance
Distance to forest cover in 1854	42.9	31.7
Mean percentage of conifers within 50m	23.3	9.1
Mean percentage of open ground within 50m	16.9	26.0
Mean percentage of trees under 20 years old within 50m	4.7	8.6
Slope	2.8	5.6
Primary tree genus	2.8	3.0
Hillshade	2.1	2.8
Mean percentage of trees 31 to 80 years old within 50m	1.3	5.0
Mean percentage of broadleaves within 50m	1.2	3.6
Mean percentage of trees 20 to 30 years old within 50m	1.1	2.1
Mean percentage of trees over 80 years old within 50m	1.0	2.4

If any spatial covariates were removed the performance of the model in terms of AIC (Appendix 1,Table A1.4) was worse, so the full model is also the minimum model. AUC is the area under the receiver operating characteristic (ROC) curve, and is a commonly used measure of model performance in distribution modelling, although with known issues (Lobo, Jiménez-Valverde & Real 2008). The fit of the model is considered better, the higher above 0.5 the value of AUC is. The mean AUC from five replicates of the full model was 0.793 with a standard deviation of 0.01. The most important variable in predicting the probability of occupancy, i.e. the likelihood that an ant nest is found at a particular location, was its distance to forest cover in 1854 (Table 2.3), with probability of occupancy decreasing the further it was from the historic forest cover (Fig 2.5a). The next most important variables were percentage of conifers within 50m and percentage of open ground within 50m (Table 2.3). The probability of occupancy is highest in the mid values of percentage of conifers within 50m, decreasing slightly as the value approaches 100% and more strongly as the value approaches 0% (Fig 2.5b). Probability of occupancy was highest for the minimum values of percentage of open ground within 50m, and decreased to almost 0 as 100% was approached (Fig 2.5c). The age classes of the forest were of fairly low importance (Table 2.3) but all showed the same trend, with probability of occupancy decreasing slightly as the percentage of that age class within 50m approached 100% (Fig 2.6). The remaining variables contributed very little to the model (Table 2.3), and so their relationships

with probability of occupancy are not presented here, but can be found in the supplementary material (Appendix 1, Figs. A1.3-A1.5).

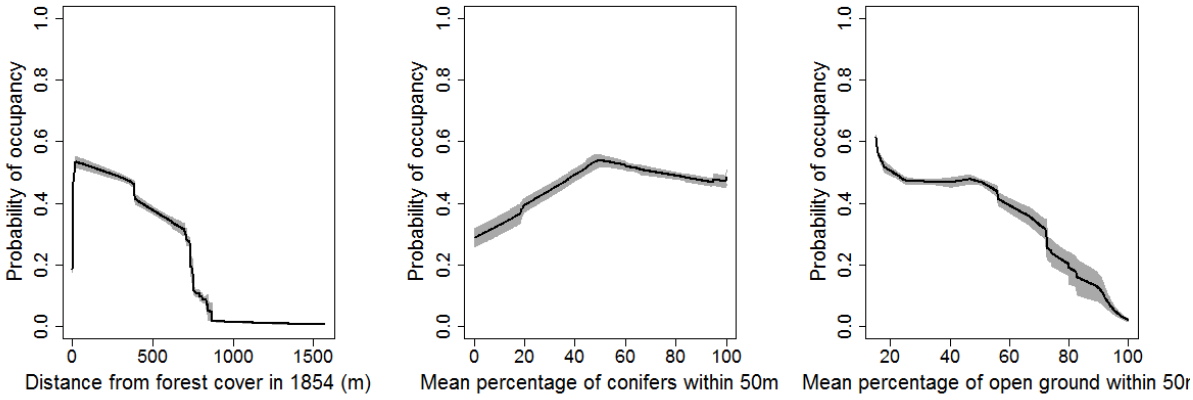

Figure 2.5. The relationships between probability of occupancy of *F. lugubris* and a) distance to forest cover in 1854, b) mean percentage of conifers within 50m, c) mean percentage of open ground within 50m. Lines are means of 5 models with the grey polygons being standard deviations of those models

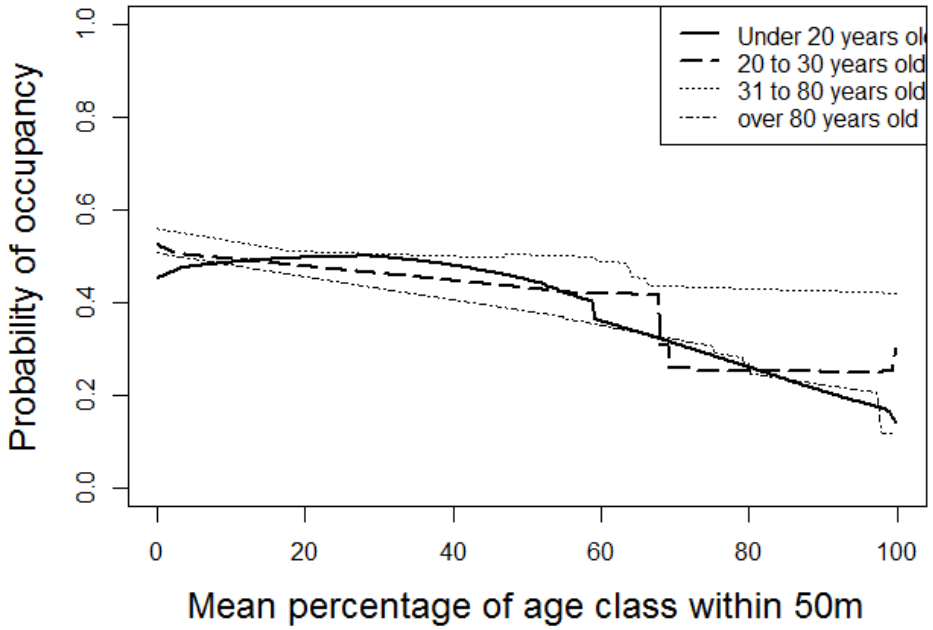

Figure 2.6. The relationship between probability of occupancy of *F. lugubris* and the percentage of 4 separate age classes within 50m. Lines are means of 5 models. See Appendix 1, Fig A1.6 for each relationship separately with standard deviations.

When the modelled relationships were predicted to the study landscape, the areas covered by two populations of *F. lugubris* of known geographical extent identified in initial surveys but not included in the model also showed high probabilities of occupancy (Figs 2.7a and 2.7b). This supports a high predictive power of our model. The initial prediction showed large areas of forest with a low probability of occupancy of *F. lugubris* (Fig. 2.7b). When the same projection is made with the effect of distance to forest cover in 1854 removed, virtually all the forest across the landscape had a medium to high probability of occupancy (Fig 2.7c).

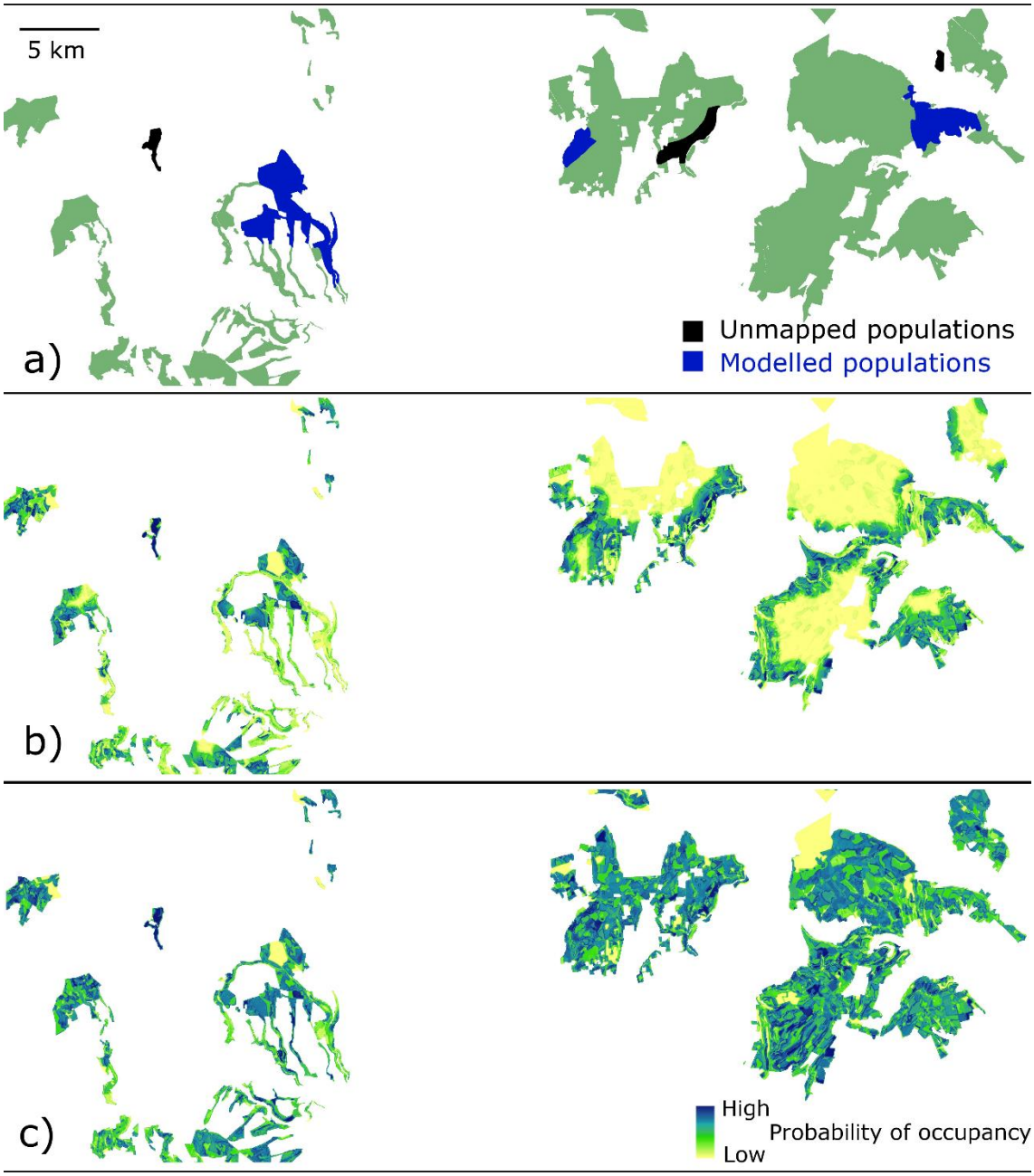

Figure 2.7 a) Green polygons display the forest area with sufficient data available to allow application of habitat suitability modelling. Blue areas denote the modelled areas and black areas are known populations not included in the model. **b)** A projection of the fitted model to the whole landscape. Darker

areas denote higher probabilities of occupancy of *F. lugubris*. **c)** A projection of the fitted model to the whole landscape with the effect of where forest cover was in 1854 removed, again darker areas denote higher probabilities of occupancy of *F. lugubris*.

2.5. Discussion

How has recent afforestation impacted the forest cover of our study landscape?

We have documented the change in forest cover across our study site caused by afforestation programs in Britain during the last 160 years, resulting in a substantial increase in forest cover. This level of increase reflects the scale of woodland expansion for Britain as a whole, where forest cover has increased from 5% in 1900 (Mason 2007) to the current figure of 13% (Forestry Commission 2013a). Currently across our study landscape, the majority of the forest consists of conifers (Table 2.2), which again, is consistent with the pattern throughout Britain, although the ratio of conifer to broadleaved is substantially more skewed towards conifers in our study site than in the country as a whole (Conifer 42% of forest area, Broadleaved 37%, the remainder consists of felled areas, mixed woodland, ground in preparation and assumed woodland of unknown structure, Forestry Commission 2013). A high proportion of conifers may not be novel conditions for the North York Moors, as archaeological evidence suggests significant numbers of Scots pine, *Pinus sylvestris*, used to occur across the North York Moors (Atherden 1976). However, the dominance of non-native species that we currently see (Table 2.2) and the management of plantation woodland certainly represents a change in habitat for forest specialists.

Do non-native conifer plantations offer suitable habitat for *F. lugubris*?

The novel and artificial habitat created by afforestation with non-native conifer plantations in the last 100 years has allowed large expansions of the forest specialist *F. lugubris*. This historical expansion indicates that non-native conifer plantations offer suitable habitat for this species, a finding that is reinforced by our habitat suitability model. All wood ant populations are directly bordered by forest that displays high probability of occupancy, therefore we expect that the historical expansion of these ant population will continue into the future.

In the past, the impact of plantation forests of non-native conifers were interpreted as being negative in terms of their effects on biodiversity but recently there have been suggestions that even intensively managed plantations of non-native species can provide an opportunity to enhance the biodiversity of the world's ever diminishing forest resource (Humphrey *et al.* 2003; Brockerhoff *et al.* 2008; Quine & Humphrey 2010). Our study supports the idea that non-native conifer plantations can offer valuable habitat for some native forest specialist species. Afforestation with non-native conifers in Britain has been shown to facilitate connections

between previously isolated forest fragments for forest specialists (Hale *et al.* 2001; Vanhala *et al.* 2014). While the impact of conifer plantations increasing connectivity of populations is positive, demonstration that non-native conifer plantations provide breeding habitat would make them even more valued habitats for supporting woodland biodiversity. Our results confirm the ability of non-native conifer plantations to support this forest specialist species.

Formica lugubris is widely distributed throughout the Palearctic (Goropashnaya *et al.* 2004) and is known to forage on both broadleaves and conifers (Robinson, Tofilski & Ratnieks 2008). *Formica lugubris* is therefore able to use a range of forest habitats, and may not be representative of species that specialise on a subset of forest habitats. Although there are a number of broader studies on community structure that do find beneficial contributions of conifer plantations to biodiversity (Moss *et al.* 1979; Humphrey *et al.* 2003), there will be species such as those dependent on broadleaved trees that have not done as well (Quine *et al.* 2007). Our results do suggest that those species that find natural conifer forest to be suitable habitat should be able to expand into recently planted conifer plantations.

Management has a large effect on forest species (Hartley 2002). Several findings from our model are informative for development of appropriate management of forests for wood ants. The variables for the 4 age classes of plantation forest within 50m represent the level of variation in structure of the forest within 50m of a nest: as each variable increases the variability of plantation within 50m decreases. Our results show that there is a lower probability of occupancy of *F. lugubris* as each of the 4 age classes of forest increases towards 100% (Fig 2.6). Therefore, as the variation in the age of trees within 50m decreases, the probability of a wood ant nest occurring also decreases. Increased heterogeneity of plantation woodland has already been suggested to increase potential biodiversity (Buse & Good 1993; Moore & Allen 1999; Nájera & Simonetti 2010). Our results support this and we also present a scale within which species heterogeneity is relevant for this species: 50m. The relationship between openness within 50m and probability of occupancy will show if *F. lugubris* benefits from opening of the canopy. Due to the strong positive relationship with the edge of plantations that we found for *F. lugubris* (Appendix 1, Fig A1.2), we expected to find that there would be an optimum level of openness above the minimum value; however, we did not find this trend (Fig. 2.5c). It appears the standard layout of plantation woodland with wide tracks allowing sunlight at the edges of the plantations is sufficient for *F. lugubris* and we would not predict further opening of the canopy at a 50m scale to increase the suitability of the habitat for *F. lugubris*.

What degree of lag is there between establishment of non-native conifer plantations and their colonisation by this forest specialist species?

Although the rate of expansion of *F. lugubris* into new habitat is substantial in terms of nest numbers, the total distance over which *F. lugubris* has expanded is remarkably short (Fig. 2.4).

Between 1854 and 2013, *F. lugubris* exhibited an average expansion rate of just 5m yr^{-1}. Each population of *F. lugubris* is bordered by at least 3km^2 of unoccupied forest that our model predicts to be suitable habitat, therefore expansion of wood ant populations is not limited by habitat availability. It is, instead, the speed at which *F. lugubris* populations expand that is limiting colonisation. *Formica lugubris* is expected to be a poor disperser, with new nests formed a short distance from the parent nest by budding (Hughes & Broome 2007). A poor disperser is an ideal study organism for this question as lag between formation and colonisation of new forest habitat should be clearly identifiable; however, the rate of expansion we found did not keep abreast with availability of new forest habitat. There are neither major roads through the connected forest, nor major water bodies that could act as barriers. The minor roads throughout the study site in many cases cut straight through populations that have expanded. As a result we have no reason to consider them to be a barrier to further dispersal. Our habitat suitability model reinforces the view that the rate of expansion of *F. lugubris* is the limiting factor in this system; we have shown that large areas of connected suitable habitat are available for *F. lugubris*, with the main limiting factor to colonisation being the distance from where historic ant populations occurred. It is well known that species responses generally lag well behind the speed of ecological change (Tilman *et al.* 1994; Ellis & Coppins 2007), and there is no reason that this should be different for creation of novel forest ecosystems. The severity of the lag we have discovered, with wood ant population expansions of under 800m in 160 years of forest expansion, demonstrate the level of lag that should be expected, at least for the more poorly dispersing forest specialist organisms.

Species may be dependent on a particular phase within the dynamic cycle of plantations; for example, over-mature stands show unique assemblages of fungi (Humphrey *et al.* 2000), clear felled areas support a distinct range of Carabid beetle species compared to mature plantations (Butterfield *et al.* 1995) and a range of bird species specialise on either young or old growth (Fuller *et al.* 2007). Specialisation on a specific part of the forestry cycle reduces the suitability of the habitat to a smaller temporal window within each cycle, which will inevitably slow expansion of woodland specialists throughout plantation forests. However, *F. lugubris* does not show specialisation on a specific stage of the forestry cycle and in plantation forests they are edge specialists (Appendix 1: Fig A1.2), likely driven by the importance of sunlight on the nest in thermoregulation (Kadochová & Frouz 2014; Chen & Robinson 2014). As a result, *F. lugubris* will most likely spread along edges and not through plantation blocks, with populations possibly ceasing to expand for a time when suitable edge habitat is unavailable and then continuing when forest management opens a new area and exposes new forest edge. Researchers studying recently created landscapes must take this colonisation lag into account in the study design, data analysis and model creation stages, or risk drawing fallacious conclusions. The colonisation lag that we display means that land managers must not expect

short-term colonisation of newly afforested land: it will take time for forest specialists to colonise. The time taken will depend on the distance from a source population and the dispersal capabilities and specificity of habitat required by each organism.

Within the 13% forest cover in Britain as a whole, the forest cover in England currently stands at 10% (Forestry Commission 2013a), with plans to increase this to 12% by 2060 (Forestry Policy Team 2013), an ambition that will require planting of large areas of new forest. It is a stated aim of the Forestry Commission to maximise the biodiversity supported by their estate (Forestry Commission 2013b) and therefore new forests should be planted in such a way as to maximise their contribution to biodiversity. The colonisation lag we have shown highlights the importance of planting new forest as close as possible to existing forest, especially historic fragments of native woodland, to allow colonisation of forest specialists as quickly as possible. For species that are extremely poor dispersers, such as *F. lugubris*, any form of gap between forest blocks greater than tens of metres wide will hinder colonisation. Our study landscape does not contain any populations that appear to have traversed gaps between fragments, so it would appear this occurs rarely if at all in *F. lugubris.* However as our study was not set up explicitly to examine this problem we cannot be sure that dispersal between separate fragments does not happen. There are a great many more mobile species that will be able to expand longer distances and across intervening habitats, however our findings are an indication of the potential lagging of important parts of the forest ecosystem behind initial colonisation.

2.6. Conclusion

We have shown a large change in forest cover over our study landscape due to afforestation, primarily with non-native conifer species. Our data lend support to the recent suggestions that non-native plantations can have positive influences on forest dependent species: non-native plantations have facilitated large population expansions of the forest specialist *F. lugubris* from existing fragments of native woodland, and provide large areas of suitable habitat into which expansion can continue. We have also shown that despite availability of appropriate habitat a considerable lag should be expected between the creation of plantation forests and their colonisation by forest specialists. This has implications for further work in recently created ecosystems, which must take into account the ability of organisms to colonise the habitat, and for land managers, who should not expect short-term responses of organisms to the availability of new habitat. We suggest future planting of forest in Britain should be as close as possible to existent forest fragments to encourage the colonisation of the new habitat by forest specialists.

Chapter 3: The importance of spatial scale in assessing habitat quality: A case study on wood ants

3.1 Abstract

The presence of an organism within a landscape is predicted to a degree by the availability of its preferred habitats. Habitat can be assessed at a variety of spatial scales, however it can be difficult to define the most relevant scale and multiple spatial scales may yield different predictions to those arising from just one. The woodland specialist ant *Formica lugubris* has expanded from ancient woodland remnants into plantations of non-native conifers, but not the entire plantation forest has been colonised, and the reasons why are unclear.

We assess the habitat preferences of *F. lugubris* using variables calculated at a variety of spatial scales potentially relevant to the ecology of the species, ranging from 10m to 500m. We compare this with a previous study conducted on a single spatial scale (50m) and ask i) does including multiple spatial scales affect the predicted relationships? ii) What features of the colonised woodland best predict the presence of *F. lugubris*?

We find only two of the 11 variables comprising the minimum model in the study assessing a single spatial scale are present in the minimum model using multiple spatial scales. Our model reveals a preference of *F. lugubris* for 20-30 year old trees which was not detected in the previous study. This age class is more abundant in conifer plantations than natural forests, which may explain the success of *F. lugubris.* We show that the variable scales that performed best are greater than that assumed from species specific knowledge previously, which suggests that colony-level interactions with the habitat are more important than individual nests. Our results show that the most important forest management decisions are at the planting stage, where proximity to ancient woodland and placement in a well-lit areas of the landscape will maximise the potential for colonisation of *F. lugubris.* Assessment of multiple spatial scales of variable can lead to a greater understanding of the organism in question and conclusions based on single spatial scale models may be drawn into question when multiple scales are assessed.

3.2 Introduction

Anthropogenic land use changes generally have detrimental effects upon natural ecosystems. The amount of natural forest worldwide shows continual decline (FAO 2010), and the loss of biodiversity attributed to forest loss is well recognised (Travis 2003; Brook, Sodhi & Bradshaw 2008; Bradshaw, Sodhi & Brook 2009). In contrast, the area occupied by planted forests is increasing worldwide, and this expansion has led to increases in forest cover in Europe in recent times (FAO 2010). In order to ameliorate the loss of natural habitat, many countries, including Britain, Finland and the USA are currently aiming to improve the ability of planted forests to

support biodiversity (Ministry of Agriculture and Forestry 2006; USDA Forest Service 2009; Forestry Commisson 2011).

Britain provides a prime example of the recent expansion of planted forests: centuries of forest removals led to a forest cover of around 5% in 1900 (Mason 2007), which has since recovered to the current figure of 13% (Forestry Commission 2013a). This expansion in British forest cover is primarily due to the planting of non-native conifers for commercial objectives carried out in the early to mid-20th century (Quine *et al.* 2013). In recent times, priorities have shifted to encompass a broader range of objectives and a recognition of the benefits of native species in providing a range of ecosystem services beyond that of timber production alone (Forestry Commisson 2011; Quine *et al.* 2013). However, the legacy of afforestation with non-native conifers is still evident in Britain today, for example the non-native conifer Sitka spruce, *Picea sitchensis*, is currently the most common tree species in British forests (Forestry Commission 2013a). A large commercial forest area offers a substantial opportunity to apply management approaches that, in addition to timber production, also aim to support the biodiversity within this habitat.

Management of planted forest ecosystems presents huge challenges. Forest ecosystems support a range of species, many of which differ in their individual habitat requirements, for example many species specialise on particular stages of the forest successional cycle (Sweeney *et al.* 2010; Burgess *et al.* 2015). Species may prefer particular habitats, but may respond to that habitat differently across a range of spatial scales. For example, many organisms benefit from access to disturbed areas (Swanson *et al.* 2010). In a forest, a disturbed area could be as small as that created by a single felled tree or as large as a vast clear-felled area. These clearly distinct scales may elicit different responses from even a specialist organism. In order to make informed decisions on how best to manage a forest landscape for multiple objectives, the requirements of a range of species requiring different habitats must be considered alongside other environmental, economic and social objectives to comply with the principles of Sustainable Forest Management (Quine *et al.* 2013).

The wood ants of the *Formica rufa* group are keystone species in woodland ecosystems, found across the temperate and boreal forests of Eurasia (Hughes & Broome 2007; Stockan & Robinson 2016). These ants are forest specialists, relying for the majority of their food on honeydew brought into their nests by worker ants tending aphids that live in the surrounding trees (Rosengren & Sundström 1991; Domisch *et al.* 2016). Wood ant nests can be as tall as 1m and consist of dead vegetation, the composition of which depends on the type of forest in which they are found. The presence of nests modifies the soil structure, increasing soil porosity (Frouz & Jilková 2008) and nests are hotspots of nutrient exchange due to the accumulation of food and detritus in and around them (Domisch *et al.* 2009; Frouz, Jilková & Sorvari 2016). Wood

ant nests support high levels of biodiversity, including many species that rely on nests as habitat (Härkönen & Sorvari 2014; Parmentier *et al.* 2014; Robinson *et al.* 2016). Their central role in the forest ecosystem, as well as their ability to support a range of other species, makes wood ants of the *F. rufa* group especially important species for management to focus on when aiming to improve the ability of managed forests to support biodiversity.

Nest building organisms, such as *Formica lugubris* demonstrate the importance of different spatial scales. In the UK, *Formica lugubris* forms new nests by budding, whereby one or more queens leave the parent nest with a subset of workers and form a nest nearby (Hughes 2006). Wood ants are quite mobile for their diminutive size, and can form nests over 100m away from their natal nest (D. Procter unpublished data), but nests are usually formed closer to the natal nest (Maeder *et al.* 2016). The precise location of the new nest is likely to depend on factors operating as small spatial scales (under 10m), such as insolation and vegetation height, which affect the thermal environment of the new nest (Kadochová & Frouz 2014; Chen & Robinson 2014). Once active, the worker force of a new nest forages a large area around the nest (Stockan & Robinson 2016). A wood ant nest foraging territory is generally 0.3-0.5 ha i.e. within 50m of the nest, but can extend further (Domisch *et al.* 2016). *Formica lugubris* is also polydomous in the UK, where multiple spatially separate nests function as a single colony (Hughes 2006), therefore the spatial scale of the colony may also interact with environmental variables. Polydomous colony organisation varies in scale from two nests to vast populations that function as a single colony {Ellis et al. in prep}. Across this landscape the largest colony we are aware of contains 47 nests spread over 200m, but we have only mapped a small subset of colonies. Polydomous colonies will forage areas beyond their nests in the same manner as single nests. The location, and persistence, of *F. lugubris* nests therefore should depend on variation in habitat from the very small scale (up to 10m), through individual dispersal and foraging scales (50-100m) up to the polydomous colony scale (possibly well over 200m).

In a recent study the habitat preferences of *F. lugubris* were assessed, using variables applied at only a single spatial scale (Procter *et al.* 2015). The results from that study have important implications for the management of forest for this wood ant species, however the limitation to a single spatial scale of variable may mean that the reported findings are inaccurate. Here we build upon the foundations of Procter et al. (2015), and extend their habitat suitability modelling to encompass multiple spatial scales, informed by ecological knowledge. In doing so, we provide evidence of how important it is to consider multiple spatial scales as opposed to just one when considering an organism's preference for habitat. Our study has implications for the management of this high conservation value species and also assesses the importance of considering a range of spatial scales when assessing habitat preferences in other species.

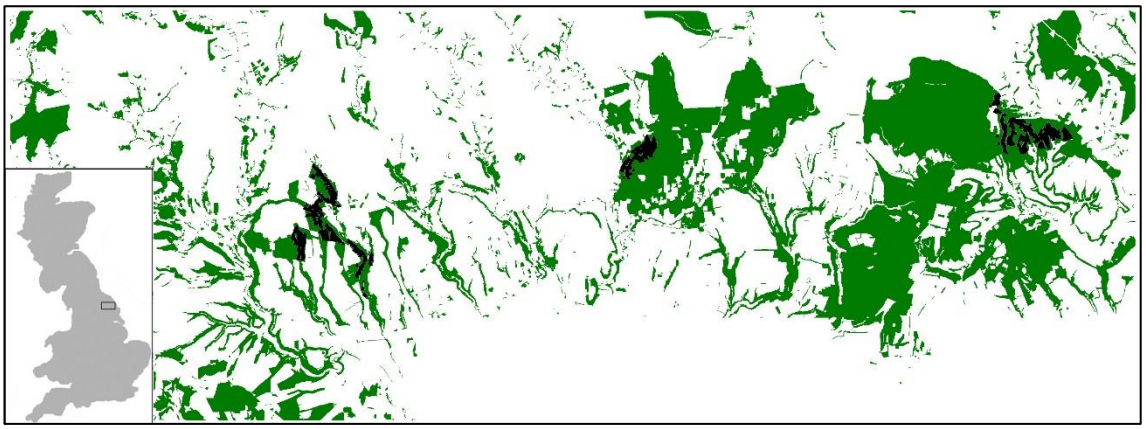

Figure 3.1. The contemporary forest cover across the study landscape (grey polygons) and the locations of the ant nests used to create the models in this paper (black points). The inset shows the location of the study landscape within Britain.

3.3 Methods

Study landscape

The study landscape covers well-mapped populations of the wood ant *F. lugubris* in the southern half of the North York Moors national park (934 km^2 total area, Long/Lat 54.289, -1.059, Fig. 3.1). The landscape has undergone substantial increases in forest cover over the last 160 years through extensive planting of non-native conifers, which has resulted in the gradual expansion of *F. lugubris* populations into recently planted forest (Procter *et al.* 2015). The Forestry Commission is responsible for publicly owned forests in England and manages the majority of the land across this landscape (total 60%), and maintains extensive records in their Forestry Commission sub-compartment database. This resource contains data on characteristics of each plantation block such as the primary species and age structure as well as a number of other variables. Access to this dataset along with a detailed knowledge of the spatial distribution of ant nests in this area has allowed us to develop relevant variables for modelling the suitability of forest habitat for *F. lugubris*. We used a previously published dataset of 2831 nest locations within Forestry Commission managed land across the study landscape (for full details of the nest dataset see Procter et al., 2015).

The forest within this landscape contains over 40 tree species, however three species are particularly abundant: Sitka spruce, *Picea sitchensis* covers the largest area (22.2% of forest area), followed by Scots pine, *Pinus sylvestris* (15.5% of forest area) and Japanese larch, *Larix kaempferi* (8.6% of forest area). Approximately one third of trees are 30 years old or younger (28.4% of forest area), a further third are 31-60 years old (32.1% of forest area), with the remainder either older than 60 years (15.7% of forest area) or undefined (23.8% of forest area).

Variables tested

We assessed 11 variables for their ability to predict the presence of *F. lugubris* nests (Table 3.1). All habitat variables were represented as rasters at 10m resolution. Of the 11 variables used, we assessed 9 at multiple spatial scales (Table 3.1). The variables 'distance to forest cover in 1854' and 'primary tree genus', were not assessed at multiple spatial scales. 'Distance to forest cover in 1854' is a measure of minimum distance to historically occupied forests. Therefore it represents minimum dispersal distance for the ants and we can see no biological reason for varying the scale at which this is assessed. Assessing means within different radii for different primary tree genera would have meant separating that single variable into one variable per tree genus i.e. 14 separate variables. This would have massively increased model complexity with little expected gain, therefore we avoided doing so. The nine other variables were assessed at 10m, 50m, 100m, 150m, 200m, 250m, 300m, 350m, 400m, 450m and 500m. The different spatial scales greater than 10m were calculated as a mean within a circle of that radius using the MaxEnt Multiscale Toolbox v2 (Bellamy *et al.* 2013).10m is the resolution of the raster data, and therefore the minimum possible scale. The scales selected represent biologically plausible interactions with habitat variables, ranging from interactions relevant to precise nest location up to interactions with the polydomous colony (see Introduction).

Table 3.1. Each variable used, what that variable represents and why we have included it.

Variable	Scale varied	Reason for use
Distance to the nearest forest cover in 1854	No	This variable has been shown to be a good predictor of the presence of *F. lugubris* (Procter *et al.* 2015) and is a representation of the minimum distance organisms have had to disperse since that date to reach their current locations
Primary tree genus	No	*Formica lugubris* has foraging preferences for certain trees (Robinson et al., 2008). Inclusion of this variable will allow us to explore whether these small scale preferences affect landscape patterns
Percentage of trees under 20 years old	Yes	These four variables allow us to determine how *F. lugubris* responds to the structural differences in forest areas that result from variation in age composition. In addition, as each age category approaches 100% it provides an indication of the response of *F. lugubris'* to homogeneity within the defined spatial scale. The age brackets were based upon functional differences between ages (Franklin et al., 2002).
Percentage of trees 20-30 years old	Yes	
Percentage of trees 31-80 years old	Yes	
Percentage of trees over 80 years old	Yes	
Percentage of conifers	Yes	Both the percentage of broadleaves and the percentage of conifers allow us to determine broader preferences than those based merely on primary tree genus and these variables allow us to infer whether pure conifer, pure broadleaved, or mixed stands are preferable to *F. lugubris*
Percentage of broadleaves	Yes	
Percentage of open ground	Yes	*Formica lugubris* is an edge specialist and so would be expected to prefer areas where there is an intermediate level of open ground. This variable allows us to test whether than is the case.
Hillshade	Yes	Hillshade is a measure of the shadedness of the landscape that incorporates both the elevation and aspect of the land as well as the position of the sun at the given point on the globe. Wood ants are strongly affected by the thermal environment (Kadochová & Frouz 2014), which may be reflected in their response to this variable. Counterintuitively, given the name, high values represent areas of high insolation and low values low insolation.
Slope	Yes	We would expect that it would be more difficult to maintain large above ground nests such as those constructed by wood ants on steep slopes compared to more level ground. Inclusion of this variable will allow us to see whether this is the case.

Ideally all possible versions of each variable would be considered together in a single model to determine which is the most important in determining the suitability of habitat for *F. lugubris* nests. However, different spatial scales of the same variable can be highly correlated (Appendix 2, Table A2.1). Highly correlated predictor variables, or multicollinearity, can reduce the ability of modelling methods to predict habitat suitability with accuracy (Elith *et al.* 2011). To address this problem we first assess each spatial scale of each variable as a univariate model, allowing us to test which spatial scale of each variable performs best as a predictor of the distribution of *F. lugubris*. We then combine versions of each variable that do not display multicollinearity into a multivariate model. Where variables do show multicollinearity we use the spatial scale of that variable which exhibits the highest predictive power in the multivariate model, following Bellamy et al. (2013).

Univariate models

We assessed the spatial scale at which each variable displays the highest predictive power in terms of the occurrence of *F. lugubris* nests. We created univariate habitat suitability models for each spatial scale of each variable in MaxEnt 3.3.3k (Phillips *et al.* 2006), therefore 11 radius size models per variable. Model performance was compared in terms of AUC, the area under the ROC (receiver operating characteristic) curve, a commonly used measure of model performance (Lobo *et al.* 2008). AUC produces a value indicating goodness of model fit, where a value of 0.5 is no better than random and the fit of the model improves the closer the value is to 1, where 1 would represent perfect explanation of the distributional data.

For habitat suitability models to be reliable, the points used to create the model must be spatially independent. Clustering of points (in our case locations of nests) within homogeneous areas leads to false inflation of model performance values and overfitting towards environmental biases (Veloz 2009; Boria *et al.* 2014). To remove spatial bias we first removed points that were replicated within the minimum scale of the habitat variables (10m). In this first step we removed 848 points using the rarefy occurrence data tool in SDMToolbox v1.1. To account for any further spatial bias, presence points can be spatially constrained i.e. neighbouring nest locations assigned to different partitions of the data. Nest locations are represented within the model as points. Points are divided into two categories; the training partition is composed of the points used to train the model, the test partition utilises the remaining points to test the fitted model. Spatial constraining of training and test points removes false inflation of AUC values compared to model cross-validation (Bellamy *et al.* 2013). We spatially constrained our nest locations by measuring the distance between each nest location and its nearest neighbour, then splitting neighbour pairs that were less than 20m apart into different data partitions, one was used as a training point and one as a test point (Parolo, Rossi & Ferrarini 2008). The remaining points that were all over 20m from their nearest neighbour were added to the training points. This

categorising approach provided 1360 training points and 623 test points i.e. approximately two thirds of the data were used to train the model and one third to test the model.

Multivariate modelling

The univariate modelling identified the most useful predictive scale of each variable, however it did not reveal anything about the importance of the variables in comparison to one another. I order to assess which variables best predict the occurrence of *F. lugubris* nests we then built a multivariate model. Including all the variations of spatial scale of each variable we had 101 potentially useful explanatory variables. We assessed the multicollinearity of variables using ENMTools v1.4.3 (Warren *et al.* 2010). All of the versions of variables with varied scale 50-500m were strongly correlated (Appendix 2 Table A2.1). We therefore followed Bellamy et al. (2013) in choosing the spatial scale with the highest predictive power in terms of AUC as the variable to put into the multivariate model. None of the original variables at 10m resolution were strongly correlated with the larger spatial scale variables, therefore these were also included (Appendix 2, Table A2.1). There were also strong correlations (>0.7 in magnitude) between the following three pairs of variables: percentage of conifers within 100m and percentage of open ground within 200m, percentage of conifers within 100m and percentage of trees aged 31-80 years old within 200m and percentage of conifers within 10m and percentage open ground within 10m, (Appendix 2, Table A2.2). We therefore removed percentage of open ground within 200m, percentage of trees ages 31-80 years old within 200m and percentage of conifers within 10m and from consideration for the multivariate model, because they displayed lower AUC in univariate models than the variables with which they were strongly correlated (Appendix 2, Table A2.2). The full multivariate model on which model selection was performed therefore contained 17 variables (Table 3.2).

We dealt with the issue of spatial autocorrelation separately in the multivariate and univariate models, because the predictions in the models were based on different combinations of variables. Once the appropriate set of variables for the multivariate habitat suitability model had been selected (see above), we assessed the level of heterogeneity in the habitat variables using the 'calculate climate heterogeneity' tools in SDM Toolbox v1.1 (Brown 2014). Repeat points within areas of spatial homogeneity were removed using the 'Spatially rarefy occurrence data' tool in SDM Toolbox v1.1. The modelled areas were separated into five categories of heterogeneity based on natural breaks in the data. The highest heterogeneity class represents areas where habitat, defined by the variables we provide, varies most. The lowest heterogeneity class represents areas with the least variation in the variables we are using. Duplicate points were removed within 10m for the highest heterogeneity class, then at 70m, 130m, 190m and finally 250m for the lowest heterogeneity class. The 10 m radius was chosen because that is the resolution of the spatial covariates, so it is not possible to have spatial heterogeneity within that

scale. The maximum value of 250 m was chosen after visual inspection of test output values, because this radius resulted in very small numbers of nest locations being within the areas of low heterogeneity. The intermediate radius sizes are simply intervals between the minimum and maximum values of heterogeneity. The number of points removed at each step was 848 at 10m, 1043 at 70m, 136 at 130m, 30 at 190m and 13 at 250m, leaving us with 761 unique occurrence points upon which to build the multivariate model. These 761 points were then spatially constrained into training and testing data sets. We measured the distance from each point to its nearest neighbour then allocated individual members of neighbour pairs that were less than 30m apart into different data sets; one was used as a training point and the second as a testing point. The remaining points that were over 30m apart were added to the training points. This resulted in inclusion of approximately a third of the data (228 points) to test points and two thirds (533 points) to training points.

Following variable selection, we tested the optimal level of regularisation multiplier to use for the multivariate model by running the full model with the following range of regularisation multipliers; 1, 2, 5 10 and 20 and comparing the model performance in terms of AIC in ENMTools 1.4.3 (Warren *et al.* 2010). The regularisation multiplier adjusts how smooth the modelled response will be, with higher values giving smoother output (Elith *et al.* 2011). A regularisation multiplier of 1 performed best so this was then used in all other runs of the model (Appendix 2, Table A2.3). The full multivariate model was pruned to remove variables that were impairing model performance. The full model plus pruned versions with each variable removed separately were compared in terms of AIC in ENMTools 1.4.3 (Warren *et al.* 2010). The model with the lowest AIC was then used and further pruned until no more variables could be removed to improve model performance. The minimum model was then re-run with logistic output, which is proportional to the probability of nest presence given a specific sampling effort (Elith *et al.* 2011). Comparisons between models can be unreliable due to the probability of nest presence varying with sampling effort, however because sampling effort is identical across our different analyses, this is not an issue in our study. The importance of the variables in the minimum model were assessed by their percentage contribution to the model and permutation importance to the model. Percentage contribution is determined by summing the increase in regularised training gain due to that variable per iteration of the model. Permutation importance is a measure of how much worse the model performs if that variable is randomised.

3.4 Results

Univariate modelling

The power of the univariate models based on each of the individual variables to predict the occurrence of *F. lugubris* nests is not consistent across the range of spatial scales of the variable (Fig. 3.2 a, b), however there are some themes. None of the variables display their strongest

explanatory power when data are considered at the finest spatial scale data (10m); all perform better when means within larger spatial scales are used (Fig. 3.2 a, b). Means within 200m provide particularly high explanatory power for a number of variables, namely percentage of trees 20-30 years old, percentage of trees over 80 years old, percentage of broadleaves, percentage of open ground and slope (Fig. 3.2). The AUC values for all univariate models were quite low, with no single variable at any scale achieving an AUC value of 0.7, therefore none of these variables would be good predictors of the locations of *F. lugubris* nests when taken alone. In a previous modelling study on the same data all variables were assessed at 50m (Procter *et al.* 2015), however our results show that this was never the variable with the highest predictive power (Fig. 3.2).

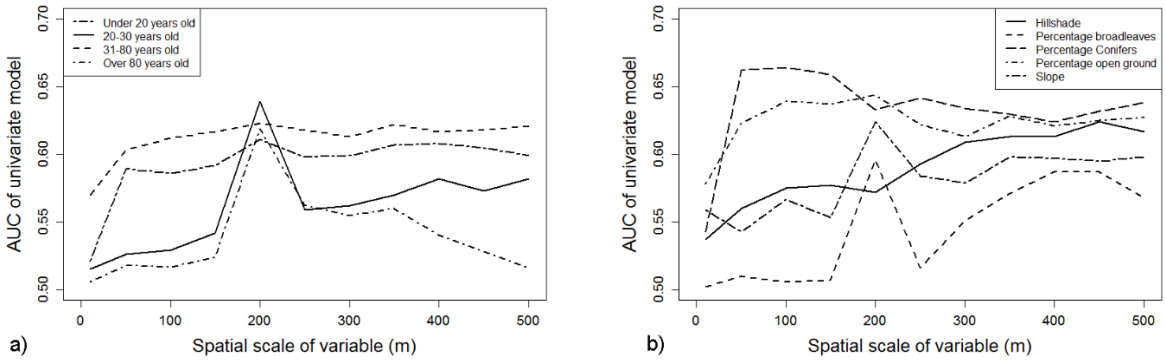

Figure 3.2. Variation in the predictive power of different univariate models in terms of AUC as they vary with spatial scale of the variable **a)** the four tree age based variables **b)** the remaining five variables

Multivariate Modelling

The full multivariate model included 17 variables prior to the pruning exercise (Table 3.2). Using AIC to compare the performance of the full model with that of the reduced versions we sequentially removed four variables that were decreasing model performance, namely: percentage of trees under 20 years old within 10m, percentage of trees under 20 years old within 200m, percentage of trees over 80 years old within 10m and percentage of trees 31-80 years old within 10m (Appendix 2, Table A2.4).

Table 3.2. Variables included in the multivariate habitat suitability model before model selection. These 17 variables are all that remain of the possible 101 that could have been included in the habitat suitability model before removing variables that showed strong multicollinearity.

Variable	**Spatial scale**
Distance to forest cover in 1854	Not varied
Primary tree genus	Not varied
Percentage of trees under 20 years old	10m
Percentage of trees under 20 years old	200m
Percentage of trees 20-30 years old	10m
Percentage of trees 20-30 years old	200m
Percentage of trees 31-80 years old	10m
Percentage of trees over 80 years old	10m
Percentage of trees over 80 years old	200m
Hillshade	10m
Hillshade	450m
Percentage of broadleaves	10m
Percentage of broadleaves	200m
Percentage of conifers	100m
Percentage of open ground	10m
Slope	10m
Slope	200m

Distance to forest cover in 1854 and percentage of conifers within 100m were the most important variables in predicting the probability of presence of *F. lugubris* nests, and together this pair of variables made a percent contribution of over 50% to the predictive power of the model and had a permutation importance of almost 50% (Table 3.3). The probability of presence of *F. lugubris* nests remains fairly constant up to a distance of approximately 600m from forest cover in 1854, beyond which probability diminishes rapidly towards zero (Fig. 3.3a). There is a positive relationship between the probability of presence of *F. lugubris nests* and the percentage of conifers within 100m (Fig. 3.3b). The next two most important variables were the primary tree genus within 10m and mean hillshade within 450m (Table 3.3). Probability of presence of *F. lugubris* nests was higher when *Quercus* (oaks) and 'other broadleaves' were present (Fig. 3.4.). The category of 'Other broadleaves' represents a combination of broadleaved genera, each of which were present in small numbers across the study landscape and so were not included individually (Appendix 2, Table A2.5). The probability of presence of *F. lugubris* nests is lower in areas of forest composed of *Betula*

(birches) and *Fagus* (beech), than of other genera (Fig. 3.4). Mean hillshade (defined in Table 3.1 where, counterintuitively, high values represent high insolation) within 450m shows two peaks, one at medium-low values of hillshade, which would indicate shaded areas of the landscape and one at very high values, which would indicate areas of the landscape with only a small degree of shade (Fig. 3.3c). Percentage of open ground within 10m showed a peak of probability of presence of *F. lugubris* nests in the low percentages and then a steady decrease in probability as the percentage of open ground increases to the maximum of 100% (Fig. 3.3d). Mean slope of the ground within 200m showed a decrease in the probability of presence of *F. lugubris* nests at higher values, with little trend at lower values (Fig. 3.3e).

Table 3.3. The relative importance of each of the variables included in the fully pruned model. Percentage contribution is determined by summing the increase in regularised training gain due to that variable per iteration of the model. Permutation importance is a measure of how much worse the model performs if that variable is randomised.

Variable	Spatial scale	Percent contribution	Permutation importance
Distance to forest cover in 1854	10m	27.8	21.2
Percentage conifers	100m	26.9	27.4
Primary tree genus	10m	12.0	6.1
Mean hillshade	450m	10.4	8.0
Percentage of open ground	10m	4.3	6.0
Percentage of trees 20-30 years old	200m	4.2	4.6
Slope of the ground	200m	4.0	6.4
Percentage of trees 20-30 years old	10m	3.3	2.2
Hillshade	10m	1.9	1.8
Slope of the ground	10m	1.6	5.7
Percentage of trees over 80 years old	200m	1.6	4.3
Percentage of broadleaves	200m	1.2	4.6
Percentage of broadleaves	10m	0.7	1.7

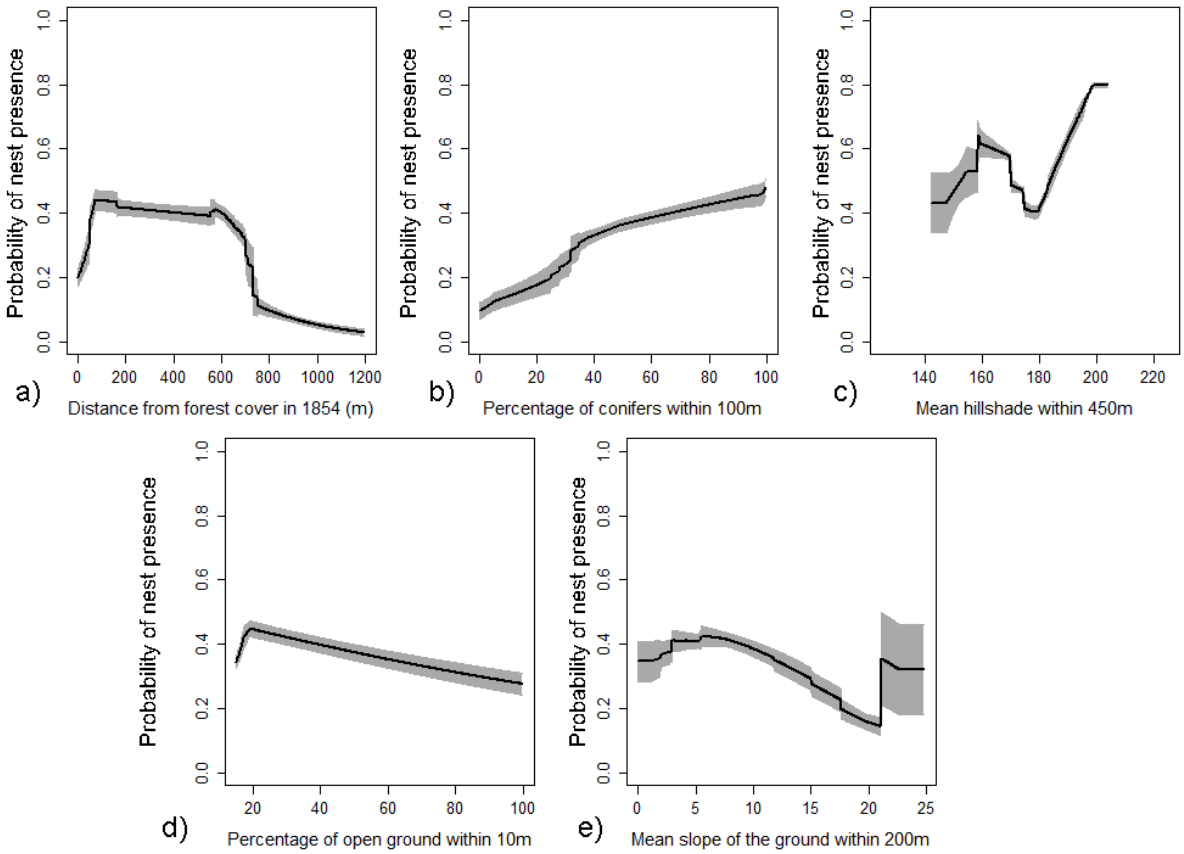

Figure 3.3. The relationships between the probability of presence of *F. lugubris* nests and the five most important variables to the model performance for the multivariate model that are not related to age structure of the forest: **a)** The distance from forest cover in 1854, **b)** percentage of conifers within 100m, **c)** Mean hillshade within 450m **d)** percentage of open ground within 10m, **e)** mean slope of the ground within 200m. Lines are means of 5 models with the grey polygons showing standard deviations.

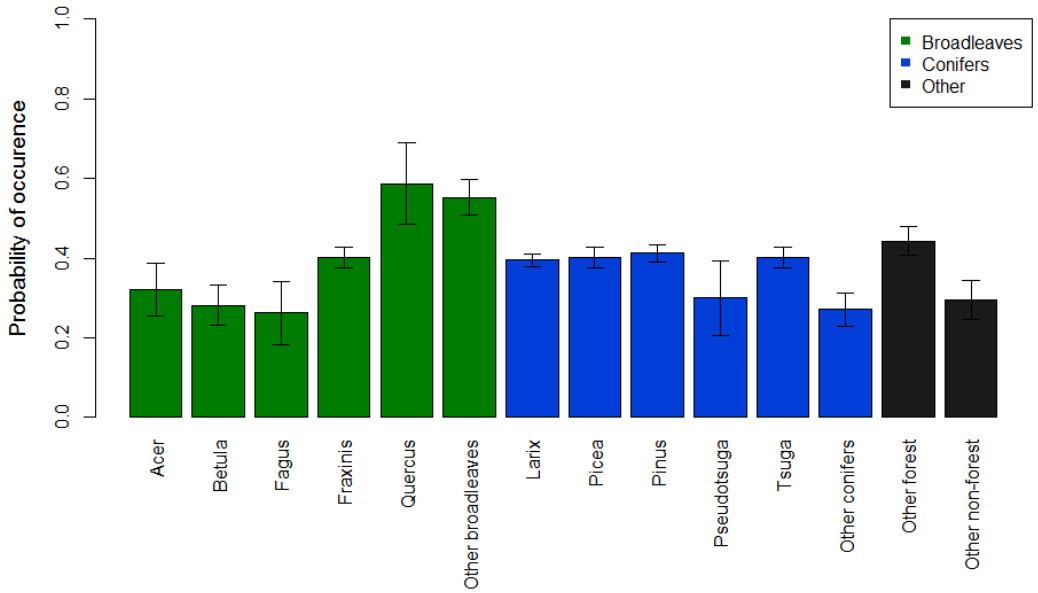

Figure 3.4. The probability of occurrence of *F. lugubris* nests in relation to the primary tree genus within the forest. Other forest comprises deer glades and felled areas. Other non-forest incorporates man-made areas within forests, such as car parks and picnic areas as well as unplanted areas, therefore non-forest does not mean tree-free.

There was not a consistent relationship between the three variables that related to the age structure of the forest and the probability of occupancy of *F. lugubris* nests: there was a positive relationship between the percentage of trees 20-30 years old within 200m and the probability of presence of *F. lugubris* nests (Fig. 3.5a). There was a positive relationship between percentage of trees 20-30 years old within 10m until approximately 80% cover, after which the probability of presence of *F. lugubris* nests decreased strongly as percentage of trees 20-30 years old within 10m approached 100% (Fig. 3.5b). The percentage of trees over 80 years old showed a weak negative relationship with the probability of presence of *F. lugubris* nests (Fig 3.5c). The remaining variables were of very low importance to the model (Table 3.3) and so are not discussed here, but can be viewed in the supplementary materials (Appendix 2, Fig. A2.1).

The 'Distance to forest cover in 1854' variable was also the most important variable when a single spatial scale was assessed, but 'Primary tree genus' was of much lower importance in that model (Procter *et al.* 2015). Furthermore, none of the other variables used here, which contributed over 60% of model performance (Table 3.3), were included in the previous habitat suitability model due to the selection of a single spatial scale. The model we find to best explain the location of *F. lugubris* nests is therefore very different when we assess multiple spatial scales than if only one spatial scale is assessed.

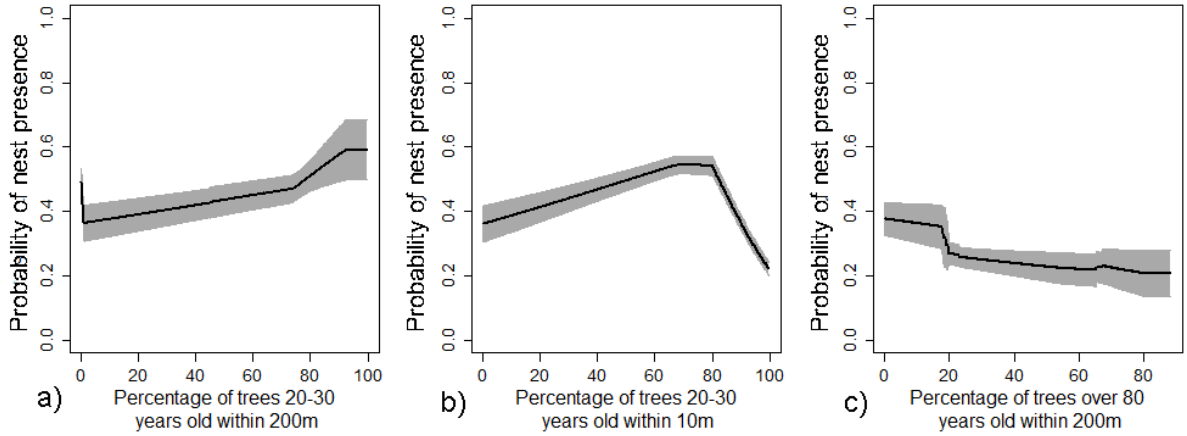

Figure 3.5. The relationships between the probability of presence of *F. lugubris* nests and the three variables related to the age structure of the forest remaining in the model after model pruning: **a)** percentage of trees 20-30 years old within 200m, **b)** percentage of trees 20-30 years old within 10m **c)** percentage of trees over 80 years old within 200m

When we project our multivariate model across all areas for which data are available across the landscape we find that the model is a good fit of where wood ant nests are currently found (Fig. 3.7 a and b). There are large areas of the forest across the landscape which are predicted to be very poor quality for *F. lugubris* (Fig. 3.7 b). However, when we remove the effect of distance from forest cover in 1854 and re-project the model we reveal that virtually all the forest across our study landscape is suitable for supporting populations of *F. lugubris* (Fig. 3.7c).

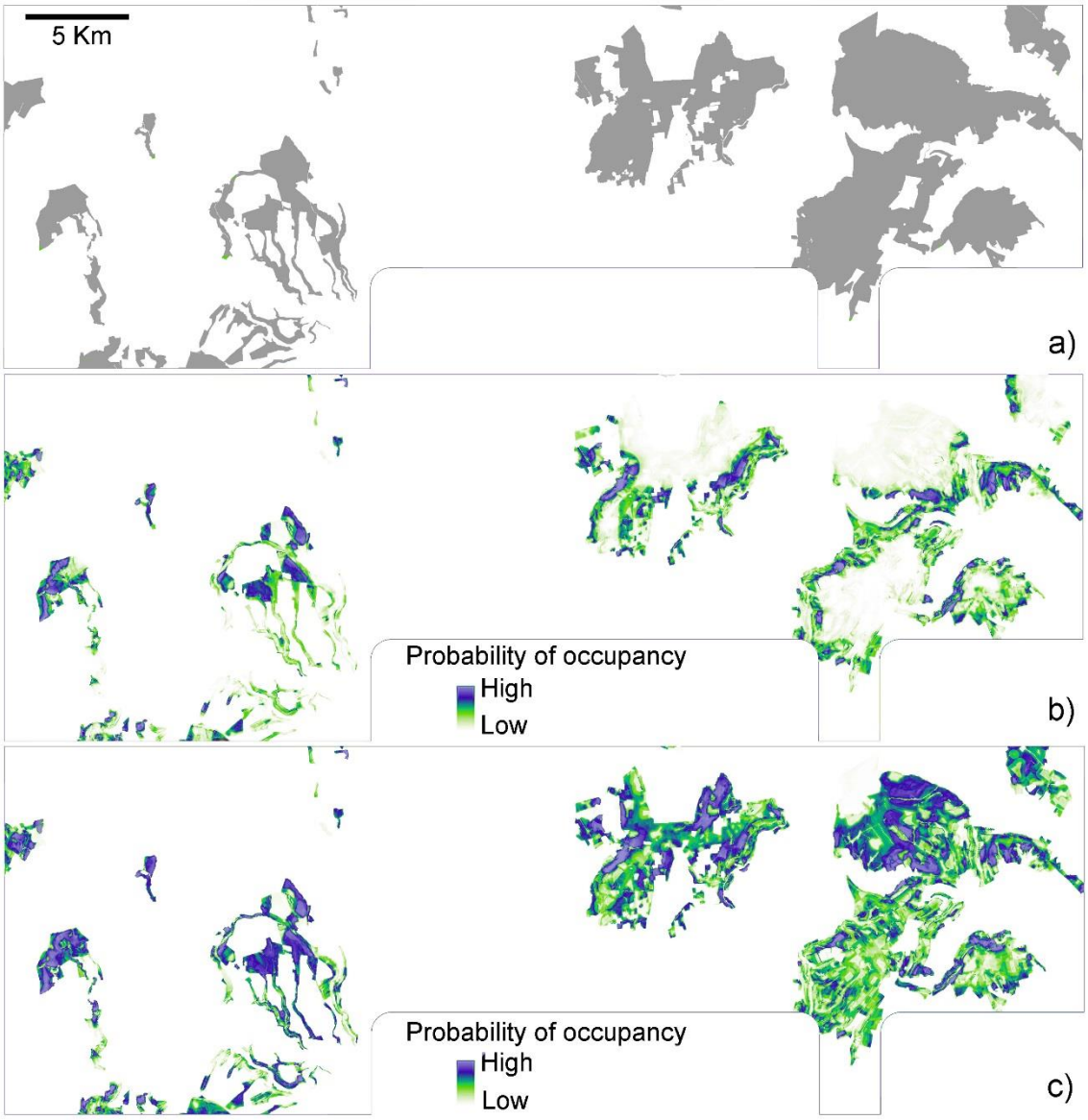

Figure 3.6. a) The areas of the landscape for which data are available, **b)** the predicted probability of presence of *F. lugubris* nests based on the full multivariate model, **c)** the predicted probability of presence of *F. lugubris* nests with the effect of distance from forest cover in 1854 removed from the full multivariate model.

3.5 Discussion

We have shown that the use of multiple spatial scales for each variable can result in a very different best fitting model being constructed than when only a single spatial scale is assessed. If a study is attempting to understand the full complexity of the relationships between an organism and its habitat, then multiple spatial scales must be assessed. Our results reveal that the spatial scale that produces the strongest response from *F. lugubris* varies, but a number of variables get the strongest response at the 200m scale. We would therefore recommend that management interventions aimed at benefiting wood ants should be implemented on a 200m

scale. Furthermore, any modifications of habitat likely to be detrimental to wood ants should avoid the 200m scale and should be implemented at a smaller spatial scale in order to reduce the negative effect of the modification.

It may be thought that using the highest resolution data possible would increase the accuracy of predictions of habitat suitability. However, our results do not support this assumption: each variable showed higher predictive power when expressed as means of radii greater than 10m compared to the 10m scale. This does not mean that there are no important characteristics at the 10m scale, simply that the variables we use do not capture them. For example access to sunlight is known to be important to wood ants, due to its role in the thermoregulation of the nest (Kadochová & Frouz 2014; Chen & Robinson 2014). We attempted to include this using the Hillshade variable, because much of the variation in sunlight intensity across the landscape will be determined by both slope and aspect of the land. However, within forests the canopy has a very strong effect on the amount of light that penetrates to the floor, which is the area that matters to the ants. It was not possible to include the effects of canopy on insolation into our model, therefore there may be small scale (<=10m) effects on positioning of the nests to optimise insolation, which we do not capture with our model.

The results from our multivariate model support the main finding of Procter et al. (2015): that the most important predictor of the presence of *F. lugubris* nests is the distance to historic forest cover (Table 3.2). We do not expect the relationship we display between the probability of presence of *F. lugubris* nests and distance to historic forest cover to be stable over time. The current distance that nests are from forest cover in 1854 is a measure of the minimum distance that populations must have dispersed in order to colonise their current locations. *Formica lugubris* is predicted to continue expanding into currently unoccupied forest (Procter *et al.* 2015), therefore in the future we would expect to find *F. lugubris* nests further from forest cover in 1854 than they currently are. A similar model assessed in the future would find a different relationship with distance to forest cover in 1854. The strong relationship we display between the presence of *F. lugubris* nests and distance to historic forest cover is further evidence that the history of both a landscape and a population must be considered in order to properly understand the current distribution. Failure to include distance to forest cover in 1854 as a variable would have meant that the most important variable in predicting the distribution of *F. lugubris* was not considered. The presence of a particular species depends both on the suitability of that habitat for that species and the ability of that species to disperse to the suitable habitat (Saunders *et al.* 1991). Further studies must take into account the potential for a species to have reached its current location or risk drawing incorrect conclusions. There are currently plans to increase the forest cover of England by a further 2% by 2060 (Forestry Commission 2013b), which will involve establishment of large areas of newly planted forest. We present further evidence that any future planting of forests should be as close as possible to existing forest fragments to

facilitate the colonisation of the new habitat by a forest dependent species. Our findings also highlight the importance of not assuming that organisms are in equilibrium with their habitat, *F. lugubris* certainly is not.

Our findings utilising multiple spatial scales to predict habitat suitability disagree with several findings with previous work on a single spatial scale: Procter et al. (2015) found a consistent, though weak, negative relationship between the percentages of any single one of the four age categories of trees within 50m and the probability of presence of *F. lugubris* nests. This relationship was interpreted as evidence for homogeneity of forest structure negatively impacting the probability of presence of *F. lugubris* nests, which is in line with suggestions from the literature (Buse & Good 1993; Moore & Allen 1999; Nájera & Simonetti 2010). However, we do not replicate these results in our current multiscale model. Of the seven variables related to the age structure of the forest initially included in the multivariate model (Table 3.2) only three remained after model pruning. Therefore, fewer than half are important in predicting the presence of *F. lugubris* nests. Of the three which do remain, only one replicates the trend shown by Procter et al (2015): there is a negative relationship between the probability of presence of *F. lugubris* nests and the percentage of trees over 80 years old within 200m (Fig. 3.5c). Our results suggest that *F. lugubris* has a preference for locating its nests in areas in which there is a high proportion of 20-30 years old trees. We base this conclusion on the model, which shows that an increase in the percentage of 20-30 years old trees within both 10m and 200m increases the probability of presence of *F. lugubris* nests (Fig. 3.5a, b), although at percentages above 80% within 10m this relationship inverts. The 20-30 years old age class is characterised by an established cohort that is beginning to close the canopy (Franklin *et al.* 2002). *Formica lugubris* requires both access to trees on which to forage (Rosengren & Sundström 1991) and uses insolation for thermoregulation (Kadochová & Frouz 2014), therefore trees that are sufficiently large to support high aphid populations but small enough to allow some sunlight on to the forest floor are likely to be what is driving this preference. The preference for 20-30 year old trees again explains observed high abundance of *F. lugubris* in commercial forests, because the planting of blocks of trees of the same age on short rotation cycles means that there will be access to a higher percentage of 20-30 year old trees than is the case in natural situations. We would recommend that very large areas of forest over 80 years old are not retained on the edge of expanding populations of *F. lugubris* because the low performance of *F. lugubris* in this age of forest may cause them to act as a barrier to population expansion.

The most important variables for predicting the occurrence of *F. lugubris* are unlikely to be affected by management of commercial forests. A short distance from historic forest cover improves the probability of occurrence of *F. lugubris* and, as we suggested earlier, this can be addressed at the onset when developing the planting design, because beyond this stage

management cannot influence proximity to historic forest cover. A higher percentage of conifers within 100m increases the probability of presence of *F. lugubris* nests, which is encouraging given the current and probable future importance of fast growing conifer species for commercial purposes in managed forests in Britain. Mean hillshade within 450m is a measure of the average shadiness of the landscape, and our model indicates higher probabilities of *F. lugubris* nest presence in less shaded areas of the landscape. Again, this could be assessed at the time of planting but not subsequently. There may also be other reasons for managers to avoid planting on the well-lit areas of the landscape because such areas generally occur on steep sided south facing slopes, where both planting and harvesting will be more difficult than on flat ground. In contrast, the primary tree genus within a stand is amenable to management, and our results show that broadleaves can also play a role in supporting the presence of *F. lugubris* nests, especially oaks (*Quercus* in Fig. 3.4). *Quercus* and *Betula* species have previously been shown to have a positive influence on the presence of *F. lugubris* (Robinson *et al.* 2008), which shows agreement with *Quercus* promoting the presence of *F. lugubris* best in our model, however areas in which *Betula* sp. dominate show lower probability of presence of *F. lugubris* nests than many other genera (Fig. 3.4). Field observations indicate that larch (*Larix*), pine (*Pinus*) and spruce (*Picea*) species are all foraged on strongly by *F. lugubris*, but beech (*Fagus*) was not foraged on nor did ants nest in close proximity to it (Robinson *et al.* 2008). Our model agrees with these fine scale foraging data: we find high probabilities of presence of *F. lugubris* for larch, pine and spruce but low probabilities of presence of *F. lugubris* nests in beech (Fig. 3.4). From our model we would recommend that larch, pine and spruce all support *F. lugubris*, which is fortunate for management as these are the most popular genera used in commercial planting. The suitability of the habitat can be improved by either maintaining or additionally planting areas of oaks and avoiding beech. Maples and sycamores (*Acer*), as well as ash (*Fraxinus*) and hemlock (*Tsuga*) could all contribute to the maintenance of a *F. lugubris* population without negative effects.

The variation in univariate model performance we see may be explained by the ecology of the study species. While we are certain that there are small scale habitat effects on nest locations, such as variation in insolation due to canopy cover, we accept that it is unlikely that the variables we include in this model will capture that variation. The larger spatial scales should be relevant to different parts of the ecology of *F. lugubris*. Both new nest formation and individual nest foraging normally take place on a much smaller scale than 200m (Domisch *et al.* 2016; Maeder *et al.* 2016). However, polydomous colonies can have an influence over hundreds of meters. The peaks of univariate model performance we see at 200m therefore may be evidence that it is the interaction between the colony as a whole and the habitat that matters, rather than between individual nests and the habitat. Polydomous colonies have only recently begun to gain research attention (Debout *et al.* 2007; Robinson 2014) {Ellis et al. in prep}, and the relative

importance of nest scale vs colony scale when interacting with habitat is unknown. There is growing evidence that interactions between polydomous nests are cooperative, and help to ensure efficient foraging and the survival of nests (Cook, Franks & Robinson 2014; Ellis & Robinson 2015a; b), therefore the colony scale may be the most important for determining habitat preferences for polydomous and species. The previous study used 50m as the spatial scale for variables, because this is the distance from a nest where the majority of nest formation and foraging occurs. Perhaps it is the importance of colony levels characteristics over nest level characteristics that leads us to such different relationships in this study.

We have shown that the best explanatory model when multiple spatial scales are assessed can differ wildly from when only a single spatial scale is assessed. Using univariate models, we show that the spatial scale which best predicts the presence of *F. lugubris* is 200m, which may suggest that the polydomous colony has greater importance for predicting the presence of *F. lugubris* than individual nests are. However, when a multivariate model is created those variables at a 200m scale are of low importance relative to other variables that were considered. Only the most important variable in the model was consistent between this study and a previous study which used a single spatial scale. It is therefore essential that multiple spatial scales are assessed where possible in further studies of habitat suitability. Forests which have been managed for commercial forestry provide high quality habitat for *F. lugubris*, and the primary tree genera used in commercial forests (larches, pines and spruce), all predict a high probability of presence of *F. lugubris* nests. The most important decision in management that aims to encourage the presence of *F. lugubris* occurs prior to planting, because consideration has to be given to the location of new plantations in terms of their proximity to historic forest cover and the ants requirement for well-lit areas of the landscape. In existing forests the popular conifer species can be augmented with oaks to improve the quality of the habitat for *F. lugubris*. Increases in forest cover in Britain in the future should allow the expansion of *F. lugubris* into new areas provided that they are placed adjacent to existing populations.

Chapter 4: Positive effects of non-native conifer plantations: evidence of connection of previously isolated populations and high genetic diversity

4.1 Abstract

Habitat fragmentation has detrimental effects on the populations that habitat supports. Detrimental effects extend to demographic, ecological, and genetic consequences of habitat fragmentation. British forests have historically been fragmented, but recently this trend is being reversed, due to increases in commercial forests comprised primarily of non-native conifers. In many locations, commercial forests have connected previously isolated forest fragments, which will have beneficial effects on forest species, if they can make use of the commercial forest habitat. The wood ant *Formica lugubris* has recently expanded from forest fragments into commercial forests, but may still suffer from the genetic consequences of historic fragmentation and isolation. We assess genetic diversity in a population of the forest specialist *F. lugubris,* and ask i) is this demographically healthy population genetically diverse? ii) is there evidence of commercial forests connecting previously isolated population fragments? Our results show that this historically fragmented and isolated population is genetically diverse, and is in no danger of extinction. Furthermore, we show evidence of commercial forest expansion connecting previously isolated population fragments. We also find strong mitochondrial divergence within the *F. lugubris* population, which warrants further investigation. Our findings suggest that this beneficial forest species should continue to thrive. Furthermore our findings add to evidence that, contrary to some expectations, there are healthy wood ant populations in the UK. We demonstrate an example of anthropogenic land use change having positive effects on a natural ecosystem: the creation of commercial forest on previously non-forest land has defragmented this historically degraded landscape.

4.2 Introduction

Loss of habitat, fragmentation of habitat and isolation of populations increases their extinction risk (Wilcox & Murphy 1985). The most obvious effect of habitat loss is the direct reduction in the population size it can support. However, reducing a previously continuous habitat into isolated fragments also has implications beyond the reduction in total population size. Subdivision of remaining habitat reduces the populations present within each fragment, making each fragment more susceptible to extinction due to stochastic demographic events and natural catastrophes (Shaffer 1981). Habitat fragments also have a higher edge to interior ratio, increasing negative edge effects on species persisting in fragments (Saunders *et al.* 1991). Habitat destruction is often accompanied by degradation of the remaining habitat, therefore

remaining populations can have a lower quality habitat in which to persist, again increasing the likelihood of population extinctions (Wilcox & Murphy 1985).

Alongside the demographic and ecological consequences of habitat destruction and isolation, there are a range of negative genetic effects that come in to play in populations vulnerable to extinction. Reduction of the effective population size of isolated populations causes increased inbreeding and stronger effects of genetic drift, which leads to reduced genetic diversity within the isolated population (Höglund 2009). Across a range of species, threatened populations exhibit significantly lower genetic diversity than non-threatened populations (Spielman *et al.* 2004). Furthermore, reduced genetic diversity can correlate with reduced reproductive success (Westemeier *et al.* 1998), and subsequent increases in genetic diversity can stimulate increases in population health (Ingvarsson 2002). Assessment of the genetic health of demographically recovering populations is therefore essential, to ensure that there is sufficient genetic variability to allow recovery to continue.

In general, anthropogenic land use change has negative effects on biodiversity (Foley *et al.* 2005). A potential exception to this is the recent increases in non-native conifer plantations in Europe, especially in Britain. Britain has undergone centuries of degradation and overexploitation of natural ecosystems, which led to a minimum forest cover of 5% around 1900 (Mason 2007). Since then there have been massive increases in forest cover, due to the planting of non-native conifers for commercial forestry, and modern day forest cover stands at 13% (Watts 2006; Forestry Commission 2013a), with further increases in forest cover planned (Forestry Policy Team 2013). Although non-native conifer plantations represent a different habitat to natural/naturalised forest there are examples of several species responding well to the recent increase in conifer cover (Hale *et al.* 2001; Vanhala *et al.* 2014; Procter *et al.* 2015). Furthermore an extensive survey of plantation forest biodiversity in Britain concluded that plantations made a significant contribution to the conservation of forest biodiversity (Humphrey *et al.* 2003; Quine & Humphrey 2010). If conifer plantations connect previously isolated populations that can make use of the plantation habitat, they are in effect defragmenting the forest landscape, reducing the negative effects of habitat fragmentation that British forests have historically suffered.

The wood ants of the *Formica rufa* group are common across the forests of Europe and Asia (Stockan *et al.* 2016). Wood ants are forest specialists, due to the majority of their diet in the summer comprising honeydew from aphids (Rosengren & Sundström 1991), and are keystone species in forest ecosystems (Hughes & Broome 2007). Wood ant nests support high levels of biodiversity, including a range of species found nowhere else (Härkönen & Sorvari 2014; Robinson *et al.* 2016). The construction of nests increases soil porosity (Frouz & Jilková 2008), and the accumulation of detritus in the nest makes the nests hotspots of nutrient exchange

(Domisch *et al.* 2009). Within the *F. rufa* group, *F. lugubris* is found across Europe. *Formica lugubris* exhibits variation in dispersal strategies across its range, but in the UK it is a poor disperser (Gyllenstrand & Seppä 2003; Ellis & Robinson 2014), and so is a good model system for the slowest dispersing components of the forest ecosystem. Expansions of British forests over the last century have allowed the expansion of populations of *F. lugubris*, but historic populations have survived through severe habitat fragmentation and isolation (Procter *et al.* 2015). It is therefore quite possible that, though the populations are now demographically healthy, they could show low genetic diversity and could be much less healthy than may be expected from current population numbers.

Here we assess genetic diversity in the wood ant *F. lugubris* in the light of recent population expansions and historic population fragmentation and ask i) is the demographically healthy (i.e. numerous) population we study equally genetically healthy? ii) is there any evidence of the increases in forest cover in this population connecting previously isolated population fragments?

4.3 Methods

Our study landscape is in the southern half of the North York Moors national park (Long/Lat 54.289, -1.059, Fig. 4.1). The landscape contains a number of wood ant populations, which have undergone recent expansions into recently planted commercial forests (Procter *et al.* 2015). Nest numbers vary between populations but several are very healthy (over 1000 nests each), and each population is associated with an area of ancient woodland. It is in these ancient fragments that we presume populations persisted, before the recent forest expansions allowed their spread to its current extent. We focussed our sampling on a single population to assess the genetic health of this recently expanded population, and attempt to assess whether increases in forest cover has connected previously isolated population fragments. The sampled population had previously been accurately mapped (Procter *et al.* 2015), which allowed us to randomly select 21 points throughout the landscape to collect ants at. At each of these points we collected 10 ants from three nests, therefore 63 nests and 630 ants in total throughout the population. Clustering nests in groups of three allowed us to assess differentiation from the very local to the population scale. Other work on the same dataset has showed that the three nests at each sample point show no genetic distinction (Chapter 6), therefore each of these 21 sample points will be taken as an independent unit in analyses, with differences between nests within triplets not assessed. We assessed nuclear genetic variation using all sampled ants at 12 microsatellite loci. A subset of nests spread throughout the population were selected for investigation of mitochondrial variation. A total of 39 out of 63 nests were chosen, to maximise coverage of the geographic area covered by the population. A single ant from each nest was chosen at random and variation was assessed by sequencing a section of Cytochrome oxidase 1.

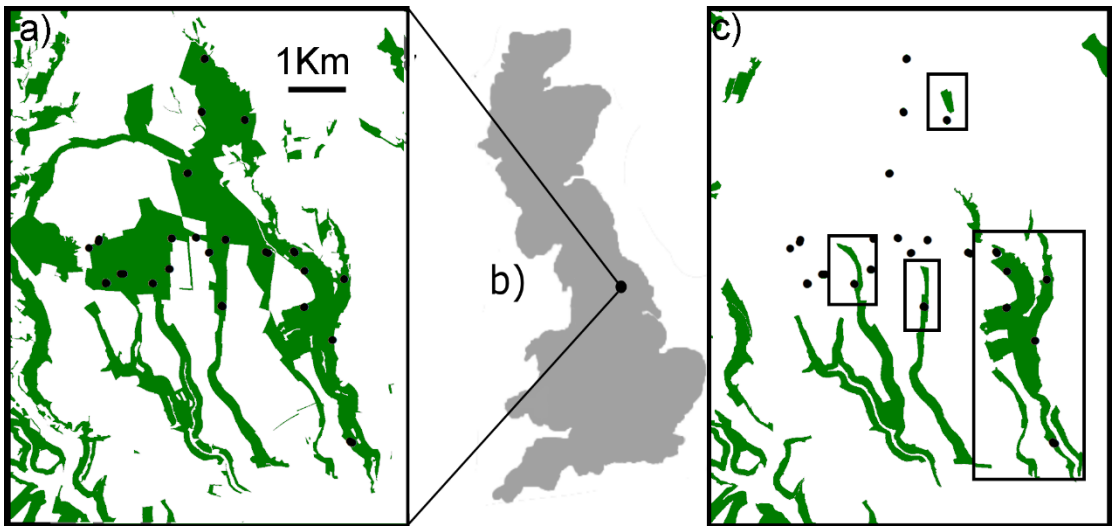

Figure 4.1. The study area, a) the sampled population, polygons show forest cover, black dots show sample locations, b) the position of the population within Britain, c) the same study area with only areas continuously forested since 1600 displayed. The boxes represent potential historic population fragments that have recently expanded. Sampling locations (black dots), shown for reference

DNA was extracted using GeneJET Genomic DNA Purification kits (Thermo Scientific). The sampled workers were each assessed for variation at the following 12 microsatellite loci: Fe7, Fe11, Fe13, Fe16, Fe17, Fe19, Fe21, Fe37, Fe38 (developed for *Formica exsecta* Gyllenstrand et al., 2002), and Fl12, Fl20 and Fl21 (developed for *Formica paralugubris* Chapuisat 1996, known as *Formica lugubris* type B at the time), using the conditions specified in those papers. Each forward primer had a 5' – AGGTTTTCCCAGTCACGACGTT – 3' M13 sequence attached for detection purposes. DNA was amplified using the following reaction mixture: 1µl DNA, 1X PCR buffer (Bioron), 5µM of each primer (Integrated DNA Technologies), 0.2mM of each dNTP (VWR International), 0.25µM M13 oligo with either 700nm or 800nm fluorescent dye attached (Li-Cor Biosciences), and 0.25U Taq DNA polymerase (Bioron). The PCR products were run on a Li-Cor 4300 (Li-Cor Biosciences, Lincoln, NE, USA) and allele sizes were scored by eye using a set of size standards for 700nm and 800nm wavelengths. Loci were tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium within samples in FSTAT 2.93 (Goudet 1995).

Observed heterozygosity and expected heterozygosity were determined using the hierfstat package of R (Goudet 2005; R Core Team 2015). We assessed the relationship between expected heterozygosity and distance to forest cover in 1854 using a linear mixed effects model (LME), with the triplet each nest came from as a random effect, in the lme4 package of R (Bates *et al.* 2014 p. 4). Both forest cover and ant populations have expanded in the last century (Procter *et al.* 2015), therefore the distance to where forest cover was in 1854 is a representation of the minimum dispersal that has been accomplished to form that nest. We assume that those

nests in areas that have been historically forested are in areas from which the population expanded (Fig. 4.1 c). The difference in expected heterozygosity between our population and a Finnish population of *F. lugubris* in undisturbed and continuous forest habitat (Gyllenstrand & Seppä 2003) was assessed using a paired t-test in R.

Comparing the observed and expected heterozygosity of specific nests should give an indication of whether there has been either a genetic bottleneck within the study population or whether there is evidence of recent connection of historically separate and diverged populations. Where observed heterozygosity is lower than expected heterozygosity then nests are less diverse than expected, supporting the idea that the nest in question has undergone inbreeding. Where observed heterozygosity is greater than expected heterozygosity then the nest is more diverse than expected, suggesting there has been connection of previously separate populations. We analysed the difference between observed and expected heterozygosity using a LME, with observed and expected heterozygosity measured per nest and a categorical variable with two levels denoting whether a value was expected or observed heterozygosity. The sample triplet (1-21) each nest came from was included as a random effect, to control for potential pseudo-replication. We repeated this model approach, also adding in a covariate denoting whether nests were in recently planted or historic forest, to test whether the age of the forest affected the difference between observed and expected heterozygosity.

Spatial principal component analysis (SPCA) combines not only the allele frequency data from the population, but also the spatial relationship between sample points to assess spatial biases present in genetic data (Jombart *et al.* 2008). The SPCA was performed in the adegenet package of R (Jombart 2008), utilising all nests and all microsatellite loci. We used the neighbourhood by distance method of defining spatial connections, using 50m for local connections, because that creates links only within sample triplets and then 2000m as the population connection, because that allowed a full connection network throughout the population. Only those principal components that showed strong positive eigenvalues were assessed further, because positive eigenvalues represent high level spatial structure, whereas negative eigenvalues represent local structure (Jombart *et al.* 2008). The selected principal components were interpolated using the interp function of the Akima package of R, and then plotted as a contour map across the population, to show the spatial structure present.

We assessed three sections of mitochondrial DNA using: COI-RLR (forward primer TTGATTTTTTGGTCATCCAGAAGT, reverse primer TAGGTGAATTTGAATTTTGTAATG, 980 bp), COI-IIa (forward primer CGACGTTACTCCGAATACCC, reverse primer TGGCCTTGAAGAAGAAATCG, 500bp) and COI-IIb (forward primer CAAAATTCAAATTCNCCNTATGA, reverse primer CCNGGNGTTGAGTCTATTTT, 500bp) from Holzer *et al.* (2009). Sequences were amplified

using PCR with the conditions specified in Holzer *et al.* (2009) and the following reaction mixture: 1µl DNA, 1X PCR buffer (Bioron, Germany), 5µM of each primer (Integrated DNA Technologies), 0.2mM of each dNTP (VWR International), 0.25µM M13 oligo with either 700nm or 800nm fluorescent dye attached (Li-Cor Biosciences), and 0.25U *Taq* DNA polymerase (Bioron). Following successful amplification of mtDNA fragments, we sequenced each section using Sanger sequencing. The mitochondrial sequence data was checked in Sequencher, and then aligned and analysed in Mega 6.06 (Tamura *et al.* 2013).

4.4 Results

Genetic health of expanded populations

No loci showed significant deviations from Hardy Weinberg equilibrium (randomisation tests, all loci P>0.05 fater adjustment for multiple tests, FSTAT 2.9.) or significant linkage disequilibrium (permutation tests, all combinations P>0.05 after adjusting for multiple testing, FSTAT 2.9.3) within samples, so all loci were used for further analyses. The 12 microsatellite loci displayed a range of variability: three loci displayed low variability (2-3 alleles, expected heterozygosity 0.16-0.51), with the remaining nine loci displaying higher variability (4-19 alleles, expected heterozygosity 0.67-0.89).

Expected heterozygosity was significantly but weakly correlated with distance from historic forest cover in 1854 (LME, df=3,1, X=17.34, P<0.001 Fig. 4.2a). Nests in old forest showed significantly higher levels of allelic richness than nests in new forest (LME, df=3,1, χ=8.01, P=0.004). Again, the difference between nests in new and old forest was small (mean ± st. dev. allelic richness of old forest = 46.2±3.2, new forest = 42.9±3.4). There was no difference between the expected heterozygosity pooled across loci from a matching subset of loci from this study and a Finnish study in a more continuous and less disturbed forest (GLM, df=10,1, F=0.002, P=0.97, Fig. 4.2b).

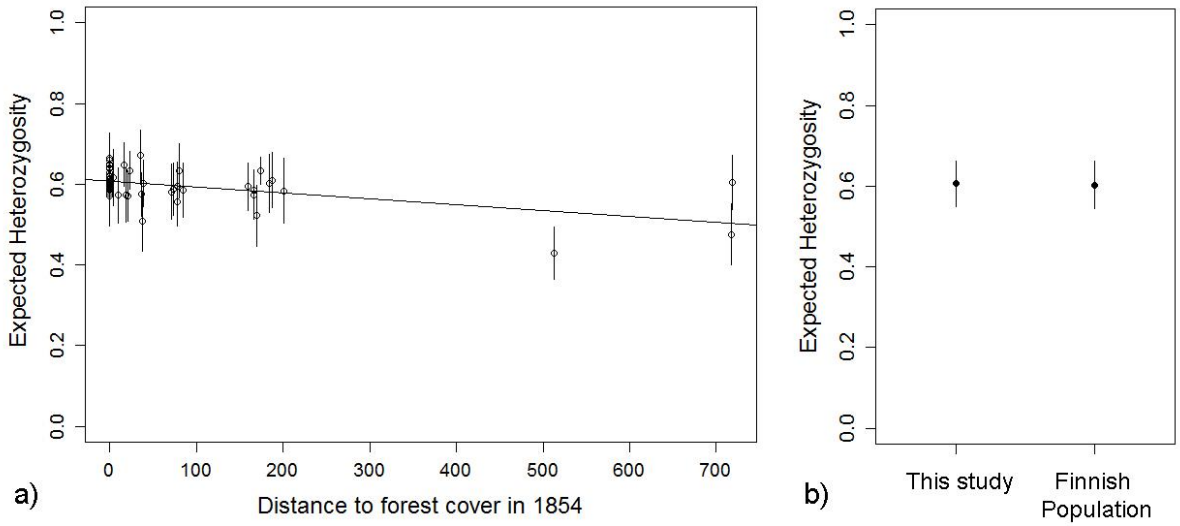

Figure 4.2. a) Mean expected heterozygosity across loci per nest and its modelled relationship with distance from forest cover in 1854, error bars show standard errors of the mean. **b)** Expected heterozygosity of a Finnish *F. lugubris* population in less disturbed and more continuous forest (Gyllenstrand & Seppä 2003) and expected heterozygosity of the same microsatellite loci from this study.

We found that observed heterozygosity was significantly lower than expected heterozygosity (LME, df=3,1, X=9.32, P=0.002, Fig. 4.3), suggesting there has been inbreeding within the population and giving some evidence to a genetic bottleneck. However, the difference between observed and expected heterozygosity was small, with a modelled difference in means of only 0.036 i.e. there is a mean difference of 0.036 between observed and expected heterozygosity (Fig. 4.3). Whether nests were in historic forest or recently planted forest had no effect on the relationship between expected and observed heterozygosity (LME, df=5,1, χ=0.088, P=0.77).

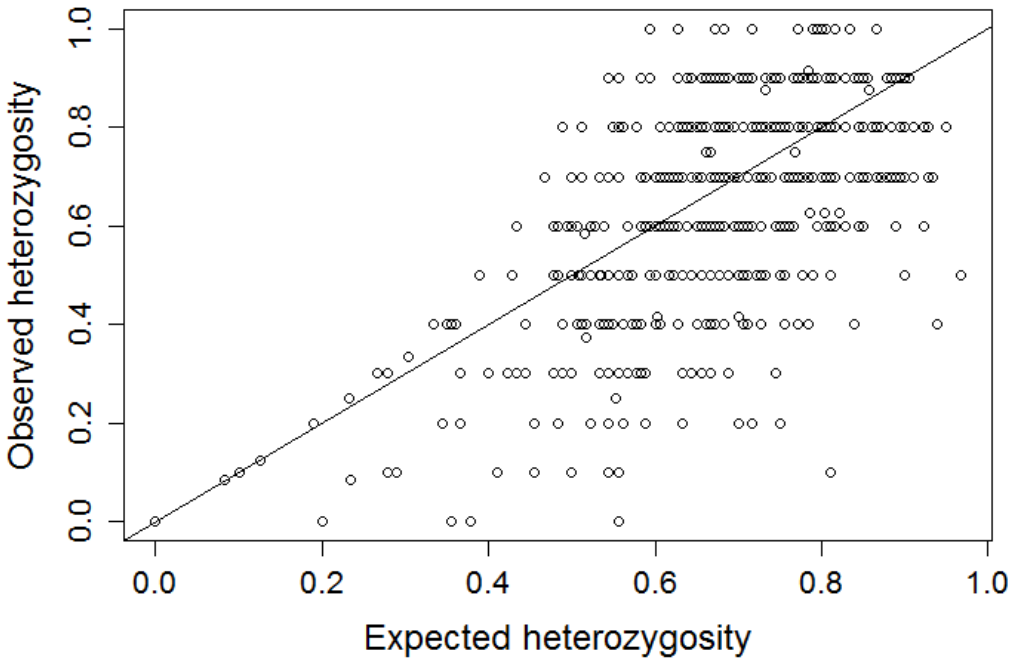

Figure 4.3. Observed against expected heterozygosity per locus and per nest i.e. there are 12 points per nest. The presented line is a perfect correlation between expected and observed heterozygosity and is for reference only. The majority of points above this line would suggest an heterozygote excess, and therefore re-connection of separate genepools, the majority below the line suggests some loss of diversity due to inbreeding.

Evidence for connection of separate populations

In order to assess whether there is evidence of previously isolated populations in our data, we conducted a spatial principal component analysis to look for spatial patterns in the data. The first two principal components showed strongly positive eigenvalues, suggesting spatial structure (Fig. 4.4a). PC1 suggests that historically separate valleys east-west support populations that are genetically distinct (Fig. 4.4 b). PC2 suggests that there is also a genetic distinction between nests at the north of the population and all nests to the south (Fig. 4.4d). The genetic groupings separated on both PC1 (Fig. 4.4b) and PC2 (Fig. 4.4d), correlate with different historic forest patches (Fig. 4.1c, 4.4c)

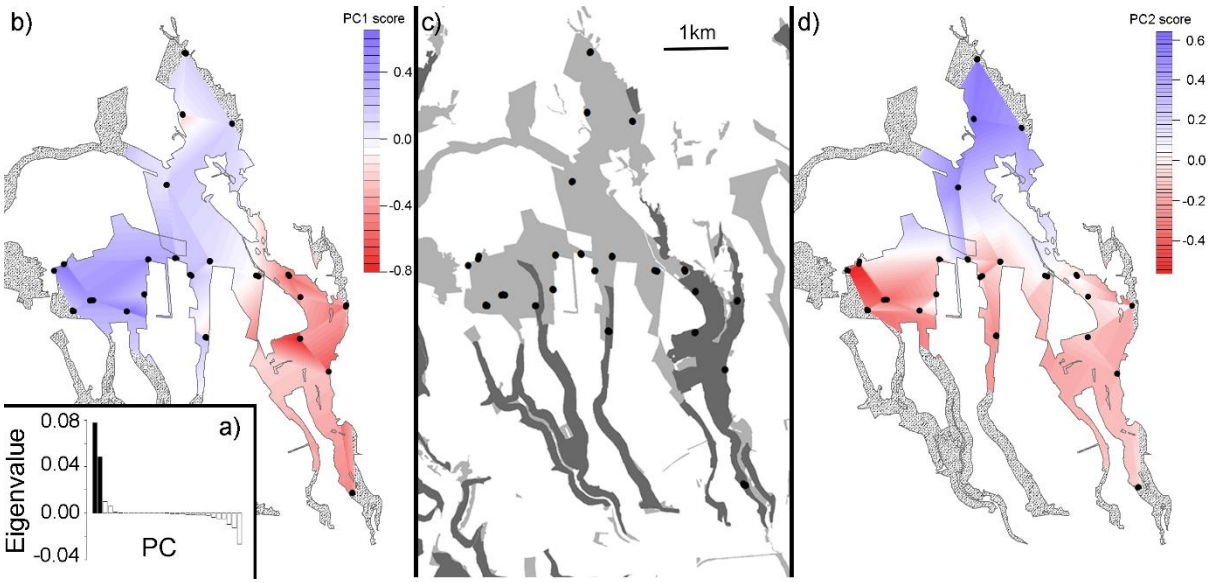

Figure 4.4 a) The Eigenvalues of each principal component (PC) of the SPCA, the two most highly postive values, suggesting spatial structure, are highlighted in black. Eigenvalues are scaled by the spatial scale of their effects, and so should not sum to 1. **b)** PC1 scores interpolated between sample points. The hashed areas we do not predict scores for because it is outside the sampled area, **c)** Modern forest cover (grey polygons) and sample points (black points), with those areas which have been continuously forested since 1600 highlighted in darker grey, **d)** PC2 scores interpolated between sample points. The hashed areas we do not predict scores for because it is outside the sampled area

We investigated the frequencies of alleles rare within the population in order to see whether they supported the patterns found in the SPCA analysis. There were 33 alleles that were represented by less than 5% of alleles in the population. Of these 33, nine supported the spatial structure presented by the SPCA: Fe16, 187bp, Fl20, 159bp, Fl20, 185bp, Fe13, 222bp supported the PC1 west grouping (Fig. 4.5b), Fe16, 199bp, FL20, 169bp, Fe21, 116bp supported the east grouping on PC1 (Fig. 4.5c) and Fe11, 161bp, Fe16, 194bp supported the north grouping on PC2 (Fig. 4.5d). The distributions of the remaining rare alleles were either too rare to draw conclusions from or showed no clear spatial structure, but all are presented in the Appendices for reference (Appendix 3).

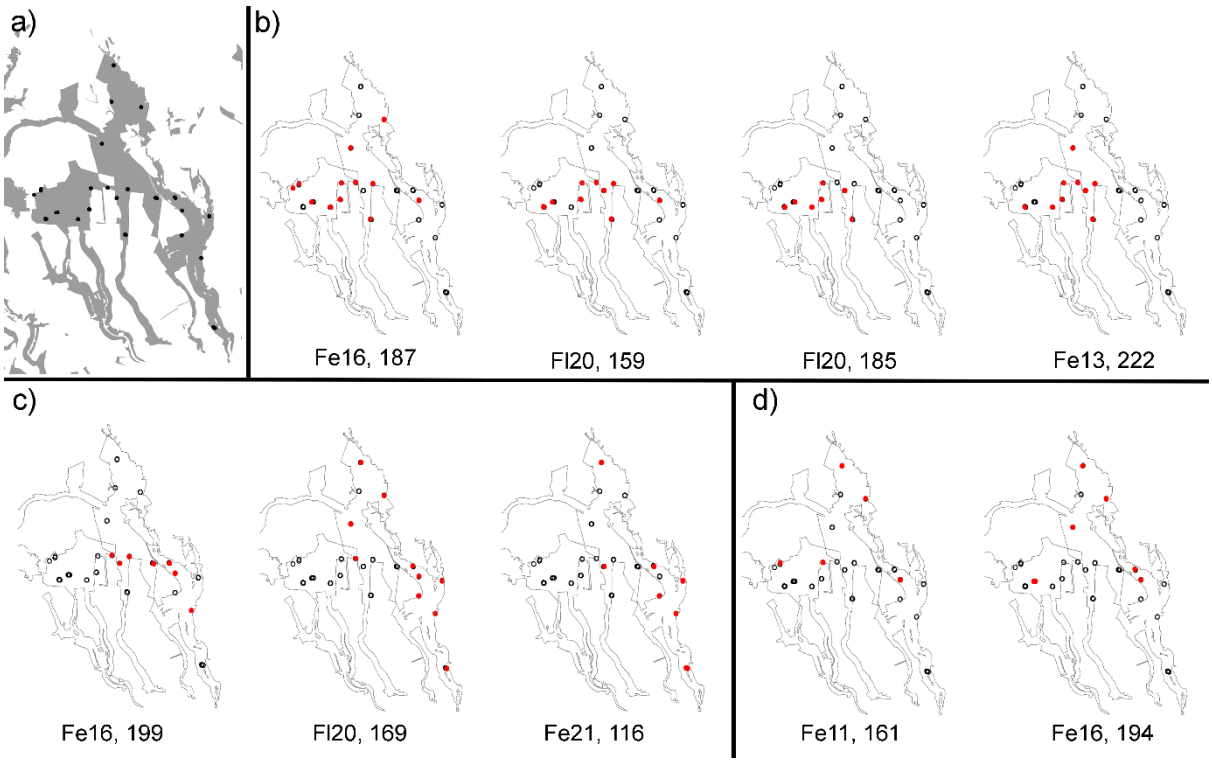

Figure 4.5. The distribution of 9 rare alleles that support the SPCA spatial structure, red points show the presence of the named rare allele, hollow points show absence. **a)** Sample locations (black points) and forest cover (polygons), for reference. **b)** Four rare alleles supporting the west grouping on PC1, **c)** three rare alleles supporting the east grouping on PC1, **d)** two rare alleles supporting the north grouping on PC2. The remainder of rare allele distributions can be viewed in the appendices for reference (Appendix 3).

All three mitochondrial sequences suffered from incomplete sequencing, reducing the complete sequence length across all samples to 471 bp for COI-RLR, 204bp for COI-IIa and 259bp for COI-IIb. The total combined sequence length was therefore 934bp. We found only two mitochondrial haplotypes throughout the population (Fig 4.6.). However, the two haplotypes showed strong divergence, differing at 10 SNPs over the 934bp length. We also sequenced samples of the sister species *Formica aquilonia* from Scotland for comparison, and found the *F. aquilonia* sample to be less divergent from haplotype 1 (blue, Fig. 4.6), than haplotype 2 was (orange, Fig. 4.6).

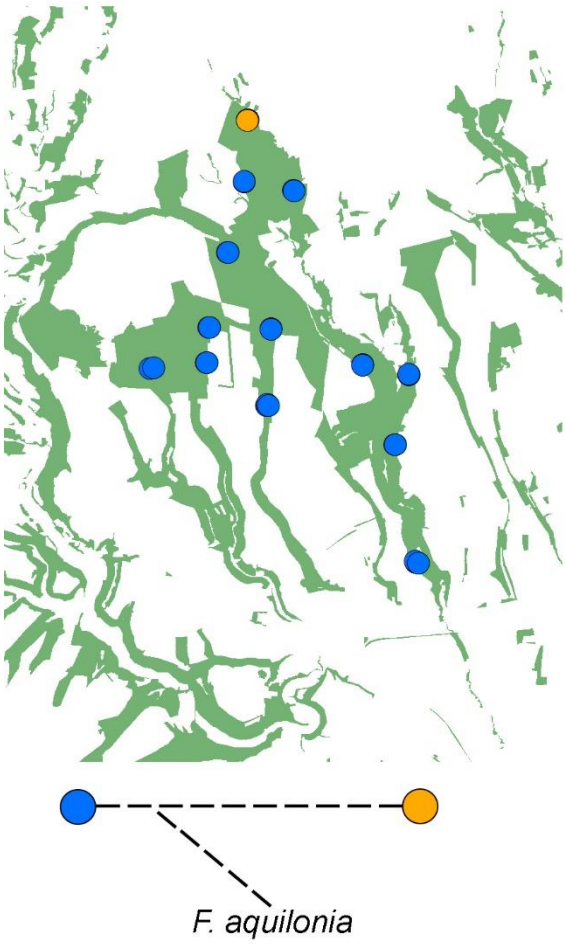

Figure 4.6. Mitochondrial DNA variation in the sequenced ants. Each circle represents three neighbouring nests which have had a single ant each sequenced at three mitochondrial sections, the colours of the nests represent their mitochondrial haplotype, the relationship between which is displayed below. Each dash between haplotypes represents a single base difference in sequence, therefore the are 10 bases between blue and organge haplotypes of *F. lugubris*, but only 7 base differences from the blue haplotype to *F. aquilonia*..

4.5 Discussion

Our results clearly demonstrate that this historically fragmented population is currently genetically diverse. There is no reason to expect that populations should not continue to thrive. Our data also suggests that increases in forest cover may not simply have allowed the expansion of a single historic population, but actually connected previously isolated population fragments (Fig. 4.4). Our results demonstrate a positive effect on conservation caused by anthropogenic land use change: the spread of non-native conifer plantations for commercial forestry have defragmented this forest landscape.

The population that is the focus of this study is one of six populations across the North York Moors National Park, which have shown large population expansions in recent times (Procter *et*

al. 2015). However, each population is centred around small sections of ancient forest from which historic record of wood ants exist (Yarrow 1955). Each ancient fragment was separated by moorland or farmland, which have subsequently been planted with commercial forests, therefore there cannot have historically been a wood ant population on this scale. These sections of ancient forest must be where populations persisted, before expansion into more recently created forest. It was quite plausible that we would see low levels of genetic diversity in the population, reflecting historically small populations and the negative effects on inbreeding and genetic drift. However, this is not what we see; the current population is just as diverse as Finnish populations in far less disturbed forest habitat. There is weak evidence of an historic bottleneck, with observed heterozygosity slightly lower than expected heterozygosity. However the effect of the apparent bottleneck is very small, and so does not appear to have had a detrimental effect upon populations. British wood ants were thought to be remnant populations heading towards extinction. This is because British forests are very fragmented, and have been even more so in the past (Mason 2007; Forestry Commission 2013a), British wood ants are sporadically distributed, and some populations have severely declined (Robinson 2001), and the Irish *F. lugubris* do appear to be both demographically and genetically vulnerable to extinction (Breen 1977; Mäki-Petäys & Breen 2007). Our results add to a growing body of evidence that, in contrast to expectations, some British wood ant populations of the *F. rufa* group are healthy and should be expected to not just survive, but thrive (Gyllenstrand & Seppä 2003; Vanhala *et al.* 2014). Healthy populations of wood ants is good news not just for the ants, but also for British forests, because of the positive role wood ants play in the forest ecosystem (Domisch *et al.* 2009; Härkönen & Sorvari 2014; Frouz *et al.* 2016).

Our results are not so straight forward when it comes to the connection of previously isolated populations. If isolated populations had been reconnected recently we would expect to see a heterozygote excess in the nests between populations, as a result of population mixing. We see no such heterozygote excess (Fig. 4.3). However, the SPCA principal component (PC) 1 suggests genetic divisions east to west in the population, which correlate with separate historic forest fragments (Fig. 4.1c) and rare allele frequencies (Fig. 4.5). Furthermore, SPCA PC2 suggests a genetic division between the extreme north of the population and everywhere south, which correlates with separate historic fragments and with mtDNA haplotype distributions (Fig. 4.6). Increases in conifer plantations in Britain have been shown to increase connectivity of populations of the red squirrel, *Sciurus vulgaris* (Hale *et al.* 2001) and allow interbreeding of *Formica aquilonia* (Vanhala *et al.* 2014). Therefore, our evidence further suggests that non-native conifer plantations, traditionally viewed as negative for biodiversity, can have positive effects on fragmented forest ecosystems, and allow defragmentation of the forest landscape.

A rare mitochondrial haplotype within the population could be explained by a rare, long distance dispersal event. However, *F. lugubris* in the UK is very poor disperser, with new nests

formed by budding, whereby one or many queens leave the natal nest with a subset of workers from that nest and form a new nest nearby (Hughes 2006). The nearest populations neighbouring the study population are 6km to the west and 7km to the east, and the majority of the land in between is not forest, therefore it is very unlikely that a queen could disperse to this population. We therefore think that is unlikely that haplotype 2 is the result of long distance dispersal.

The divergence we show in the two mitochondrial haplotypes is interesting in itself. Across a total of 934 base pairs, the two haplotypes are divergent at 10 bases (Fig. 4.6), and *F. aquilonia*, which had been included as an outgroup, was actually less divergent from haplotype 1 than haplotype 2 was. The taxonomy of the *F. rufa* group of wood ants is not simple, with species very similar in terms of morphology (Yarrow 1955; Collingwood 1979; Seifert 1996; Stockan *et al.* 2016), but *F. lugubris* and *F. aquilonia* were clearly separate in a phylogeny based on mitochondrial sequences (Goropashnaya *et al.* 2012). Hybridisation is common in the *F. rufa* group, with stable hybrid nests and populations well known in several locations (Czechowski & Radchenko 2006; Seifert *et al.* 2010), therefore it is possible that we have stumbled on evidence of hybridisation within this *F. lugubris* population. If hybridisation is at play here, it is likely to have happened some time ago, because all individuals within this population appear morphologically to be *F. lugubris*. Furthemore, there is only a single nest of another species (*F. rufa*) within the North York Moors (D. Procter unpublished data), which is several kilometres away from the nearest *F. lugubris* nest, and so unlikely to have had the opportunity to hybridise with *F. lugubris*. Further investigation of this pattern could be illuminating, for instance this could be evidence of an historical hybridisation event, or a cryptic species. Two such cryptic species have recently been separated from *F. lugubris* in Switzerland (Seifert 1996; Bernasconi *et al.* 2011), but no such detailed assessment has taken place in the UK. However, it is beyond the scope of this paper to assess evidence for either possibility.

Planted forests are thought to have negative effects in many areas of the world (Kanowski *et al.* 2005; Fitzherbert *et al.* 2008), where they have been replacing primary forest. The situation in Britain is quite different, with plantations rarely replacing ancient forest fragments. Instead, planting of non-native conifer plantations primary took place on upland areas (Ratcliffe 1986). Furthermore there are no pristine forest ecosystems in Britain, all forest is to some degree affected by human activity or actively managed (Peterken 1993), therefore there is not such a valuable habitat to compare plantation forest with. As a result the biodiversity supported by plantation forest makes a significant contribution to the overall biodiversity of forest ecosystems in Britain (Humphrey *et al.* 2003). The management of commercial forests in Britain is also moving away from dominance of commercial objectives, to involve a range of priorities including the management of biodiversity, recreational space, and an increase in the proportion of native species planted (Quine *et al.* 2013). In the future we therefore expect a greater positive

impact of plantation forestry on biodiversity in Britain. We do not think our findings of the beneficial effects of plantation forests are relevant to high biodiversity primary forest ecosystems in some parts of the world, however we support the idea that man-made plantations for commercial forestry can support biodiversity in countries where there has been significant historic degradation of forest ecosystems.

We have shown that this population of beneficial forest species are not only demographically, but also genetically healthy. Furthermore we have found patterns that suggest that the increase in non-native conifers that has occurred across this landscape over the last century has defragmented the landscape, and allowed the connection of previously isolated populations.

Chapter 5: Greater mitochondrial variation within *Formica lugubris* across a landscape than between species within the *F. rufa* group

5.1 Abstract

Species are fundamental units of biological organisation, but the boundaries between them are often unclear. Extreme variation within a single species can sometimes be explained by hybridisation, which can play an important role in speciation. In haplodiploid organisms, genes acquired via hybridisation are more common in mitochondrial DNA. Genetic diversity within populations is important for population persistence. In ants mitochondrial variation is often at a smaller spatial scale than nuclear DNA. The *Formica rufa* group of mound building red wood ants are keystone species in forest ecosystems, and therefore of high conservation value. Moreover, the *F. rufa* group are taxonomically close, and often hybridise in the wild.

We assess mitochondrial diversity and divergence, across a series of previously mapped populations of the wood ant *F. lugubris* in the North York Moors National Park, UK, and ask i) is there evidence of hybridisation within these populations? ii) Are the populations genetically diverse?

We find that there is stronger divergence within *F. lugubris* populations across the landscape, than there is between *F. lugubris* and other species within the *F. rufa* group. We find multiple haplotypes within populations; therefore there is high genetic diversity within the studied populations. Our results are further evidence that this landscape contains genetically diverse populations, of high conservation value. The divergence shown could be explained by either cryptic species or ancient hybridisation. We suggest ancient hybridisation is more likely. A further taxonomic revision of the *F. rufa* group may be required with more detailed data collection.

5.2 Introduction

One of the most well-known fundamental units of biological organisation is the species. However, drawing boundaries between species is much less simple than it might seem, and there is still disagreement in precisely how this should be done (De Queiroz 2007). Evolution does not always fit organisms into easily identifiable units; speciation can take place over long time periods, and examples are often found that challenge precisely where a species boundary should be drawn. The most famous example of this is ring species, where there is interbreeding of individuals throughout the range, but individuals from either edge of the range are reproductively isolated (e.g. *Ensatina eschscholtzii*, Moritz *et al.* 1992; *Phylloscopus*

trochiloides, Irwin 2005). However, species identification is important for ecological studies, to ensure the results of the study are assessed in the proper context.

Overlaps in the ranges of some species abound with hybrids, often sharing morphological traits to a degree that makes them impossible to tell apart (Mavárez *et al.* 2006; The Heliconius Consortium 2012). If there are not serious deleterious effects of hybridisation, then hybrid populations can be long lived in themselves (e.g. Czechowski & Radchenko 2006, Kulmuni *et al.* 2010). Hybridisation can either speed up or slow down the process of speciation, and can allow the acquisition of beneficial alleles through adaptive introgression, the acquisition of genes from another species via hybridisation (Song *et al.* 2011; Abbott *et al.* 2013). Hybridisation can therefore play an important role in the process of speciation.

Haplodiploid reproduction, in which one sex is diploid and the other haploid, is found in approximately 15% of animals (de la Filia, Bain & Ross 2015), and has interesting implications for hybridisation and speciation. Firstly the fact that one sex is haploid stops any alleles being masked from selection (Kulmuni & Pamilo 2014). The visibility of the effects of all alleles in one sex makes deleterious alleles more obvious and strongly selected against. Secondly, in haplodiploids, introgression of hybrid genes is expected to be much more likely in mitochondrial DNA than nuclear DNA, because only female offspring are hybrids in the first generation (Patten, Carioscia & Linnen 2015). Hybrid males can occur only through backcrossing of a hybrid female to a non-hybrid male. This reduced rate of introgression of nuclear DNA in haplodiploid species may be a reason for such great richness in haplodiploid species; without introgression of nuclear genes, speciation may occur more easily (Lohse & Ross 2015). Hybridisation is therefore also much more likely to be detected in haplodiploids using mitochondrial markers as opposed to nuclear markers.

Ants are amongst the most widespread of haplodiploid organisms, and are the dominant invertebrate in most terrestrial ecosystems (Hölldobler & Wilson 1990). The Formicidae contain vast diversity, with over 10,000 species described. One of the most successful groups of ants in temperate regions are the *F. rufa* group of mound building red wood ants, which are the dominant invertebrate in forest ecosystems across much of Eurasia. The *F. rufa* group have long been a challenge for taxonomy, because they are incredibly morphologically similar (Yarrow 1955; Collingwood 1979; Seifert 1996; Skinner & Allen 1996). Cryptic species have been discovered on more than one occasion (Seifert 1996; Bernasconi *et al.* 2011) and hybrids between species are frequently found (Czechowski & Radchenko 2006; Seifert *et al.* 2010). The close taxonomic relationships within the *F. rufa* group (Goropashnaya *et al.* 2012), means that they are excellent study species when evaluating diversity within and between species.

Maintenance of genetic diversity within a species is of conservation value in itself. Reduced genetic diversity can cause reductions in fitness (Westemeier *et al.* 1998), and is often a sign of

a population having undergone inbreeding and low population sizes at some point (Templeton *et al.* 1990). Low genetic diversity therefore has negative effects on the species that exhibit it, and enhances the chance of those species going extinct. Conservation of healthy populations requires the assessment of both ecological and genetic issues (Höglund 2009). Ants often show sex biased dispersal, with nuclear genetic variation at a larger spatial scale than mitochondrial variation (e.g. Doums*et al.* 2002; Clémencet *et al.* 2005; Soare *et al.* 2014, Sundstrom *et al.* 2003). Therefore, there is the potential for mitochondrial DNA to reveal groupings of specific haplotypes that nuclear DNA does not.

The *F. rufa* group are keystone species in forest ecosystems, and have strong effects on invertebrate community structure as well as providing a food source for predators (Hughes & Broome 2007; Wardle *et al.* 2011). The presence of *F. rufa* nests has positive effects on both soil structure and nutrient cycling (Frouz & Jilková 2008; Domisch *et al.* 2009; Frouz *et al.* 2016). The nests themselves also support high levels of biodiversity (Härkönen & Sorvari 2014; Robinson *et al.* 2016). The *F. rufa* group are therefore an excellent group for conservation to concentrate on, due to the positive effects on forest ecosystems when they are present.

A recent study found very strong mitochondrial divergence within a single population of *F. lugubris* (Chapter 4). The population in question is one of six, which are distributed throughout the landscape, therefore here we assess mitochondrial divergence across the landscape, and compare the sequence with further examples from within the UK, and from other species, to put the strong divergence in context. Using the same data we ask: does *F. lugubris* exhibit genetic variability in mitochondrial haplotypes, and if so, should that inform conservation priorities?

5.3 Methods

Sampling

The primary site for this study was the North York Moors National Park, UK. Across this landscape there are six geographically separated populations of known extent, which have been historically isolated from one another (Procter *et al.* 2015). We took samples from across this landscape, spread in such a way to cover the whole geographic extent of all populations (Fig. 5.1). We sampled 105 nests spread unevenly between populations, due to the differences in extent of each population. The populations are labelled HH, CHB, LN, CrW, CrE and Bx from west to east (Fig. 5.1). We sampled five ants from HH, 45 from CHB, five from LN, 15 from CrW, 15 from CrE and 20 from Bx (Fig. 5.1). The 45 samples from CHB are clustered in groups of three, the locations of which were determined by another study (Chapter 6). In order to gain broader geographical perspective on any variation we find, we took five ants from a previously studied population of *F. lugubris* in the Longshaw Estate of the Peak District

National Park, UK (Ellis *et al.* 2014), 10 *F. lugubris* and two *F. aquilonia* from a previous study in Scotland (Vanhala *et al.* 2014) and a single sample of Scottish *F. exsecta* as an outgroup.

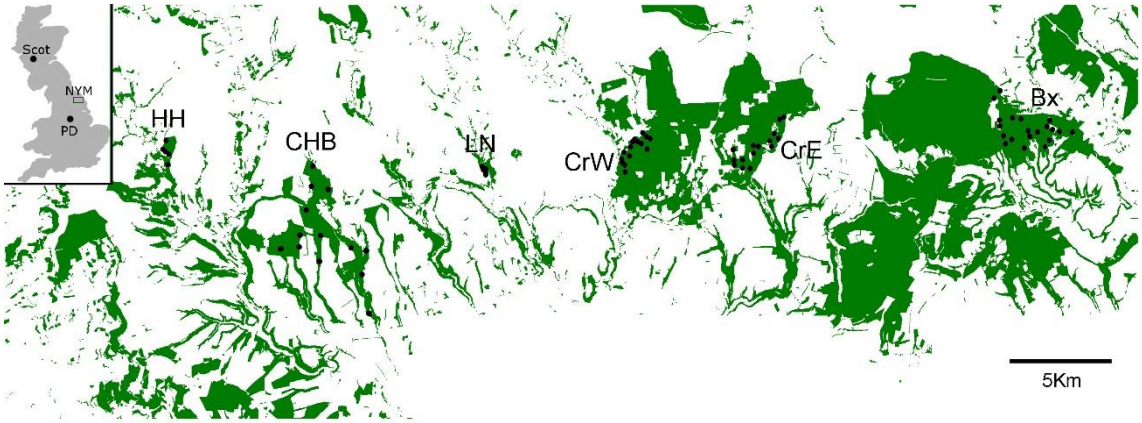

Figure 5.1. The samples collected from the North York Moors National Park. Green polygons are forest cover in 2015, black points are sample locations. The inset shows the location of the study site within Britain (NYM), and the areas where the peak district (PD) and Scottish (Scot) samples came from

DNA extraction and sequencing

DNA was extracted using GeneJET Genomic DNA Purification kits following manufacturer's instructions (Thermo Scientific). We sequenced three sections of mitochondrial cytochrome oxidase using primers COI-RLR (forward primer TTGATTTTTTGGTCATCCAGAAGT, reverse primer TAGGTGAATTTGAATTTTGTAATG, 980 bp), COI-IIa (forward primer CGACGTTACTCCGAATACCC, reverse primer TGGCCTTGAAGAAGAAATCG, 500bp) and COI-IIb (forward primer CAAAATTCAAATTCNCCNTATGA, reverse primer CCNGGNGTTGAGTCTATTTT, 500bp), from Holzer et al. (2009)(Holzer *et al.* 2009)(Holzer et al. 2009). Sequences were amplified using PCR with the conditions specified in Holzer *et al.* (2009) and the following reaction mixture: 1µl DNA, 1X PCR buffer (Bioron, Germany), 5µM of each primer (Integrated DNA Technologies), 0.2mM of each dNTP (VWR International), 0.25µM M13 oligo with either 700nm or 800nm fluorescent dye attached (Li-Cor Biosciences), and 0.25U *Taq* DNA polymerase (Bioron). Following successful amplification of mtDNA fragments, we sequenced each section using Sanger sequencing.

Upon delivery the mitochondrial sequence data was checked in Sequencher, and then aligned and analysed using maximum parsimony trees in Mega 6.06 (Tamura *et al.* 2013). We created 1000 trees using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei & Kumar 2000), and present the consensus of the 1000 trees, reporting the percentage of trees that support each branch. Any branches supported by less than 50% of trees are collapsed.

Several other studies have sequenced the first of the three sections we sequenced, COI-RLR. To compare the variation in our samples with a wider context, we took all sequences from Genbank that overlapped with COI-RLR in the genus *Formica*. This included *F. paralugubris* (Accession:EU600788, Holzer *et al.* 2009, 58 nests sampled), *F. pratensis*, *F. truncorum and F. yessensis* (Accessions AB103363, AB103355, AB103357, AB103362, AB103360, Hasegawa and Imai, unpublished, unknown sample sizes), *F. fusca* (Accession: FJ824419, Jansen & Savolainen 2010; LN607805, Babbucci et al. 2014). We analysed this section using maximum parsimony trees as above.

5.4 Results

All three mitochondrial sections suffered from incomplete sequencing; therefore the final sizes of each section for analysis were as follows: COI-RLR 471 bp, COI-IIa 204bp and COI-IIb 259bp. The total combined sequence length analysed was therefore 934bp. In this 934bp we found variation at 11 nucleotides, which separated the *F. lugubris* samples into five haplotypes (Fig. 5.2). The five haplotypes were strongly supported by repeated tree creation, with all branches but one found in every tree of 1000 created.

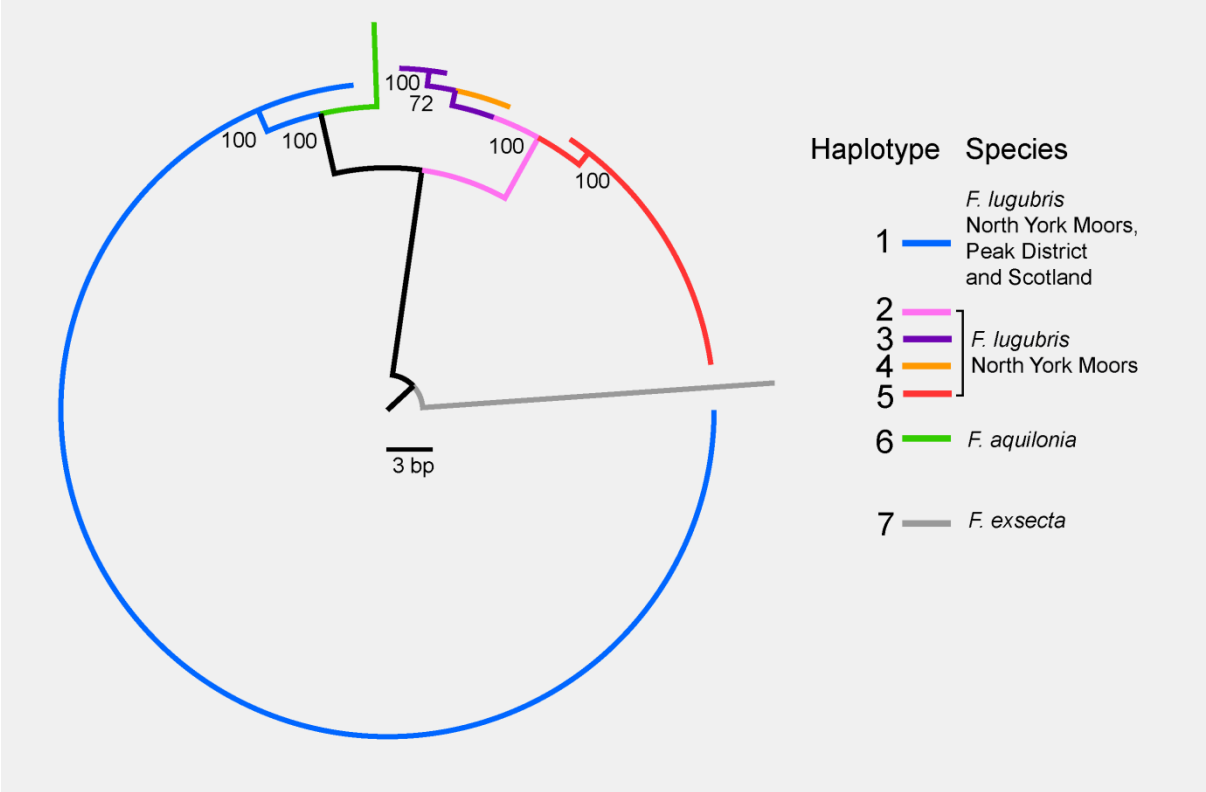

Figure 5.2. Consensus tree of 1000 maximum parsimony trees of relationships between sequenced mtDNA haplotypes. Numbers on each branch represent the percentage of trees that support that branch. The tree is drawn to scale using the average pathway method (Nei & Kumar 2000).

Haplotype 1 (blue in Figs. 5.2 and 5.3) was more common than all others, and found in all populations except HH (Fig. 5.3). There was only one haplotype found in all samples in the Scottish *F. lugubris* samples and the Peak District *F. lugubris*. There was a strong division between *F. lugubris* haplotype 1 (blue in Figs. 5.2 and 5.3) and *F. lugubris* haplotypes 2-5 (Pink, purple, orange and red in Figs. 5.2 and 5.3), with 10 SNPs separating the grouping of 2-5 from haplotype 1. The samples of Scottish *F. aquilonia*, which were expected to be closely related to *F. lugubris*, fell out between the two *F. lugubris* haplotype groupings (Fig. 5.2), grouping more closely with haplotype 1 than haplotypes 2-5. As expected, the outgroup sample of *F. exsecta* was strongly divergent from all other sequences. Haplotype 3 was not found in any location other than the extreme north of the CHB population.

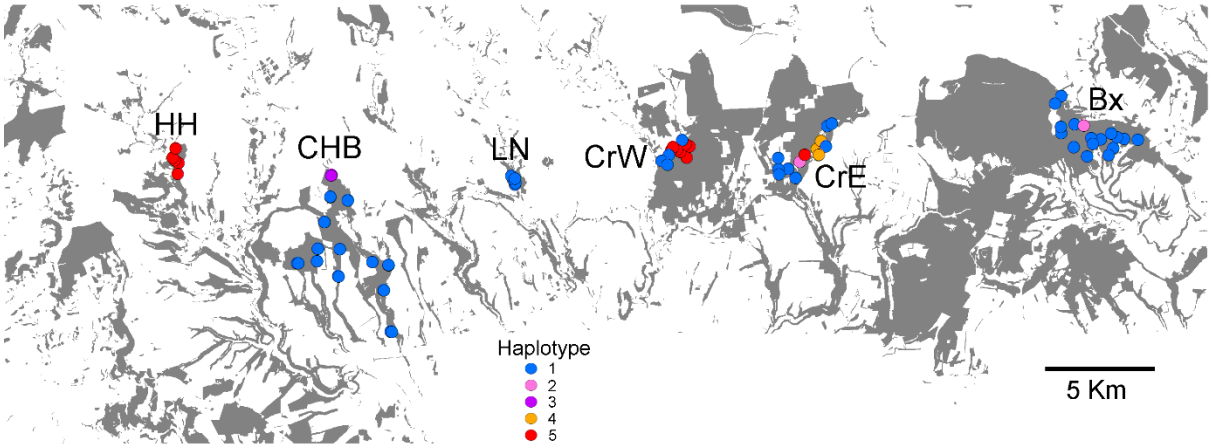

Figure 5.3. The distribution of *F. lugubris* mitochondrial haplotypes across the North York Moors

Comparing the sequence COI-RLR with a wider variety of studies which have sequenced the same section of mtDNA, we find that further species fall out between the two groupings of *F. lugubris*, with *F. aquilonia*, *F. paralugubris*, *F. truncorum*, *F. yessensis* and *F. pratensis* all showing lower divergence from the *F. lugubris* haplotypes than they show from one another. (Fig. 5.4). The expected outgroups of *F. fusca* and *F. exsecta* show strong divergence from all other species.

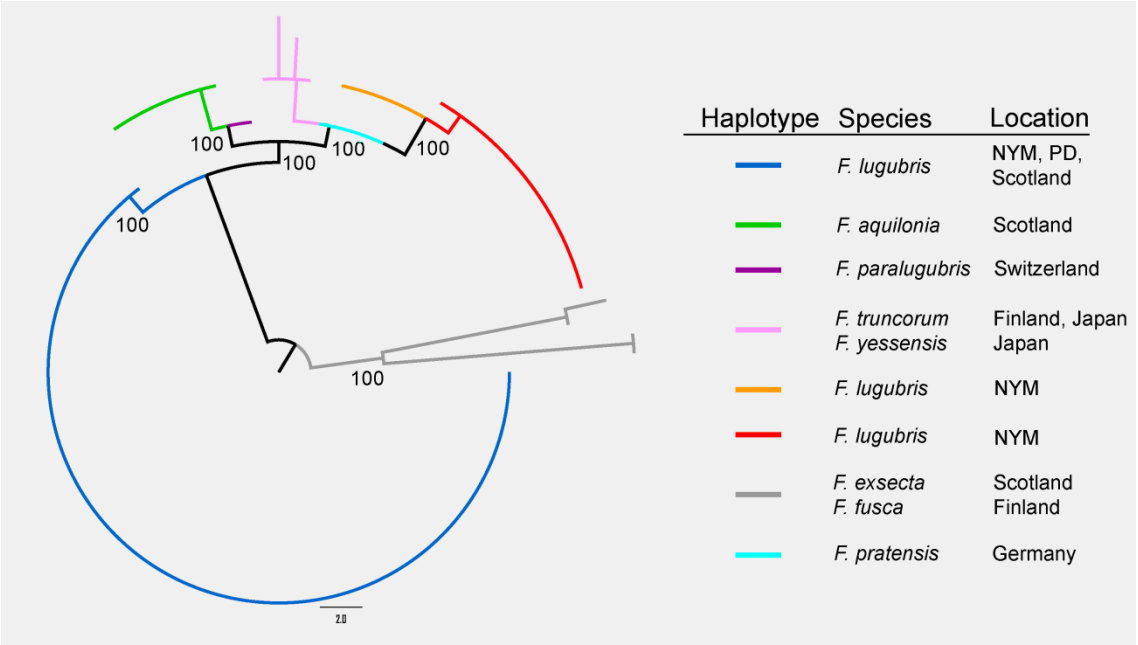

Figure 5.4. A consensus tree from 1000 maximum parsimony trees of the COI-RLR sequence (total 471 bp), including extra species data from Genbank. Numbers at branches represent the percentage of trees which replicate each branch. Abbreviations: NYM – North York Moors, PD – Peak District

5.5 Discussion

Our results clearly show strong divergence within a species across a single landscape in the UK. This divergence is greater than that between the study species and other members of the *F. rufa* group. We therefore suggest that this could either be evidence of cryptic species within this landscape, or evidence of historic hybridisation. Our results also support the idea that, although population sizes are large throughout the landscape (Procter *et al.* 2015), the ants are not genetically homogenous, and there are pockets of rare mitochondrial haplotypes, which may be of higher conservation concern.

Mitochondrial divergence

Formica lugubris has previously shown limited phylogeographic structure across Europe (Goropashnaya *et al.* 2004). In contrast, our findings show strong variation within populations across the North York Moors in an area less than 1% of the area of Europe. We note that a recent phylogeny of the *F. rufa* group, based on mitochondrial DNA, used a total of 35 individuals across Europe (Goropashnaya *et al.* 2012), our data suggests a more complex picture may emerge with greater sampling of each species. Two cryptic species close to *F. lugubris* have been identified in recent times in Switzerland (Seifert 1996; Bernasconi *et al.* 2011). We suggest another reappraisal of the species currently known *F. lugubris* may be in order. Unfortunately, the variable mtDNA section we discovered did not cover the same sequence as

Goropashnaya *et al.* (2012), therefore we cannot directly compare the variation we display across this landscape with that of the full *F. rufa* group.

Whilst it is possible that our results are evidence of two cryptic species living alongside one another in the UK, they could also be the result of an historic hybridisation event. Stable hybrid populations are known between a range of species in the *F. rufa* group (Seifert & Goropashnaya 2004; Czechowski & Radchenko 2006; Seifert *et al.* 2010). Another study assessed nuclear DNA in part of this landscape, and found that nests sharing strongly divergent mitochondrial haplotypes were no more divergent than nests sharing the same haplotype (Chapter 4). Therefore there does not appear to be reproductive isolation between the different mitochondrial haplotypes. Hybridisation may be what has caused such strong patterns of mitochondrial divergence. However, if this is the case, then it probably happened thousands of years ago, before deforestation fragmented the forest across the North York Moors (Atherden 1976). The locations of wood ant nests across the North York Moors are remarkably well known (Yarrow 1955; Procter *et al.* 2015), and currently there is only a single nest of any species that is not *F. lugubris* known from the North York Moors. This single *F. rufa* nest is over two kilometres from the nearest *F. lugubris* nest, and the intervening habitat has been thoroughly searched for further wood ants. The next extant population of wood ants that is not *F. lugubris*, is outside of the borders of the national park and therefore well beyond potential dispersal distance. It is therefore very unlikely that hybridisation can have been recent. When combined with the fact that haplotypes 2-5 are genetically close and broadly geographically spread (Figs. 5.2, 5.3), we suggest that hybridisation resulting in these patterns would have been before the historic fragmentation of the forest across this landscape. Analysis of the COI-RLR sequence suggested the closest species to haplotypes 2-5 is *F. pratensis*, therefore this is a potential hybrid candidate, although there are several other members of the *F. rufa* (particularly *F. rufa* and *F. polyctena*) for which we do not have data that may also be candidates. We did not set out to assess differences between species, rather to assess variation within a single species. Our results are therefore lacking some contextual information that an intended phylogenetic study would of course have included.

An interesting point is that *F. lugubris* varies in social organisation across its range: populations in Ireland, Switzerland and Finland are monogynous (one queen per nest), whereas populations in the UK, and other parts of Switzerland are polygynous (Gyllenstrand & Seppä 2003; Mäki-Petäys & Breen 2007; Bernasconi *et al.* 2010). *Formica paralugubris* was recently taxonomically separated from *F. lugubris* (Seifert 1996) and shows a similar social organisation to the *F. lugubris* populations studied here (Chapter 6), i.e. polygyny and polydomy. Recently genetic distinctions within ant species have been shown to explain differences in social organisation (Ross & Keller 1995; Purcell *et al.* 2014). The hypothesis that an ancient hybridisation that has caused this variation within the landscape, and the potential hybrid is no

longer present within the landscape, would make it likely that there has been introgression of more genes than just the section we have discovered. Hybridisation with a polygynous species, such as *F. polyctena*, could explain the polygynous and polydomous organisation found in *F. lugubris* in the UK. As far as we know there is no evidence of different haplotypes showing different social organisation, haplotypes 1 and 3 are both certainly polygynous and polydomous (Chaper 6). However haplotypes 2, 4 and 5 have not directly had their social organisation assessed, there may be greater variation across the landscape than we realise.

Genetic diversity

We found that there was much more mtDNA variability than we were expecting across the landscape. The high diversity we display is further evidence that the *F. lugubris* populations on the North York Moors, which have historically been restricted to small fragments, contain high genetic diversity within restricted populations (Chapter 4). Due to the very large population sizes of *F. lugubris* across this landscape (Procter *et al.* 2015), conservation of specific nest clusters may not be deemed important. However, conservation of genetic diversity, as well as healthy population sizes, must be taken into consideration to ensure species persistence (Höglund 2009). Our data shows that *F. lugubris* is not genetically homogenous across this landscape, indeed there are several localised haplotypes with very limited distribution (Fig. 5.3). Further investigation may also find further diversity in areas we have not yet sampled. It is therefore not the simple story that all wood ant nests are equal within this landscape, conservation efforts should seek to protect genetic diversity as well as large population sizes.

There is not a consistent pattern of haplotype diversity between the different populations across the landscape: two show a single haplotype (HH and LN, Fig. 5.3), three show two haplotypes (CHB, CrW and Bx, Fig. 5.3) and one population shows particularly high variability, with four haplotypes present within one population (CrE Fig. 5.3). The CrE population is centered on one of the largest areas of ancient woodland across this landscape. It is tempting to claim that the CrE population was probably the largest in the landscape, before the recent expansion of plantation forest. Small population sizes increase the chances of reduced genetic variation and increased inbreeding (Höglund 2009), which we see no evidence of. Therefore the historical population may have been large. Unfortunately this is the only population in this landscape that has not been mapped in detail, due to difficult terrain (Procter *et al.* 2015), therefore we are uncertain of even the current population size, let alone the historic population size. Furthermore, as detailed below, we would need to sample the landscape in more detail to be sure of a relationship between the availability of historic forest and present day genetic diversity.

Limitations

Whilst the patterns we see are interesting, we acknowledge that the level of sampling within this study limits our conclusions. Firstly we have sampled 105 nests out of a known total of over 5000 within the North York Moors (Procter *et al.* 2015). Therefore, we suspect that it is likely that we have under-sampled the real diversity in the landscape, and also the distribution of that diversity across populations. Secondly we have sampled a single ant per nest. *Formica lugubris* is polygynous in the UK, with an estimated 20 reproducing queens per nest (Gyllenstrand & Seppä 2003). It is therefore quite possible that multiple haplotypes could be found within each nest. Thirdly we have not thoroughly sampled the remainder of the UK. A well-studied Peak District population contains over 1000 nests (S. Ellis pers. com.) and is one of several *F. lugubris* populations within the Peak District. We have sampled 10 nests from Scotland, but *F. lugubris* is found throughout much of Scotland, and so probably exhibits a great deal more diversity than we have sampled (Stockan *et al.* 2016). There are also unsampled populations in Wales and the North East of England that may show further diversity. We only find haplotype 1 in both the Scottish and Peak District populations, however, as is clear from above, there could be much more variation within those populations that we have missed with low sampling effort. Despite this suspected under-sampling of diversity, we show strong patterns, but the limitations of the study sampling means that our results are suggestive rather than conclusive.

5.6 Conclusion

This study was an accidental discovery when investigating other patterns, and therefore lacks the depth that a true phylogenetic study would have, however our findings highlight interesting patterns. 1We have shown that *F. lugubris* across the North York Moors shows remarkable variation in mtDNA. Haplotypes found within the same population show stronger divergence from each other than from other species. This could be evidence of cryptic species or hybridisation. From the available evidence we suggest hybridisation is more likely, though further work is necessary for a clearer answer. In either case, our findings suggest further work is justified, in order to assess whether there is a cryptic species, hybridisation event, or some other phenomenon at work across this landscape. Our study is further evidence of high genetic diversity within recently expanded *F. lugubris* populations in the fragmented landscape of the North York Moors.We emphasize that the large populations that are known from this area actually contain genetic variation within them, which is of conservation value. This must be taken into account during decisions affecting population persistence.

Chapter 6: Does cooperation mean kinship between spatially discrete ant nests?

6.1 Abstract

Eusociality is one of the most complex forms of social organisation, characterised by cooperative and reproductive units termed colonies. Altruistic behaviour of workers within colonies is explained by inclusive fitness, with indirect fitness benefits accrued by helping kin. Members of a social insect colony are expected to be more closely related to one another than they are to other conspecifics.

In many social insects, the colony can extend to multiple socially-connected but spatially separate nests (polydomy). Social connections, such as trails between nests, promote cooperation and resource exchange, and we predict that workers from socially-connected nests will have higher inter-nest relatedness than those from socially unconnected, and non-cooperating, nests.

We measure social connections, resource exchange and inter-nest genetic relatedness in the polydomous wood ant *Formica lugubris* to test whether i) socially-connected but spatially separate nests cooperate, and ii) high inter-nest relatedness is the underlying driver of this cooperation.

Our results show that socially-connected nests exhibit movement of workers and resources, therefore are cooperating, whereas unconnected nests are not. However, we find no difference in inter-nest genetic relatedness between socially-connected and unconnected nest pairs, both show high kinship.

Our results suggest that neighbouring clusters of connected nests show a social and cooperative distinction, but no genetic distinction. We hypothesize that the loss of a social connection may be the first step in the formation of separate colonies. Genetic divergence between neighbouring nests may build up only later, as a consequence rather than a cause of colony separation.

6.2 Introduction

Understanding how and why animal societies are organised in the way they are has long been a focus of biological research. Eusocial societies, characterised by cooperative brood care, overlapping generations and division of labour, are amongst the most complex forms of social organisation. Eusociality is found throughout the animal kingdom, for example: in mammals and crustaceans (Jarvis & Bennett 1993; Duffy, Morrison & Rios, R. 2000), but is particularly widespread in the insects (Stern 1998; Inward, Vogler & Eggleton 2007; Smith *et al.* 2009; Johnson *et al.* 2013). In eusocial organisms, the colony is a fundamental unit of social

organisation; this reproductive and selective unit competes with other colonies within a population (Hölldobler & Wilson 1990). Furthermore, the colony is also a cooperative unit; workers cooperate within colonies, collaboratively collecting resources and tending young, in order to produce the next generation.

Within a social insect colony, the workers do not themselves reproduce, and are therefore behaving altruistically by helping the queens reproduce. This altruism can be explained by inclusive fitness theory, with indirect fitness benefits to the workers accrued via the enhanced reproduction of kin (Hamilton 1964; Bourke 2011). Positive relatedness between interacting organisms is required for the evolution of altruism, and as such members of a social insect colony are expected to be more related to one another than they are to other individuals within the population. The positive effects of inclusive fitness can be further enhanced by ecological factors which give higher benefits or lower costs of altruism (Bourke 2011).

The traditional view of an ant colony is a single nest which contains a single queen and highly related workers, however this is increasingly being shown to be too simple (Heinze 2008). Ant colonies can contain multiple reproducing queens at any one time, a trait known as "polygyny" (e.g. Pedersen & Boomsma 1999; Tsutsui & Case 2001; Holzer *et al.* 2006). In addition, the number of nests that comprise an ant colony can differ. Spatially discrete nests can operate functionally as a single colony, a situation termed polydomy (Debout *et al.* 2007). Polydomy is found in widespread ecologically-important species (Ellis & Robinson 2014), and is a feature of some of the world's most damaging invasive species (e.g. *Pheidole megacephala* Fournier *et al.* 2012; *Linepithema humile* Gordon and Heller 2014; *Anoplolepis gracilipes* Hoffmann 2014). The suggested benefits of polydomy to the colony include: risk spreading (van Wilgenburg & Elgar 2007b), efficient resource acquisition and exploitation (Schmolke 2009; Cook *et al.* 2013), escape from the limitations of a single nest site (Cao 2013), or release from the inefficiency of a very large nest (Robinson 2014; Kramer *et al.* 2014). All of these potential benefits of polydomy follow logically from the assumption that the colony is a cooperative unit, and this is reinforced by empirical evidence of cooperation in the form of resource exchange between nests (Buczkowski 2012; Gordon & Heller 2014; Ellis *et al.* 2014; Ellis & Robinson 2016).

Polydomous colonies are defined as consisting of spatially separate nests linked by a social connection (Debout *et al.* 2007). Some ant species connect spatially separate nests with trails along which workers continually move back and forth, forming a clearly visible social connection (McIver 1991; Gordon & Heller 2014; Ellis *et al.* 2014). The strength of social connection between nests can be dramatic, with strong connections between nests involving hundreds of workers moving in either direction every minute (Skinner 1980). Wood ants of the *F. rufa* group, which includes *F. lugubris*, do use aboveground trail networks extensively

(Rosengren 1971) but no examples of subterranean trail networks are known.. Polydomous trail networks are structured to allow efficient transport of resources within the colony (Cook *et al.* 2014). In the wood ant *Formica lugubris*, pairs of nests which exhibit a higher flow of resources moving through them are more likely to grow, reproduce and survive from year to year than those with a lower resource flow (Ellis *et al. in Review*). Polydomous trail networks therefore represent connections between cooperating nests, sharing workers and resources, in line with the expectations of a social insect colony. In populations of *F. lugubris*, colonies connected by trails are often bordered by other nests to which they have no social connection, although the distance between unconnected nests can be similar to that between connected nests (D. Procter pers. obs.). Wood ant trails are long lived (Rosengren 1971). Furthermore, during mapping of trail networks of *F. lugubris* in the UK over multiple years, neighbouring trail networks were never observed to connect (Ellis *et al.* in Review), therefore trail networks do correspond to a consistent connection. *Formica lugubris* exhibits variation in dispersal strategies across its range but in the UK new nests are formed by budding, whereby one or several queens split off from the parent nest with a subset of the workers and form a new nest nearby (Hughes 2006). Budding nest formation could result in neighbouring nests with high genetic relatedness, allowing the formation of polydomous colonies.

We predict that the social connections between nests correlate with genetic distinctions, because members of a social insect colony are expected to be more related to one another than to other members of the population. While strong trails between nests are evidence of a social connection, there may be subtler social connections between nests unconnected by trails. We predict that nests connected by trails exchange workers but, more importantly, nests unconnected by trails do not exchange workers. Existing evidence suggests that polydomous colonies defined along social lines display resource cooperation, therefore we expect that social connections between nests correlate with resource movement between nests. In order to assess these predictions we measure i) worker movement, ii) carbohydrate resource exchange and iii) genetic relatedness between neighbouring nest pairs, which are either connected or unconnected by trails

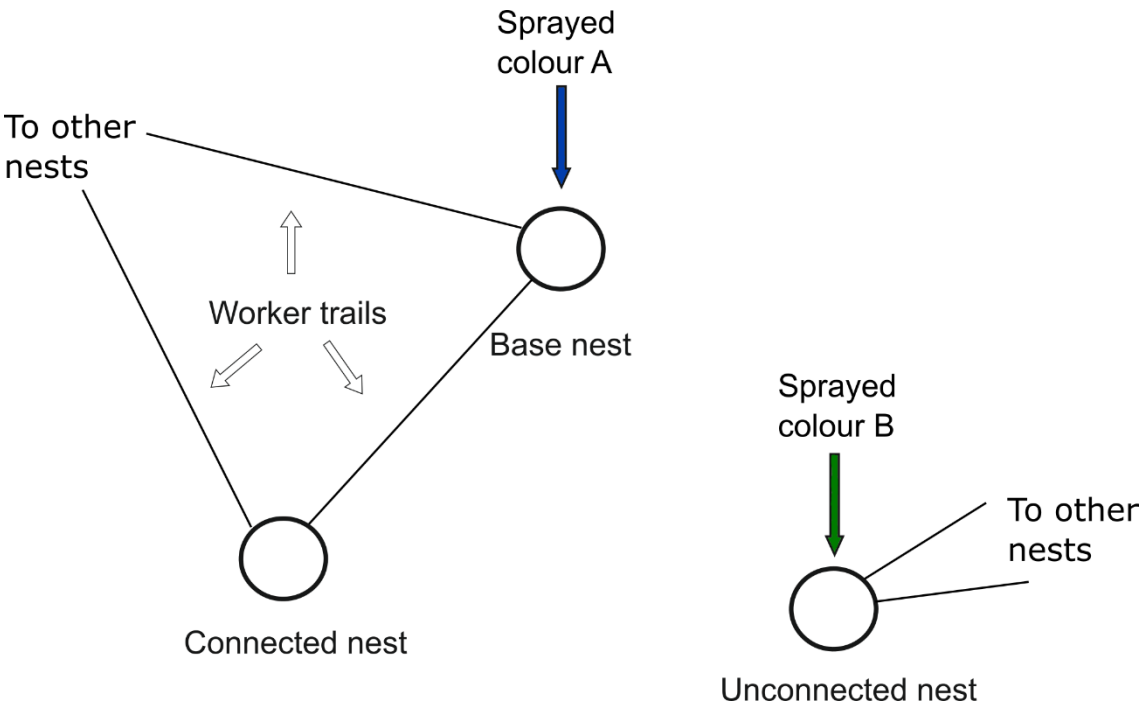

Figure 6.1. A schematic of the design for triplets used in this study; two nests connected by trails (arbitrarily termed 'base' and 'connected' nest) and a third nest (termed 'unconnected'), a similar distance away but not connected by a trail. Spraying the base nest colour A and the unconnected nest colour B allows us to track worker movement from the base to connected nest, from the base to the unconnected nest and from the unconnected nest to the base or connected nest. The unconnected nest was in some, but not all, cases connected to a separate nest network.

6.3 Methods

Study species and population

Formica lugubris Zetterstedt, 1838, is a member of the mound-building red wood ants of the *Formica rufa* group, common across the temperate and boreal forests of Europe and Asia (Goropashnaya *et al.* 2004; Stockan & Robinson 2016). The species exhibits variation in social structure throughout its range but populations in Britain are polygynous and polydomous (Gyllenstrand & Seppä 2003; Hughes 2006; Ellis & Robinson 2014). Red wood ants are ecologically dominant, a trait they share with many other polydomous species (Fournier *et al.* 2012; Gordon & Heller 2014; Hoffmann 2014). *Formica lugubris* forms strong trails both between neighbouring nests and from their feeding grounds in aphid colonies in nearby trees to nests (Sudd 1983; Ellis *et al.* 2014). The majority of the nutrient intake during the summer comes from honeydew from aphids (Rosengren & Sundström 1991).

The study population is located in the southern half of the North York Moors National Park, in the North East of England, UK (Long/Lat 54.289, -1.059, Fig. 6.2). This landscape has undergone large increases in forest cover in the last 160 years, which has allowed concomitant

expansions of the wood ant populations (Procter *et al.* 2015). The investigated population of *F. lugubris* contains approximately 3000 nests, across an area of 10.4km^2 (Procter *et al.* 2015). This population was chosen for this study because prior knowledge of its extent and the location of nests allowed the selection of randomly distributed sampling points throughout the population, with sufficient spacing that any given polydomous colony, defined by social connections between spatially separate nests, did not span multiple sample points.

The forest is dominated by non-native conifer plantations adjacent to sections of ancient broadleaf woodland. Commercial forests dominated by non-native conifers represent a much more dynamic habitat than that provided by ancient woodland, due to relatively short harvest cycles, early canopy closure and frequent management interventions. The more dynamic nature of commercial forests may cause faster nest turnover than in ancient woodland. Our sampling points cover both ancient woodland and commercial forestry plantations, allowing us to assess whether there was an effect of forest age on the inter-nest genetic relatedness patterns we see within nest pairs. The age of the forest had no effect on these patterns, therefore we present analyses only in the Appendices (Appendix 4).

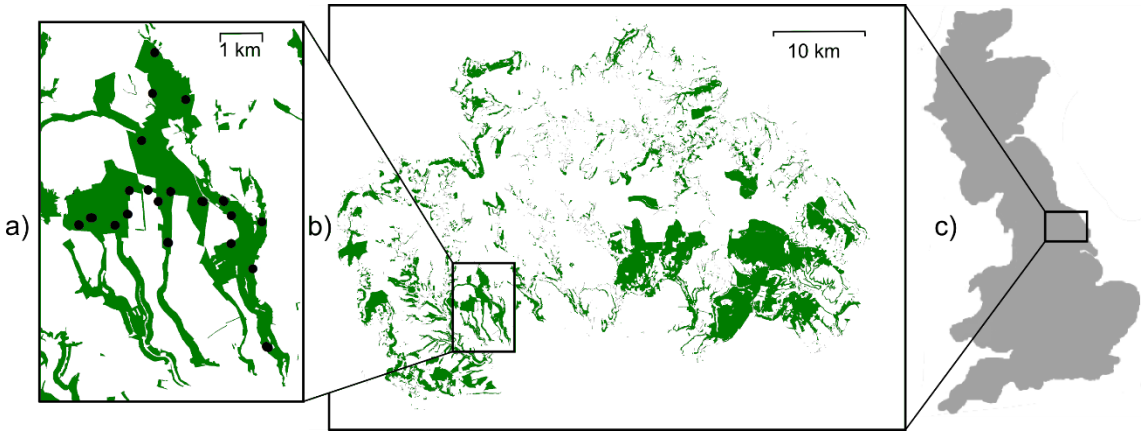

Figure 6.2. a) The study *F. lugubris* population: green polygons are forest cover and black circles are sampled triplet locations. Boxes denote **b)** the population's location within the North York Moors National Park (again grey polygons are current forest cover) and **c)** the location of the North York Moors within Britain.

Mapping test triplets

The specific arrangement required for this study was a series of groups of three nests, where two nests in each triplet were connected by a trail of workers (arbitrarily termed the 'base' and the 'connected' nests) and the third nest was not connected directly or indirectly to either of the other two nests (termed the 'unconnected' nest, Fig. 6.1). In order to locate appropriate triplets, we began by randomly choosing 40 nests from previous survey data. Taking each randomly selected nest in turn, we mapped all nests to which the selected nest was connected by trails,

either directly or indirectly (via one or more other nests), which resulted in a mapped network of nests connected by trails. We then searched the area immediately surrounding the mapped network of connected nests to find a nest close by that had no trail connection to any of the mapped nests (Fig. 6.1 unconnected nest). If no appropriate unconnected nest was found, we moved on to the next randomly chosen nest and began again. We found the desired triplet arrangement on 24/40 occasions. The mapping took place in April and May 2014. .

We attempted to find connected and unconnected nests for each triplet that were a similar distance from the base nest; however overall, unconnected nests were significantly further away from the base nest (connected mean 8.9m ± 8.3 SD, unconnected mean± SD = 15.8m ± 9.3, paired t-test, $t=-4.59$, $df=23$, $P<0.001$). To account for this difference in distance between the base nest and the connected or unconnected nest, the Euclidean distance, i.e. straight line distance, between nests was included as a covariate in generalised linear mixed models during analysis.

It could have been possible that nest size explained presence or absence of trails within triplets. For example, trails might only form between nests that are over or under a certain size. We therefore recorded nest volumes using the methods of Chen and Robinson (2013), which have been shown to correlate with worker populations i.e. the number of workers within the nest (Chen & Robinson 2013), and tested for size effects on the presence of trails. No size effects were statistically significant (Appendix 4), so nest volumes were not included in further analyses.

Worker movement

We assessed worker movement between nests by mass marking ants on the nest surface with a single light application of spray paint (Painter's touch multi-purpose paint, Rust-oleum, Durham, blossom white and spa blue) on two nests in each of the 24 mapped triplets in June 2014. The paint brand was chosen because colours did not wear off, and the application of paint did not affect worker behaviour (D. S. Procter, pers. obs.). The paint colours were chosen because they were both distinguishable from one another and clearly visible on the ants themselves. The ants on the base nest (Fig. 6.1) were sprayed one colour and those on the unconnected nest were sprayed a second colour. The third nest within the triplet ('connected' in Fig. 6.1), was not mass marked, because we could only find two paint colours that were both easily visible on the ants and distinguishable from one another. Nest surfaces were agitated before spraying, so that many workers from the interior came out onto the nest surface and were also marked. Colours were alternated between base and the unconnected nests in different triplets. We then returned to the sprayed triplet 1, 2, 3, 14 and 30 days after marking, and counted the number of workers of each colour on each of the three nests within the triplet by systematically scan-sampling the surface of each nest. From this we ascertained the relative

level of worker movement from the base nest to the connected nest, the base nest to the unconnected nest and the unconnected nest to the base nest (Fig. 6.1). We tested whether the number of workers moving between nest pairs was significantly greater than zero using Wilcoxon rank tests in R (R Core Team 2015).

Resource movement

We cannot assume that carbohydrate resource movement correlates with worker movement; therefore, we assessed inter-nest resource movement independently of worker movement in 10 of the mapped triplets in July 2014. We restricted the resource movement assessment to 10 of the triplets containing smaller nests. The larger nests in our mapped triplets contained so many workers that we could not be confident of detecting the marked food even in the baited nest with only 100 workers sampled, and it was logistically impractical to sample more than 100 workers per nest. Ants transfer sugar solution between colony workers via trophallaxis, the exchange of food mouth to mouth or mouth to anus (Hölldobler & Wilson 1990). There is a large amount of ant activity around nests that does not occur along the inter-nest trails: therefore, trophallaxis between workers of different nests could hypothetically be independent of the trails of workers between nests. Using a food bait approach, we assessed resource movement within the triplets by mixing sugar solution with Rabbit Immunoglobulin IgG (Sigma-Aldrich) using the methods of Buczkowski and Bennet (2006). We focussed on the transfer of resources from the base nest to others within the triplet using a single label. Sucrose solution (70%) in 1.5ml volumes with 0.5mg/ml IgG was placed in feeders made from inverted micro-centrifuge tubes placed on top of the base nest of each triplet. We used 10 feeders per baited nest. Feeders were topped up 24 hours after initial placement on the nest surface. Samples of 100 workers per nest from each nest within the triplet were collected 48 hours after sugar solution was initially provided and sampled ants were placed in a chilled cool box. Upon arrival at the laboratory, the chilled workers were killed by placing them in the freezer at -20°C, where they were retained prior to analysis. Each sampled worker was assayed for IgG presence using an ELISA assay, carried out as follows: a 96 well PCR plate was coated with 100µl of anti-rabbit IgG, diluted 1:500 in distilled water and incubated at 4°C for 2 hours. Once incubation was complete, the primary antibody was discarded and 280µl of 1% non-fat dry milk was added to each well as a blocker of any remaining non-specific binding sites. After 30 minutes the milk was discarded. Individual ant samples were homogenised in 200µl phosphate buffered saline, vortexed, and 70µl of each sample was added to a well in the prepared plate and incubated for 1 hour at room temperature. Samples were then discarded and each well was washed three times with PBS Tween 20 (0.05%) and then twice with phosphate buffered saline. Anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:1000 in 1% non-fat dry milk was added to each well, after which the plate was incubated at room temperature for 1 hour. All wells then received the five washes described above before adding 50µl of TMB (tetramethylbenzemidine) HRP

(horseradish peroxidase) substrate (New England Biolabs) and incubated for 30 minutes at room temperature. Samples were analysed on a BMG Labtech POLARstar OPTIMA microplate spectrophotometer set at an obsorbance of 650nm. Six negative controls which contained ants without IgG and six blanks which contained no ant sample were run on each plate. Individual wells were scored as positive if their absorbance value was more than three standard deviations higher than the mean of the negative controls (Buczkowski & Bennett 2007). We analysed differences in the number of workers testing positive for IgG between connected and unconnected nest pairs using a generalised linear mixed model (GLMM). The response variable was the number of workers testing positive for IgG and we used a Poisson error structure. The explanatory variables were whether or not the nest pair was connected by a trail and the Euclidean distance between nests. The triplet the nest pair came from was included as a random effect. We used the glmer function in the lme4 package of R (Bates *et al.* 2014).

Aggression

Aggression bioassays are a commonly used determinant of colony boundaries (e.g. Denis *et al.* 2006; Garnas *et al.* 2007; Hölldobler 1983; Kenne and Dejean 1999), based on the assumption that workers will behave aggressively towards workers from neighbouring colonies, but not their own colony mates. We conducted preliminary aggression studies in May 2014 (see Appendix 4 for details) on *F. lugubris* in our study landscape, but found that aggression levels were so low that aggression tests could not even distinguish behaviourally between populations that were separated by tens of kilometres, let alone neighbouring colonies. We note that lack of aggression does not necessarily imply lack of colony-mate recognition (Holzer *et al.* 2006; Björkman-Chiswell *et al.* 2008). However we found no difference in antennation duration between tested workers from different locations (Appendix S4 for details). We therefore decided not to deploy aggression bioassays to the full study, because they were unlikely to be informative.

Genetic distinctions between connected and unconnected nest pairs

We collected 10 workers per nest from each nest within 20 of the 24 triplets throughout the landscape in July 2014. We excluded four of the triplets used to assess worker movement, due to damage during the study period. All 10 triplets used to assess resource movement were included within the 20 sampled for genetic work. DNA was extracted using GeneJET Genomic DNA Purification kits following manufacturer's instructions (Thermo Scientific). The sampled workers were each assessed for variation at the following 12 nuclear microsatellite loci: Fe7, Fe11, Fe13, Fe16, Fe17, Fe19, Fe21, Fe37, Fe38 (developed for *Formica exsecta* Gyllenstrand et al., 2002), and Fl12, Fl20 and Fl21 (developed for *Formica paralugubris* Chapuisat 1996, known as *Formica lugubris* type B at the time), using the primers and PCR conditions specified in those papers. Each forward primer had a 5' – AGGTTTTCCCAGTCACGACGTT – 3' M13

sequence attached at the 5' end for subsequent detection purposes. DNA was amplified in a total volume of 20µl using the following reaction mixture: 1µl DNA, 1X PCR buffer (Bioron, Germany), 5µM of each primer (Integrated DNA Technologies), 0.2mM of each dNTP (VWR International), 0.25µM M13 oligo with either 700nm or 800nm fluorescent dye attached (Li-Cor Biosciences), and 0.25U *Taq* DNA polymerase (Bioron). PCR products were diluted with formamide loading buffer and run on a Li-Cor 4300 (Li-Cor Biosciences, Lincoln, NE, USA). Allele sizes were scored by eye using a set of size standards for 700nm and 800nm wavelengths. Analyses based on genetic differentiation assume that loci are at Hardy-Weinberg equilibrium and there is no linkage disequilibrium between loci, therefore loci were tested for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium within triplets in FSTAT 2.93 (Goudet 1995).

We calculated pairwise genetic relatedness between all sampled workers in each triplet using the Triadic likelihood estimator of relatedness of Wang (2007) in the Coancestry 1.0.1.5 program (Wang 2011), allowing for inbreeding in the population. Differences in inter-nest genetic relatedness between workers from connected and unconnected nest pairs were analysed as a generalised linear mixed model (GLMM) with binomial errors, because response values are constrained between 0 and 1. The response variable was the pairwise inter-nest genetic relatedness between workers with explanatory variables being the nest pair on which the inter-nest relatedness value was based (connected or unconnected) and the Euclidean distance between the pair of nests. Triplet identity was included as a random effect. The GLMM used the glmer function in the lme4 package of R (Bates *et al.* 2014).

We could not expect to see any differentiation between adjacent nests if there is no differentiation in the population as a whole. In order to confirm that there was differentiation within the population we assessed isolation by distance for the 60 sampled nests within the population as a whole by measuring all pairwise F_{ST} scores between nests using the fst.pp function of the hierfstat package of R (Goudet 2005). We then assessed whether there was a significant relationship between genetic distance ($F_{ST}/1\text{-}F_{ST}$) and Euclidean distance between nests using a Mantel test with 9999 permutations, using the mantel.rtest function in the ade4 package of R (Chessel, Dufour & Thiulouse 2004). We also analysed genetic differentiation between connected and unconnected nest pairs using hierarchical F-statistics in the hierfstat package of R (Goudet 2005). We separated the data into three hierarchical levels. Firstly the differentiation among workers within nests, which we term F_{Nest}, secondly the differentiation between nests connected and unconnected by trails within triplets, termed F_{Trail}, and lastly the differentiation between triplets within the population, termed $F_{Trip.}$ F_{Trail} is the differentiation between those nests that share a social connection or do not, which is the value we are interested in in this study. Statistical significance of the different hierarchical levels was determined by permutation tests with 1000 permutations (Goudet 2005).

Non-significant results indicate that there is no effect greater than that which is possible to detect given the experimental design employed. We conducted a power analysis in order to test the minimum level of difference in genetic relatedness we would be able to detect between connected and unconnected nest pairs. We simulated inter-nest relatedness for the two treatments (pairs of connected and pairs of unconnected nests) based on characteristics of preliminary genetic data (mean relatedness 0.131, standard deviation = 0.055). We varied the difference in mean inter-nest relatedness between connected and unconnected nest pairs between 0.001 and 0.1, at steps of 0.001. We simulated 1000 variables per level of difference in treatments. Using 20 repeats, we achieved a power of 80% whenever the difference in relatedness between treatments was greater than 0.05, in other words, a significant difference (P<0.05) between treatments was found in 80% of simulations. We were therefore confident that we could detect a significant difference in inter-nest genetic relatedness between connected and unconnected nest pairs whenever the magnitude of the difference in relatedness was 0.05 or greater.

6.4 Results

Worker movement

The number of ants detected to have moved between the base and connected nests in each triplet (Fig. 6.1), was significantly greater than zero on all counting visits: 1, 2, 3, 14 and 30 days after paint marking (Wilcoxon rank test, W=171-253, all P<0.001, Fig. 6.3a). In contrast, the number of ants that moved from the base nest to the unconnected nest did not significantly differ from zero on any counting visit (Wilcoxon rank test, W=0-1, all P=1, Fig. 6.3b). Similarly, the number of ants moving from the unconnected nest to the base nest did not differ significantly from zero on any counting visit (Wilcoxon rank test, W=0-3, P=0.346-1, Fig. 6.3c). Therefore, the presence of trails between nests does indicate a greater movement of workers and the absence of trails does appear to mean a lack of social connection. The number of workers detected to have moved between connected nests on different days did not significantly differ (Kruskal-Wallis, df=4, χ=1.46, P=0.83, Fig. 6.3).

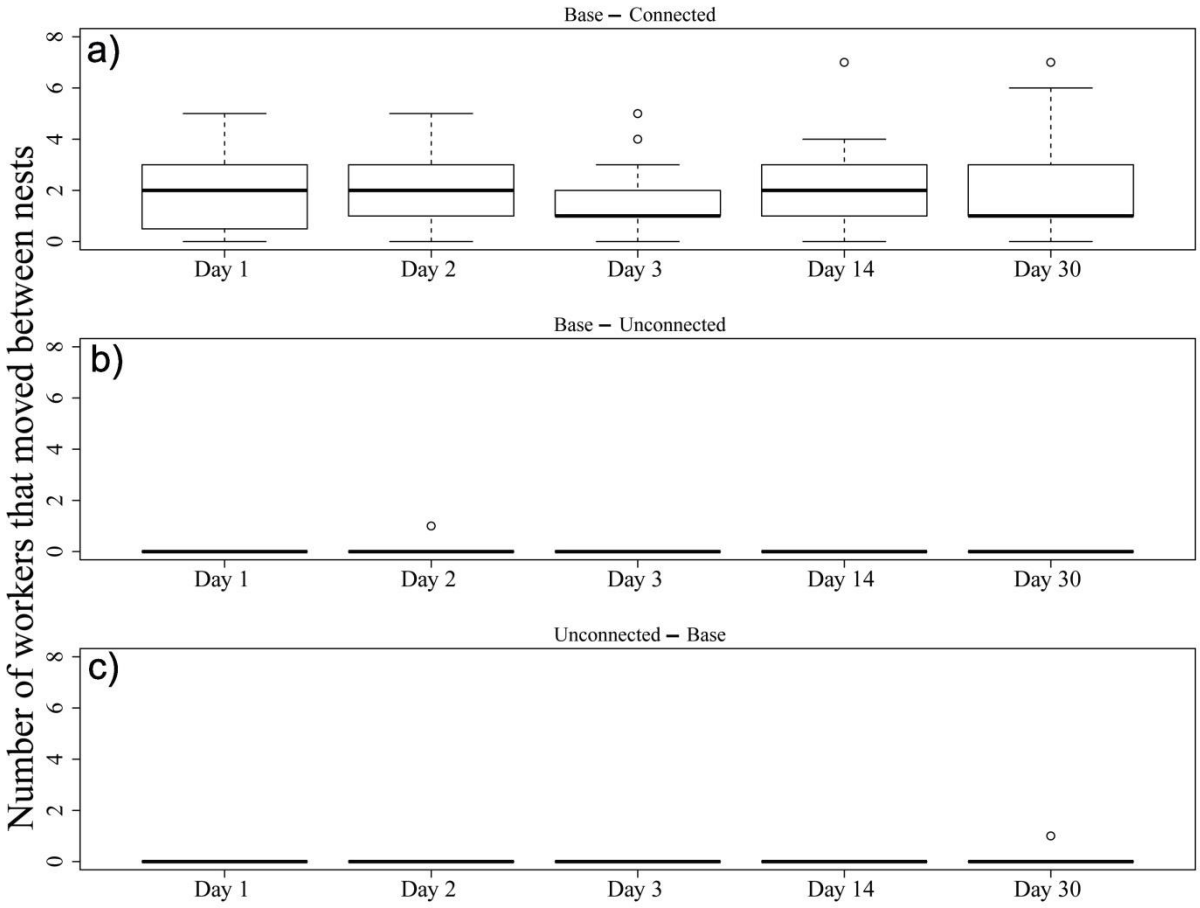

Figure 6.3. Number of workers that had moved from **a)** the base nest to the connected nest, **b)** the base nest to the unconnected nest, **c)** the unconnected nest to the base nest, for each day of re-counting for 24 triplets of nests. Boxes display 1st quartile, median and 3rd quartile, whiskers extend to 1.5 IQ, and outliers are displayed as points.

Resource movement

After 48 hours of IgG marked sucrose being made available for ant feeding on the base nest, we detected a total of 279 out of 3000 collected workers positive for IgG. Of these, 252 were found on the baited base nest themselves, 22 on the connected nest and only 5 on the unconnected nest. There were significantly more workers that tested positive for IgG on the connected nest than on the unconnected nest (GLMM, df=1,4, χ=9.34, P<0.001, Fig. 6.4a). There was no significant effect of Euclidean distance between nests on the number of workers testing positive for IgG (GLMM, df=1,4, χ=0.24, P=0.62)

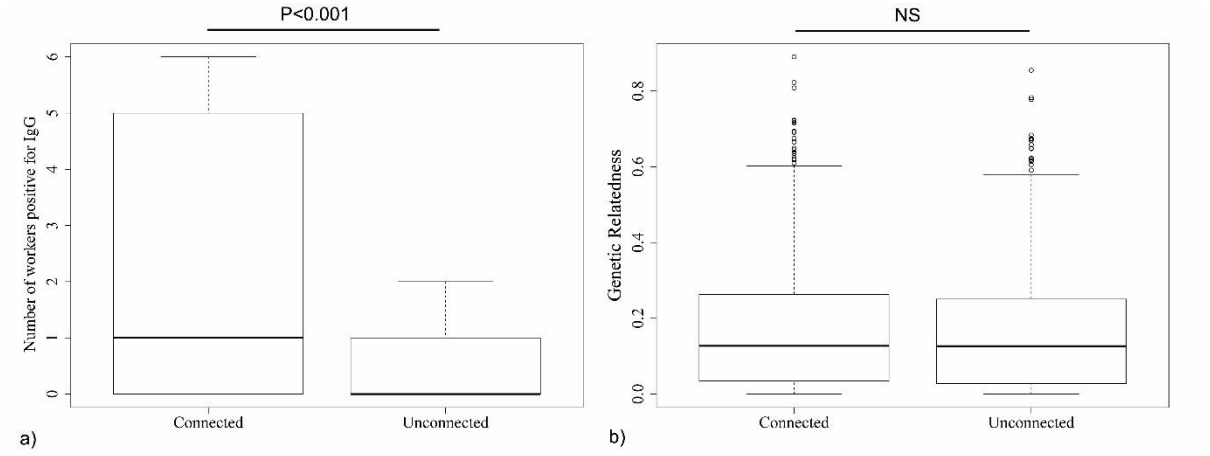

Figure 6.4. Comparisons between the connected and unconnected nest pair for **a)** The number of workers testing positive for IgG (10 triplets) **b)** Inter-nest genetic relatedness (20 triplets). Boxes display 1st quartile, median and 3rd quartile, whiskers extend to all points within 1.5 IQ, outliers are displayed as points.

Genetic distinctions between connected and unconnected nest pairs

Diversity across the 12 microsatellite loci used ranged from low to high. Three of the loci displayed low variability (2-3 alleles, expected heterozygosity 0.16-0.51), with the remaining nine loci being more variable (4-19 alleles, expected heterozygosity 0.67-0.89). None of the loci showed significant deviations from Hardy-Weinberg equilibrium or significant linkage disequilibrium within samples, so all loci were retained for the analysis. The 60 nests making up the 20 triplets of nests in which workers were genotyped, displayed significant isolation by distance, with genetic distance, measured by $F_{ST}/1\text{-}F_{ST}$, increasing significantly as distance between nests increased (Mantel test, r=0.36, P<0.001, Fig. 6.5).

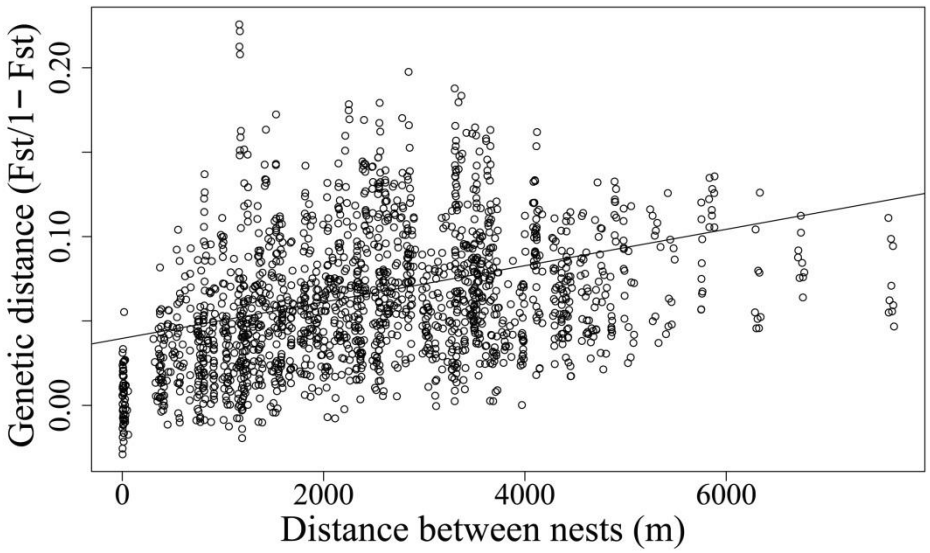

Figure 6.5. Genetic distance, measured by $F_{ST}/1\text{-}F_{ST}$, against distance between all the sampled nest pairs in the population as a whole. The line displays a linear relationship between genetic distance and Euclidean distance between nests, significance was tested using a Mantel test.

Inter-nest genetic relatedness between workers from connected nest pairs did not differ significantly from inter-nest genetic relatedness between workers from unconnected nest pairs (connected pair mean= 0.17, unconnected mean= 0.16, GLMM, df=1,3, χ=0.122, *P*=0.73, Fig. 6.4b). There was no relationship between inter-nest genetic relatedness and Euclidean distance within triplets (GLMM, df=1,3, χ =0.81, *P*=0.36). Instead, the majority of differentiation was explained by the highest hierarchical level of organisation of the data: the differentiation between different triplet groups i.e. the differentiation due to landscape patterns, which was significantly greater than zero (F_{Trip}=0.055, *P*=0.001). There was negligible differentiation between connected and unconnected pairs within triplets (F_{Trail}=0.001), or within nests (F_{Nest}=0.004), neither of which were significantly greater than 0 (F_{Trail} *P*=0.683, F_{Nest} *P*=0.087). The negligible value of F_{Trail} supports the lack of difference in relatedness between connected and unconnected nest pairs, and high within triplet relatedness, that our relatedness analyses report.

6.5 Discussion

A social insect colony is expected to be a cooperative, reproductive and selective unit, where members are more related to one another than to other members of the population. However, our results clearly show that workers from the nests of *F. lugubris* that cooperate are no more genetically related to one another than workers from nests that do not cooperate. Cooperation

between ant nests involves the exchange of workers and resources. We have shown both workers and resources can move between connected nest pairs, whereas workers do not move between unconnected nest pairs, and significantly fewer resources are exchanged. Nest pairs with a cooperative connection neither differ in their inter-nest genetic relatedness from unconnected nest pairs, nor do they display significant genetic differentiation from unconnected nests. The difference we observe in cooperation can therefore not be explained by a genetic difference.

A social insect colony is expected to be a cooperative unit. Here, our results suggestthat spatially separate nests in *F. lugubris* are cooperative units when connected by trails. Firstly, we have confirmed that trails between nests do constitute a social connection, because workers do move between connected nest pairs but, more importantly, workers are not exchanged between unconnected nest pairs. Substantially rarer movement between unconnected nests than connected nests is consistent with previous findings in a related species (O'Neill 1988), and is expected if nests are solely cooperating within one colony. Secondly, we have shown that connected nests exchange significantly more resources than unconnected nests. Movement of resources between nests could be interpreted as either cooperation or stealing, but with stealing, we would expect competitive interactions. The strong social connections we observe, without aggression, suggest cooperation rather than competition. Existing evidence from other ant species suggests that new nests within polydomous colonies are placed near food sources (Holway & Case 2000; Lanan, Dornhaus & Bronstein 2011). In *F. lugubris* this does not appear to be the case; however, nests with workers that forage are more likely to survive than non-foraging nests (Ellis & Robinson 2015a). In *F. lugubris* workers appear to use nests they are connected to by trails as a foraging resource, which could be interpreted as a form of intraspecific kleptoparasitism (Ellis & Robinson 2016). However, polydomous nest networks across ant species are structured to allow efficient transport of resources (Cook *et al.* 2014). In *F. lugubris* colony level characteristics related to the flow of resources through a nest predict nest survival (Ellis *et al.* in Review), and nests within a network that do not forage are more likely to be abandoned (Ellis & Robinson 2015a). There is, therefore, an advantage to be connected to multiple nest, which should elicit competition between nests if connections are not cooperative. However we find no aggression between neighbours in our population (see Appendix 4 for details), and therefore there is no detectable competition. Resource movement between spatially separate nests therefore suggests active cooperation between socially connected nests, as we predicted.

Our results clearly demonstrate that there is significantly higher resource transfer between nests connected by trails of workers than between unconnected nests. However, in three out of ten trials we did see carbohydrate resource transfer between unconnected nests, albeit at a low level. The few workers that were found to be positive for IgG on the unconnected nest may have

acquired resources from the baited nest via non-cooperative means. The non-cooperative acquisition of food could involve stealing from the baited nest or possibly inducing trophallaxis from workers from the baited nest. Trophallaxis is a standard method by which resources are transferred between workers of the same colony and is normally thought of as a sign of cooperation, but trophallaxis can also occur between species which do not cooperate (Bhatkar & Kloft 1977). Under these circumstances, trophallaxis acts as a means of reducing inter-species aggression. Therefore, the exchange of resources seen in this study, could be an activity that reduces aggression between colonies, analogous to reducing aggression between species. Resource movement can either correlate well with social connections (Heller, Ingram & Gordon 2008; VanWeelden, Bennett & Buczkowski 2015) or can operate at a different spatial scale (Buczkowski 2012); therefore, the slight disparity between worker movement and resource movement in our results agrees with the literature: future studies should be cautious in assuming that social connections and resource movement are always closely correlated.

Workers themselves must also be considered resources for ant colonies, because they are the workforce and contribute to the production of the next generation. Our data supports worker movement, which could be genuine worker exchange if the workers perform beneficial acts such as brood care or foraging for the recipient nests. Our current study does not investigate the behaviour of the workers that move. Therefore, worker movement may also be a form of resource exchange, and arguably more important than the exchange of carbohydrate, because carbohydrate maintains only the current generation of ants. Total resource exchange between nests is therefore a combination of worker exchange and exchange of food. Viewed in this way, the resource exchange between socially connected nests far exceeds the resource exchange between socially unconnected nests, and represents a real cooperative distinction if the workers are behaving beneficially in the recipient nest.

We have shown that the cooperative distinction we show is not reflected by a genetic distinction, however, we are not claiming that genetic factors are not important within ant colonies. The altruistic acts of workers within an ant colony are explained by inclusive fitness (Hamilton 1964; Bourke 2011), which includes both a benefit and cost term, as well as genetic relatedness. Genetic relatedness between the unconnected, non-cooperative, nest pairs is remarkably high (mean = 0.16), indeed higher than is often observed within single nests of other ant species (e.g. in another *Formica* species as low as 0.01: Pamilo *et al.* 2005; and in other ant species 0.04: Goodisman and Ross 1997; and 0.05 Pedersen and Boomsma 1999). There is therefore, no genetic reason why cooperative interactions should not occur. In *F. lugubris*, interactions between nests appear to be based on the movement of resources through the colony; ant nests that differ most in the amount of foraging that they perform are linked by stronger trails than those nests that had a more equal foraging effort (Ellis *et al.* 2014). In this study we did not assess foraging in sufficient detail to determine the costs and benefits to each nest. If

both nests within an unconnected pair forage sufficiently to support their worker force, then there may be no benefit to be gained from the presence of a trail between nests, and therefore no reason to maintain a trail. Alternatively, because aphids are abundant in the vicinity of wood ant colonies, the exchange of carbohydrate between neighbouring nests may incur only a tiny cost. With a tiny cost of resource exchange, there will be minimal evolutionary pressure to eliminate trails that are remnants of the nest formation event. Some trails may be lost by chance, while others are maintained, without a penalty to those that remain connected. We assume that the cost of the trail between nests is proportional to the length of that trail and account for trail length in our analyses. However, there may be other factors, such as desiccation or predation risk, that mean that trails between unconnected nest pairs are more costly than between connected nest pairs and preclude trail formation. We therefore suggest the distinction between connected and unconnected nest pairs is not caused by a genetic distinction, but by some unmeasured ecological or stochastic process.

Ants use cuticular hydrocarbons (CHCs) for nestmate recognition (Hölldobler & Wilson 1990). The extent to which genetic and environmental patterns affect hydrocarbon profiles varies between ant species (Buczkowski & Silverman 2006; van Zweden, Dreier & D'Ettorre 2009) but in wood ants, experimental separation has been shown to alter CHC profiles (Sorvari *et al.* 2008). It may therefore be that once a social connection has been lost for long enough for hydrocarbon profiles to diverge, genetically similar ants will no longer recognise one another as colony-mates and the division becomes more permanent. Further studies may wish to assay CHC profiles alongside social connection methods to ascertain whether this is the driving factor.

The study landscape is dominated by commercial forests, which are both recently planted and highly dynamic in comparison to natural woodland. The addition of these commercial forests has benefitted the wood ants, allowing large population expansions (Procter *et al.* 2015). Due to these recent population expansions, we cannot expect the ant populations to be at equilibrium. It is possible that the recent range expansions of *F. lugubris* on the North York Moors have resulted in neighbouring colonies exhibiting the high inter-nest relatedness that we see. However, our sampled triplets were located in both ancient woodland and recently planted conifer plantations, and all showed the same lack of genetic distinction between connected and unconnected nest pairs (Appendix 4). We therefore think it is unlikely that the dynamic landscape will have masked any possible distinctions, but it would still be interesting to compare our results with a similar study in a less disturbed forest system.

The genetic patterns we report are based solely on nuclear DNA variation. Many ant species are known to exhibit sex-biased dispersal, whereby males disperse larger distances than females. This results in differentiation in biparentally inherited nuclear genetic differentiation at a larger

spatial scale than is seen for maternally inherited markers such as those located on mitochondrial DNA (e.g. Clémencet et al., 2005; Doums et al., 2002; Soare et al., 2014). If there is a similar pattern of sex-biased dispersal in this population, the division between connected and unconnected nest pairs may become exposed if mitochondrial DNA markers are used, because different matrilines within the connected and unconnected nest may be resolved. However, preliminary surveys of fragments of mitochondrial COI DNA showed only two haplotypes within this population, with variation never present within a sampled triplet (D. S. Procter unpubl. data).

In the studied population, *F. lugubris* colonies reproduce by budding; this method of dispersal often results in strong spatial genetic structuring of populations, meaning that nests close to one another are more genetically similar irrespective of colony divisions (Sundström *et al.* 2005). Budding dispersal could therefore mean that all three of the nests in each of our triplets share common descent. Wood ant trails can be stable over long time periods (Rosengren 1971). The trail structures within this population have not been mapped over multiple years, so we do not know how long the unconnected nests have been unconnected. However, in another *F. lugubris* population in the UK, trails have been mapped over multiple years: trail turnover does occur but new connections were not formed between separate trail networks, nor did trail networks separate and then reconnect (Ellis *et al.* in Review). Therefore there does appear to be a genuine separation between neighbouring nest networks in *F. lugubris*. If unconnected nest pairs were connected until recently then our results indicate there has been insufficient time for genetic distinctions to build up between unconnected nests.

A social insect colony is expected to be a cooperative, reproductive and selective unit, which should apply whether the colony occupies a single nest or multiple spatially separate nests. In a polydomous species, we suggest that there are cooperative divisions within genetically homogenous groupings. In some eusocial insects, social organisation is to a degree controlled by environmental factors (Eickwort *et al.* 1996; Richards 2000). Similarly, we suggest that it is ecology rather than genetics that is driving the polydomous nest organisation that we observe here. Our findings support the polydomous colony as a cooperative entity, but not one that is genetically distinct from its neighbour. Our study suggests that ecology also plays a large role in determining social organisation in this, and likely other, ant species.

Chapter 7: Discussion

7.1 Thesis overview

In this thesis I have used multiple approaches in order to assess how non-native conifer plantations have impacted the wood ant, *Formica lugubris*. In Chapter 2, I extensively mapped wood ant populations across the North York Moors and compared current distributions to historic forest cover, showing that current populations are in areas where there was no forest in the past, therefore ant populations must have expanded with the forest. I then modelled the suitability of the habitat, showing that it is only the dispersal ability of *F. lugubris* that is limiting their expansion into suitable habitat, and there is no reason the expansion in to non-native conifer forest should not continue. In chapter 3 I further develop the habitat models used in chapter 2, assessing whether varying the spatial scale of variables affects the patterns we see. I present the most effective potential scale for management interventions if they are attempting to help *F. lugubris*. However, I also show that most management decisions that affect *F. lugubris* are at the planting stage and, once established, *F. lugubris* is only weakly affected by the structure of the forests. In chapter 4 I assess the genetic diversity of the expanded *F. lugubris* populations, and find them to be just as diverse as Finnish populations in undisturbed forest. Furthermore I show evidence of spatial structure in the genetic data, which would suggest that the expansion of populations with increases in forest cover has connected previously isolated population fragments. In chapter 5 I investigate mitochondrial variation across the landscape, showing that there are two haplotype groupings, which show stronger divergence than either does to other species within the *F. lugubris* group. Furthermore I show that, even with limited sampling, there is high mitochondrial variability across this landscape, again suggesting populations are genetically healthy. In Chapter 6 I move on to assess social organisation in *F. lugubris*, testing whether the social connections between nests within polydomous colonies are cooperative, and whether they are explained by greater relatedness between connected nests. I show that social connections are cooperative, but not explained by higher relatedness. I suggest that the loss of a social connection may be the first step in the formation of new colonies.

7.2 The impacts of forest cover change

Whereas anthropogenic land use change generally has negative effects on ecosystems, our results show that the creation of non-native conifer plantations on previously non-forest land has had beneficial effects the wood ant *F. lugubris*. We show that current populations have expanded thanks to the expansion of non-native conifer plantations, and should continue to do so (Chapters 2, 3). We show that the tree species used in commercial forestry are high quality habitat for *F. lugubris* (Chapter 3), and therefore presumably the aphids they feed on too.

Furthermore, the populations of *F. lugubris* that have expanded from historic fragments are genetically diverse (Chapter 4, 5), and show evidence of multiple, historically separated, population fragments being connected by the expansion of populations. From the evidence presented, we conclude that non-native conifer plantations have the potential to support some forest specialists, and so provide a valuable contribution to the maintenance of biodiversity in a degraded system such as the UK.

Research into the effects of non-native conifer plantations in Britain initially focussed on the change in community structure, once commercial forests were planted on previously non-forest ground. As a plantation on marginal agricultural land matures, the community slowly changes to greater resemble a forest community (Ratcliffe 1986). Therefore it is unsurprising that afforestation had negative effects on the biodiversity characteristic of the land upon which it was planted, primarily upland moor (Thompson *et al.* 1988; Moore & Allen 1999). Whilst this was a valuable step in research, it does not assess the value of commercial forests as a forest habitat. Studies that have tried to compare commercial and native forest biodiversity have not displayed clear results: commercial forests can display lower species richness or diversity than native broadleaved woodland (Fahy & Gormally 1998; Pedley *et al.* 2014), or the opposite can be true (Day *et al.* 1993), or there can be no difference between the two habitats (Bibby *et al.* 1985; Fuller *et al.* 2008; Pedley *et al.* 2014). The studies above have each been case studies, on too small a scale to generalise. The only research at a country-wide scale is the Forestry Commission's Biodiversity Assessment Project, which concluded that that plantations provide a significant contribution to the maintenance of biodiversity (Humphrey *et al.* 2003; Quine & Humphrey 2010). No similar assessment has taken place for native broadleaved forest, therefore it is impossible to directly compare the biodiversity supported.

Assessment of broad measures of biodiversity do allow a quick description of a community, however when it is the effects of forests that we are interested in, then surely it is forest specialist species that should be studies in greater detail. Studies of individual forest species and the effects of forest cover on them are far less common, there are only two that we are aware of. The afforestation that created Kielder forest, in the north east of England, increased gene flow between previously isolated populations of the red squirrel, *Sciurus vulgaris*, therefore defragmenting the landscape (Hale *et al.* 2001). Secondly, connection of ancient forest fragments by commercial plantations, has allowed gene flow between populations of the wood ant *F. aquilonia* in Scotland, although it was unclear whether *F. aquilonia* could also make use of the intervening plantation habitat (Vanhala *et al.* 2014). As a result of the combined research in this area, there is a general move towards seeing plantation forest as a potential benefit for forest biodiversity (Quine & Humphrey 2010; Bremer & Farley 2010). Our results give strong support to this view. Certainly for the wood ant *F. lugubris* recent increases in commercial

forests have had a very beneficial effect. Only further research will say whether this pattern is more general.

We have shown that the expansion of *F. lugubris* populations are limited by their poor dispersal capacity (Chapter 2). Therefore, if poor dispersing forest specialists, such as *F. lugubris* are to be able to spread to novel forest habitat, it must be within dispersal distance. However the forest cover of the UK is highly fragmented (Peterken 1993), therefore there will be a great many forest fragments that poor dispersers such as *F. lugubris* will be unable to reach. There are currently plans to further expand the forest cover of England (Forestry Policy Team 2013), therefore our results highlight the importance of placing the new forest as close as possible to existing forest fragments. Obviously there will be a variety of issues that must be taken into account when deciding on the location of these forests, but if the purpose of the forests is to support forest ecosystems and forest diversity, then proximity to existing forests is essential.

We have shown that an artificial forest habitat, which is composed of monocultures, high density planting, and non-native species, all of which would be predicted to have negative effects on native species, actually has massively positive effects on *F. lugubris*. This is one of very few examples of anthropogenic land use changes improving the natural environment for native species.

7.3 Advice for forest managers

There are a number of implications from this thesis that are relevant for forest management. Firstly that wood ants are an excellent species to manage forest for. Wood ants promote biodiversity and nutrient cycling in forest systems (Laakso & Setälä 1997; Hughes 2006; Domisch *et al.* 2009; Wardle *et al.* 2011; Härkönen & Sorvari 2014; Stockan & Robinson 2016), and so have a positive role within the woodland ecosystem. Added to that they feed upon any organism that does not adequately defend itself, therefore they can be an excellent defence against extreme defoliators such as some of the Lepidoptera that can outbreak on vast scales, for example *Oporinia autumnata* (Laine & Niemelä 1980). It is worth mentioning that wood ants also tend aphids, and attack predators of aphids, therefore they massively increase aphid abundance on trees (Warrington & Whittaker 1985a; b; Whittaker & Warrington 1985). The beneficial effects of wood ants on trees therefore depend on circumstances, but in a world where invasive pests are becoming an ever growing problem, the presence of wood ants may defend against some extreme impacts on forests.

Formica lugubris should require minimal extra management in order to thrive in commercial forests, because it displays preferences for larch, spruce and pine, which are by far the most popular tree genera in commercial forests (Chapters 2, 3). Therefore there is no need to radically change forest composition to suit *F. lugubris*. It also shows a preference for oaks (Chapter 3),

therefore the addition of native oak species to diversify species within plantations stands can further benefit *F. lugubris*. Insolation is important to wood ants in general (Kadochová & Frouz 2014; Chen & Robinson 2014), therefore dark areas of forest are poor habitat for *F. lugubris*. This probably explains why *F. lugubris* is less likely to be present as the percentage of trees over 80 years old nearby increases (Chapter 3). Old forest creates a high and dark canopy, and may completely shade openings within the forest. Therefore, large areas of mature and over-mature plantations may have negative effects on *F. lugubris*.

The organisation of commercial forests is ideal for *F. lugubris*, because it is an edge specialist (Chapter 2), therefore the edges of wide tracks through commercial forests, built for the access of forestry vehicles, provide perfect habitat for *F. lugubris*. Widening of the edges of these tracks would likely further benefit *F. lugubris*, as would minimising the damage to these track edges during forest operations. Clear-cutting of forest patches has negative effects on the wood ants that inhabit that forest, however the ants are able to move nest locations (Sorvari & Hakkarainen 2005, 2007). We would therefore recommend clear-cut areas under 200m in width from our data (Chapter 3), which should minimise negative effects on wood ants.

It may seem that the loss of a small number of *F. lugubris* nests to clear-fell an area is of no concern when populations are numerous, and so there is no danger of population extinction. However, findings from this thesis show that not all nests are equal within *F. lugubris* populations. In order to conserve species, genetic diversity, as well as substantial populations, must be conserved (Höglund 2009). I have shown that populations contain high levels of genetic diversity (Chapters 4, 5), and that this genetic diversity is not evenly spread within populations (Chapter 5), therefore population subsets are still of value, and nest should not be destroyed unless it is completely unavoidable.

Due to the poor dispersal ability of *F. lugubris* it is unlikely that it will reach a forest naturally unless it is already present in an area connected to said forest (Chapters 2, 3). It therefore may be an option to transplant *F. lugubris* nests to the currently unoccupied forest in order to facilitate colonisation, however translocation of healthy wood ant nests is recommended only as a last resort (Hughes 2008). Translocation of *F. lugubris* nests has been attempted a number of times in the past for reasons related to the rearing of pheasants (Yarrow 1955) forestry (Wellenstein 1973), research (Sorvari, Huhta & Hakkarainen 2014) or conservation (Catherine 2015). *Formica lugubris* is even present in Canada due to a translocation hoping to improve forestry conditions (Storer *et al.* 2008), although subsequent investigations suggest this was actually *F. paralugubris* (Seifert 2016). In any case translocations are a substantial undertaking (Hughes 2008) and, though they may allow the spread of *F. lugubris*, they should not be undertaken lightly.

7.4 Implications for *F. lugubris*

Our results on the demographic and genetic health of the populations of *F. lugubris* in the North York Moors are nothing but positive. Populations already display very large numbers of nests and high genetic diversity and so should not be in risk of extinction (Chapters 2, 4, 5). Furthermore, there are vast areas of forest in the North York Moors, which have been shown to be suitable for *F. lugubris* and to which current populations are connected (Chapters 2, 3). Therefore we expect current populations of *F. lugubris* in the North York Moors to thrive in the future. In the recent past some populations in the UK and Ireland have been shown to be decreasing to probable extinction (Robinson 2001; Mäki-Petäys & Breen 2007), therefore there was concern that other wood ant populations in the UK may also be in decline. However, evidence from this thesis, along with similar evidence from the Peak District (Gyllenstrand & Seppä 2003), suggests that, at least in some areas, British wood ant populations are very healthy.

The next question is whether the species present across this landscape truly is *F. lugubris*. All populations were morphologically identified as *F. lugubris* using the UK key (Skinner & Allen 1996), therefore, at least morphologically they appear to be *F. lugubris*. However, recently there have been two cryptic species separated from *F. lugubris* in Switzerland (Seifert 1996; Bernasconi *et al.* 2011), and it is possible that this species, that appears morphologically to be *F. lugubris*, may actually be a cryptic species. The wood ants of the *F. rufa* group are difficult to distinguish on morphological grounds, due to high intra-specific variation in morphology (Bernasconi *et al.* 2010). Recently there have been advances in morphological methods (Seifert 1996; Seifert & Goropashnaya 2004), but these methods are very time consuming and require considerable expertise. We did not attempt to apply these complex morphological assessments to our populations, at least in part because we had no reason to suspect that what we were studying was not *F. lugubris*. However, our findings of strong genetic divergence within populations probably warrant a more in depth taxonomic assessment of the *F. rufa* in the North York Moors, and probably the UK as a whole.

UK populations of *F. lugubris* are already divergent from most of the European populations in ecology: in Ireland and the majority of mainland Europe *F. lugubris* is monogynous and monodomous, however in Britain and parts of Switzerland *F. lugubris* is polygynous and polydomous (Gyllenstrand & Seppä 2003; Bernasconi *et al.* 2005; Mäki-Petäys & Breen 2007). The social organisation of *F. lugubris* in the UK is therefore closer to some of the other members of the *F. rufa* group than to the majority of *F. lugubris*. Most notably *F. paralugubris*, one of the cryptic species recently taxonomically separated from *F. lugubris* in Switzerland, displays very similar social organisation to *F. lugubris* in the UK. To back up this social distinction between British and European *F. lugubris*, we showed very strong divergence

between mitochondrial haplotypes within the landscape of the North York Moors, with greater divergence between the haplotypes than between species within the *F. rufa* group with which we could compare them (Chapters 4, 5). This could be evidence of either an ancient hybridisation event or cryptic species present within the landscape. However, in either case, our evidence clearly shows that further investigation is required.

7.5 Polydomous colony organisation

We have shown that the cooperative divisions that correlate with social connections between nests in *F. lugubris* are not explained by genetic distinctions (Chapter 6). As ever, different parts of our work agree and disagree with different parts of the literature. The idea that nest networks are cooperative networks is completely in line with a variety of findings. For example: in *F. lugubris* the strength of a trail between nests is correlated with their difference in resource collection, suggesting exchange between the nests (Ellis *et al.* 2014). Formation of nests within polydomous colonies is near food sources in some species (Holway & Case 2000; Lanan *et al.* 2011). In *F. lugubris* this does not appear to be the case, but nests are more likely to survive within polydomous colonies if they do forage. Nest networks across polydomous species are also structured to facilitate efficient resource acquisition from the environment (Schmolke 2009; Cook *et al.* 2014), therefore resource acquisition is an integral part of the polydomous system.

Social insect colonies are not only expected to be cooperative units, but also reproductive and selective units. The altruistic actions of many non-reproducing workers contributing to the reproduction of queens within a colony is explained by inclusive fitness (Hamilton 1963, 1964): the workers are related to the queen and therefore gain fitness as the queen reproduces. The gain in fitness of the workers is scaled by their relatedness to the queen, therefore in more highly related systems the evolution of altruism happens more easily. Extending this to multiple nest colonies we get a clash of terminology. Workers within an ant colony can be seen to be altruistic, because without reproducing, they can get no direct fitness benefit from an interaction. However in polydomous colonies, we speak about cooperation, because we can see bi-directional exchange of both workers and resources. In reality we do not know if this is altruism or cooperation in the evolutionary sense, because we have no idea what the costs or benefits to either partner is in terms of fitness. Whether it is truly an altruistic interactions between nests or a cooperative one, the evolution of both altruism and cooperation are more likely with higher relatedness of the interacting pair (Bourke 2011). Certainly workers within polydomous colonies are still behaving altruistically by collecting resources for the colony, because they can only gain fitness through indirect fitness. Therefore we still expect relatedness greater than zero between members of the same polydomous colony. This is precisely what we found (Chapter 6).

We would also expect to see that members of a polydomous colony are more related to one another than they are to their neighbours, however that is not what we see (Chapter 6). The question then arises, where does a polydomous colony boundary end? If we were to assess the population in Chapter 6 using genetic methods for colony delineation (Chapter 1A), then the three nests within each triplet would all be considered part of the same colony. However using methods based on social connections and resource transfer, they are separate colonies. I would argue that the second is more useful. The relatedness between unconnected, and non-cooperating nests is greater than zero, therefore there is the potential for altruistic and cooperative actions between unconnected nest pairs. However, without any evidence of social interactions or resource exchange, interactions do not occur. Colonies of *F. lugubris* tracked over time show no evidence of neighbouring nest networks connecting (Ellis *et al.* in Review), therefore social connections between nests do appear to represent genuine distinctions. Genetic divergence works on a much longer timescale than ecological divergence. Therefore, what we see at the borders of these polydomous colonies, may be a relatively recently founded second colony, that has not yet had the time to diverge from its neighbour. Aggression bioassays are an obvious tool that may backup a functional distinction between genetically distinct nests, however in our populations aggression does not distinguish either neighbouring colonies or distant colonies (Chapter 6 and Appendix 4).

It has been suggested, quite reasonably, that the social organisation present within our study site is remarkably close to *F. paralugubris* in Switzerland. *Formica paralugubris* is deemed to be unicolonial, that is there are no colony boundaries within populations (Holzer *et al.* 2006), which is generally considered to be a level of sociality that exceeds polydomy (Helanterä *et al.* 2009). However, I would dispute whether there is a fundamental difference between the social organisation found in *F. paralugubris*, and the social organisation we find in *F. lugubris* in the UK. Comparison is not simple because different studies have been done on either species, so I will summarise both separately and then compare.

Formica paralugubris is thought to have no colony divisions within populations because there is little to no aggression between workers within populations (Chapuisat *et al.* 2005; Holzer *et al.* 2006) and foreign queens are accepted into nests (Fortelius *et al.* 1993; Holzer *et al.* 2008b). However, although there is not aggression within populations, workers do recognise one another (Holzer *et al.* 2006), and though foreign queens are accepted, their reproductive output is significantly lower than resident queens (Holzer, Chapuisat & Keller 2008a). There is also significant genetic differentiation within populations of *F. paralugubris* (Chapuisat *et al.* 1997; Chapuisat & Keller 1999; Holzer *et al.* 2006, 2009), therefore populations are not genetically homogenous, as would be expected under free movement of reproductives. Mating and dispersal within populations is very local (Chapuisat & Keller 1999), further suggesting queens do not move, even if they are technically able to.

Formica lugubris in the UK has, in the main, has been investigated more in a functional manner than *F. paralugubris* i.e. the measurement of worker and resource movement, and inference of cooperative structures that define colonies. In one population trail networks have been tracked over multiple years, and so we know that there have been no instances of separate nest networks forming connections between one another (Ellis *et al.* in Review). Nests within a polydomous colony are more likely to survive if they forage, therefore the acquisition of resources appears important in polydomous colony organisation (Ellis & Robinson 2015a). Also the strength of a trail between two nests within a polydomous colony correlates with a difference in the amount of foraging each nest does (Ellis *et al.* 2014), therefore it seems that there is active cooperation within polydomous colonies. We have reinforced the evidence that connections between colonies are cooperative (Chapter 6), but we also show that this cooperative distinction is not mirrored by a genetic distinction at the local level. There is also genetic differentiation within *F. lugbris* populations (Chapter 6).

From the studies completed on *F. lugubris* in the UK and *F. paralugubris* in Switzerland, it seems that the main difference between the two species is the way in which they have been studied. Most of the studies on *F. lugubris* have been functional, and interested in how polydomous colonies are organised; only with the single chapter in this thesis are we starting to ask why. In contrast most of the studies on *F. paralugubris* have approached this population that is open to movement and asked why this exists. However, it would appear that in the process, those studies asking why there is free movement within the population, have actually answered that there is not free movement within the population. There is the potential for free movement within the population, it would seem, but it does not actually happen. Using genetic methods for colony delineation (Chapter 1A), we would certainly conclude that there are multiple colonies within the population, because there is significant genetic differentiation. We have shown that genetic and functional methods for colony delineation can show different results (Chapter 6), therefore it would be interesting to measure worker movement and resource flow within *F. paralugubris* populations. It would be interesting to exchange methodologies, and potentially personnel, and see how the other group interprets the other population. Personally from the evidence discussed above I think they will be very similar, but there is substantial speculation in that opinion. This argument is, in the main, semantic and therefore somewhat minor. However, because of the semantics, these different studies are treated in different lights, rather than contributing to a shared pool of knowledge. There is the potential for greater understanding if we discover precisely how similar and how different these well studied systems are.

There has been considerable debate over how the colony term applies to polydomous species (Helanterä *et al.* 2009; Suarez & Suhr 2012; Gordon & Heller 2012; Lester & Gruber 2012; Moffett 2012a; b; Pedersen 2012). To a large degree this debate is semantic, and therefore not that useful. Evidence from our study adds to a growing body of evidence that, although some

species appear to not differentiate between conspecifics over large regions (Giraud *et al.* 2002; Holzer *et al.* 2006; Ugelvig *et al.* 2008), there are divisions within these populations which function as smaller colonies (Holzer *et al.* 2009; Gordon & Heller 2014). Functional divisions are what matters when assessing interactions within colonies. Without relatedness between nest-mates the colony concept begins to break down (Helanterä *et al.* 2009). However, as long as there is sufficient genetic relatedness that the colony concept does not break down, which there is in our system (Chapter 6), and within other polydomous species (Keller 1995; Chapuisat & Keller 1999), then the unit that functions as a colony is the unit worth studying. Neighbouring nests with high genetic relatedness represent potential colony connections, but only those that actually are connected are within a functional colony.

7.6 Limitations and Further Work

The most obvious limitation of the work presented in this thesis, is that it is all based around a single study landscape, the North York Moors National Park. Although there are many advantages to the North York Moors as a landscape (see Chapter 1), the limitation to a single location limits generalisation of conclusions. The positive effects of non-native conifer plantation I have shown (Chapters 2, 3 and 5), certainly seem to be in effect in this landscape, however there are other areas, such as Kielder Forest, which exist as the result of massive increases in forest cover, but do not display the same massively positive story for wood ants as far as we are aware. It is possible that there is something specific about the landscape I have worked in that is causing such a positive response from wood ant populations. The work on social organisation (Chapter 6), may also be influenced by the fact that the populations show such recent expansion, and the same pattern may not be observed in a less disturbed ecosystem such as in the well-studied Finnish or Swiss populations of wood ants. To solve this problem with further work is equally obvious; replicate the studies over multiple different landscapes, preferably across Europe, to reduce confounding of site specific characteristics.

All of the studies here also involve a single study species, providing that the strong mitochondrial differentiation in Chapter 6 does not turn out to be a cryptic species. *Formica lugubris* was a carefully chosen study species for this work, which, as a forest specialist, short range disperser and keystone forest species, should be representative of a wide range of species characteristic of forest habitat. The short range dispersal and polydomous organisation are particularly essential for the social organisation work in Chapter 6. However, there are a huge number of other forest specialist species that we could have chosen and ideally multiple species would have been studied at the same time to ensure that conclusions generalise or gain a more holistic view of the situation. Due to logistic constraints assessing multiple species was not possible in this PhD, but can only improve our understanding if assessed elsewhere.

No matter the current wealth of data, more always seems to be needed to fully understand systems. Due to working in Forestry Commission land during this project we were able to use the sub-compartment database to create relevant habitat variables for use in habitat suitability modelling. However, even at the 10m resolution of variables we were able to deploy, we will have missed certain characteristics known to affect *F. lugubris.* For example *F. lugubris* is known to be affected by canopy cover, due to the importance of insolation in thermoregulation (Kadochová & Frouz 2014; Chen & Robinson 2014). I considered assessing the habitat preferences of *F. lugubris* at a finer scale than Chapters 2 and 3, but, after initial assessment, found that the data collection would have been prohibitively time consuming. Perhaps a method of assessing forest structure using remote sensing data such as LiDAR would be a method for collecting such data more efficiently.

A limitation of Chapter 6 was that we were not able to track trail networks within the population over multiple years to assess how long they have existed. To a certain extent we know from other work that trail networks are stable in the ways important to the study i.e. separate trails networks repeatedly mapped over multiple years have never been seen to merge (Ellis *et al.* in Review). A possible solution to this problem is potentially coming into existence at the moment. The trail networks assessed in variety of other studies (Ellis *et al.* 2014; Ellis & Robinson 2015a; b), have now been mapped for four years, if this repeated mapping can go on then sampling for genetic work can take place at some point in the future with information on the history of each nest, which may reduce some uncertainty present in Chapter 6.

Microsatellites were chosen as the method of assessing nuclear genetic variation for both Chapters 4 and 6, because they are highly variable and because they are neutral markers i.e. should not be under selection (Hamilton 2009). These markers allowed us to assay 630 ants, which is a considerable sample size, however with a large number of individuals comes a relatively small amount of data per individual. Relatively recently there have been examples of segments of the genome determining social organisation in ant species (Ross & Keller 1995; Purcell *et al.* 2014), therefore it would be interesting to see whether there were very specific sections of the genome that differed between polydomous colonies. The neutrality of the markers also was not necessarily an advantage when assessing the genetic effects of forest cover change. The populations are expanding into novel habitat, presumably with novel selective environments. The ability to detect genes under selection in the historically forested and novel forest areas would have been fascinating. However this would most likely have involved a trade-off, as sequencing of all 630 individuals would have been impractical. More detailed genetic work on these population would be a possible future step.

The mitochondrial sections we sequenced were not pre-planned. We assessed variation across these segments as an initial assessment of mitochondrial variability using only a few individuals

at first. When we stumbled across the highly divergent haplotypes shown in Chapters 4 and 5 we deployed these markers at a larger scale. However they are not the most extensively sequenced areas of the mitochondrial genome in related species (e.g. Goropashnaya *et al.* 2004, 2012), which did limit our ability to compare our findings with wider literature. We would recommend a good first step in further work would be to assess the haplotypes we display at the sections of mtDNA used in the most recent mitochondrial phylogeny (Goropashnaya *et al.* 2012). Ideally we would also have sequenced a larger section of the genome, because the patterns we show in Chapter 6 are only based on approximately 1 kilobase of DNA. Further sequence may shed further light on the patterns we show. I would have undertaken this work as a next step to improve the work in chapter 5, but did not have sufficient time or money to do so.

I am no longer sure whether it is *F. lugubris* that I have been working on for the last three years, thanks to the findings of Chapter 5. Further work is needed to assess whether it is. I would recommend a combined morphological and genetic investigation similar to the identification of *F. paralugubris* (Seifert 1996). However, due to the expense of genetic work, and the time and expertise required for the morphological studies, this is no small undertaking. Given the variation I show across the landscape, a large number of samples need to be assessed. If more work is to be done on these populations it is essential that we know what species it is, in order to give the results proper context. Furthermore if this is a novel species in the UK, then there is conservation imperative. There are few endemic species in the UK, let alone endemics that appear to be thriving due to anthropogenic land use change. It would a be a substantially more major conservation story if the expansions I have documented are in a species found nowhere else.

One notable omission in the current work that has been done on polydomous colony organisation is the inclusion of cuticular hydrocarbon studies. Nest-mate recognition in ants is mediated by cuticular hydrocarbon profiles (Martin & Drijfhout 2009), and in *F. exsecta* they have been shown to explain aggression far better than either spatial or genetic distance (Martin, Shemilt & Trontti 2014). If neighbouring nests, which show no genetic distinction, differ in their cuticular hydrocarbon profiles, then that may explain why we see no social connection. Assessment of cuticular hydrocarbons was beyond the scope of this project, but would be an interesting avenue to investigate. Studies utilising CHCs will need to take place in species where CHC recognition systems are well understood, such as *F. exsecta* (Martin & Drijfhout 2009), or spend some time determining this system for the species in question before meaningful results can be produced.

The studies in this thesis were observation and correlational. It would have been excellent to have an opportunity to deploy manipulative experiments. Obviously it would not be feasible in three years to plant new areas with coniferous plantation, and track the colonisation by forest

species, though that would be a fascinating study. However the polydomous colony organisation appears more amenable to manipulation. The studies I would have done next would be to see whether I could create connections between currently unconnected, but genetically related nests. I would have done this either by trying to stop the unconnected nest from foraging at all by greasing tree trunks, or by baiting the area between unconnected nests to force social connections. These projects may have been utterly impossible. Our group has tried baiting wood ant nests before, but they have such abundance of food available in the trees they essentially ignored the baits (S. Ellis pers. com.). Exclusion of wood ants from foraging has been done in the past (Fowler & Macgarvin 1985), but stopping a nest from foraging at all would require excluding that nest from all trees within approximately 50m. In plantation forest that is a lot of trees, which may make it impractical. However, manipulation is an obvious next step in the investigation of polydomous nesting.

7.8 Conclusion

In this thesis I have presented a rare example of anthropogenic land use change, and the creation of a novel and non-natural ecosystem, having huge positive effects on a native species. Thanks to the expansion of commercial forests, *F. lugubris* has expanded its range substantially. Furthermore, I have shown that this positive effect should continue into the future: there is a vast area of further habitat into which *F. lugubris* can expand. Our findings support a changing view of plantation forest in Britain; plantation forest can make a valuable contribution to forest diversity.

The wood ant populations I have studied are both demographically and genetically healthy, and in no danger of extinction. However there is some danger of division, due to possible taxonomic rearrangement, but this in itself could be another positive story. I have also shown that cooperative connections between nests are not explained by genetic distinctions. Our evidence suggests that ecology may be more important in dividing polydomous species than genetics is, contrary to popular thought.

Appendices

Appendix 1: Materials for Chapter 2

Table A1.1 Reasons for inclusion of each variable within the habitat suitability model

Variable	**Reason used**
Distance to forest cover in 1854	Colonists of a new habitat have to disperse from a source. Sections of historic forest are the closest potential sources from which wood ants could spread. Therefore any effect of dispersal ability on current nest locations should be revealed by this variable
Hillshade	This variable combines aspect and topography to give a measure of how light that part of the landscape is. As insolation is known to be important to wood ants for thermoregulation, this could have a potential effect
Slope	It is unlikely to be as easy to maintain substantial nests on steep sided slopes as on flat ground; including slope will account for this
Primary tree genus	Any difference in the suitability of tree genera will be reflected by this variable. If non-native conifers are unsuitable habitat they should display lower probability of occupancy
Zonal statistics within 50m	Ants forage extensively within 50m of their nest, therefore characteristics within those 50m may affect the ants more than at the specific point over the nest.
Age classes	This variable should reveal whether there is specific age class within the forestry cycle wood ants have a preference for. As a percentage within 50m, this also measures the level of homogeneity of the local area, which may affect the probability of nest formation.
Openness	*Formica lugubris* is an edge specialist and so may have a preference for large amounts of open ground within 50m of its nest.
Percentage of broadleaves	Broadleaves are more characteristic of the historic areas wood ants have survived in; they may prefer a certain level of broadleaf cover to spread.
Percentage of conifers	Conifers are characteristic of plantation woodland; the relationship between probability of occupancy of wood ants and percentage of conifers within 50m should be a strong reflector of the affinity of wood ants for plantation woodland, if there is little signal within genera

	Percentages within 50m										
Variable	Age under 20	Age 20-30	Age 31-80	Age over 80	Openness	Percentage broadleaves	Percentage conifers	Distance to forest cover in 1854	Hillshade	Slope	Primary tree genus
Age under 20	-	-0.112	-0.321	-0.133	-0.242	-0.069	0.365	0.070	0.001	-0.080	-0.194
Age 20-30		-	-0.198	-0.006	-0.144	0.062	0.131	0.189	0.002	-0.085	-0.119
Age 31-80			-	-0.127	-0.366	0.227	0.404	-0.218	0.014	0.170	-0.382
Age over 80				-	-0.120	0.231	-0.002	0.011	0.011	0.071	-0.130
Openness					-	-0.182	-0.535	-0.184	-0.074	-0.031	0.457
Percentage broadleaves						-	-0.390	-0.324	-0.141	0.295	-0.628
Percentage conifers							-	0.171	0.114	-0.114	-0.205
Distance to forest cover in 1854								-	0.021	-0.302	0.195
Hillshade									-	-0.121	0.023
Slope										-	-0.207
Tree genus											-

Table A1.2. Correlations between spatial variables

Table A1.3. Performance of models with variations in regularisation multiplier and feature types. Hinge features are combinations of lines with a slope of 0 up to a point and then non-zero, in effect allowing a linear relationship to begin part way through the full range of data value.

Regularisation multiplier	Features	AIC
1	All	38213.0
1	Hinge	38446.2
1	Linear	39562.8
1	Linear + Quadratic	38942.1
1	Linear, Quadratic + Hinge	38428.5
5	All	38395.2
5	Hinge	38653.2
5	Linear	39596.4
5	Linear + Quadratic	38969.6
5	Linear, Quadratic + Hinge	38555.0
10	All	38543.6
10	Hinge	38856.8
10	Linear	39665.0
10	Linear + Quadratic	39052.2
10	Linear, Quadratic + Hinge	38703.8
20	All	38782.3
20	Hinge	39194.3
20	Linear	39761.2
20	Linear + Quadratic	39245.6
20	Linear, Quadratic + Hinge	38921.4

Table A1.4. All variations of variable removal from the full model and their relating AIC scores

Model			AIC
Full model			38213.0
Variable removed	Mean percentages per 50m	Age under 20	38382.9
		Age 20-30	38244.6
		Age 31-80	38257.7
		Age over 80	38263.3
		Openness	38444.8
		Percentage Broadleaves	38233.0
		Percentage conifers	38247.9
	Distance to forest in 1854		38988.7
	Hillshade		38256.2
	Slope		38299.0
	Primary tree genus		38238.0

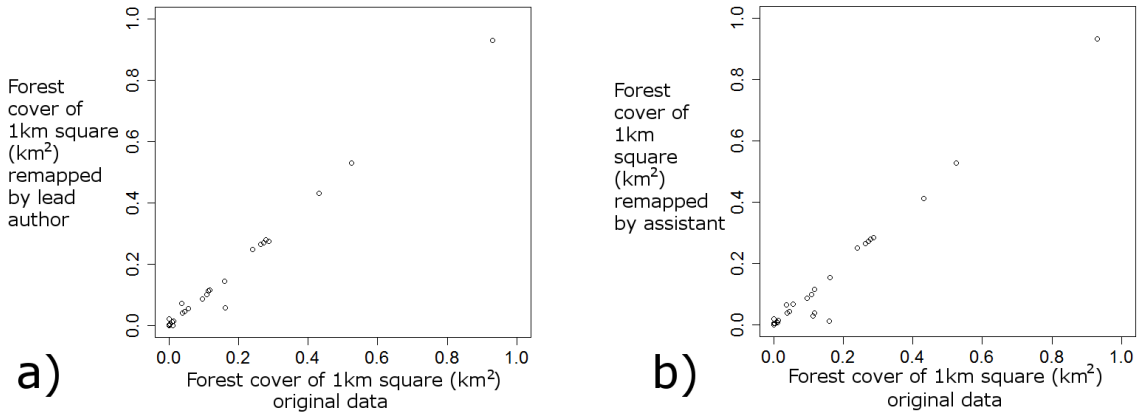

Figure A1.1. Repeatability of forest cover data with comparisons between original data and **a)** remapping by first author and **b)** original data and repeated by an assistant

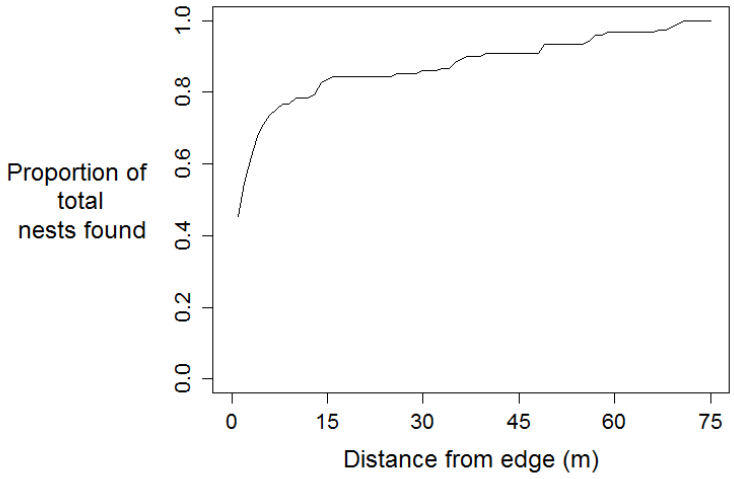

Figure A1.2. The relationship between the distance from the edge of a conifer plantation and the proportion of total nests found with 75m transects

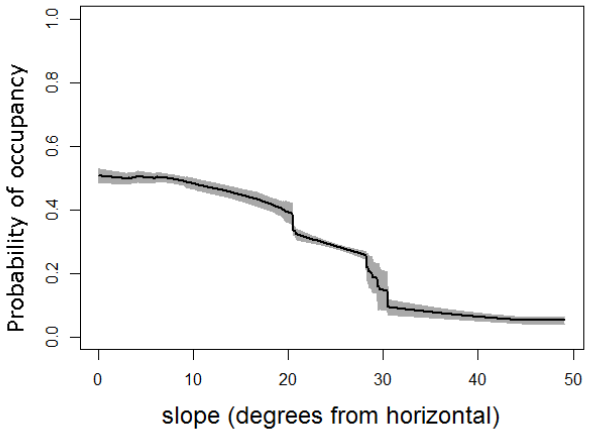

Figure A1.3. The predicted model relationship between probability of occupancy of *F. lugubris* and slope. The black line and grey polygon are the mean and standard deviation of 5 models respectively

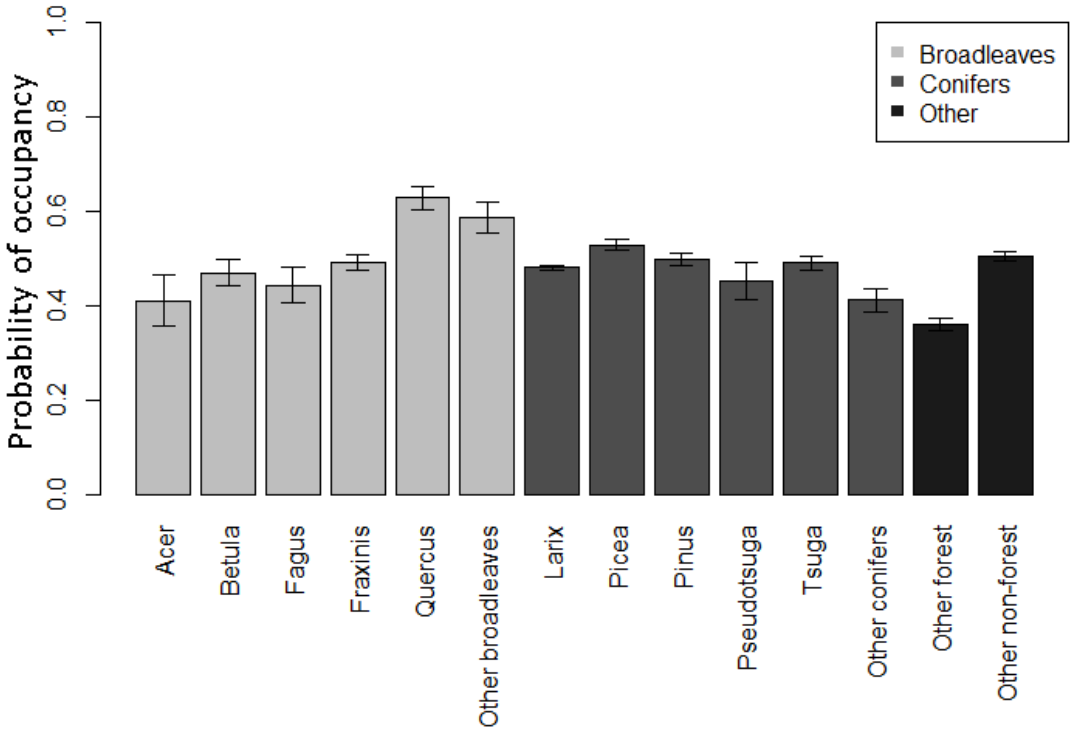

Figure A1.4. The predicted model relationship between primary tree genus and probability of occupancy of *F. lugubris*, bars and error bars are means and standard deviations of 5 model runs respectively

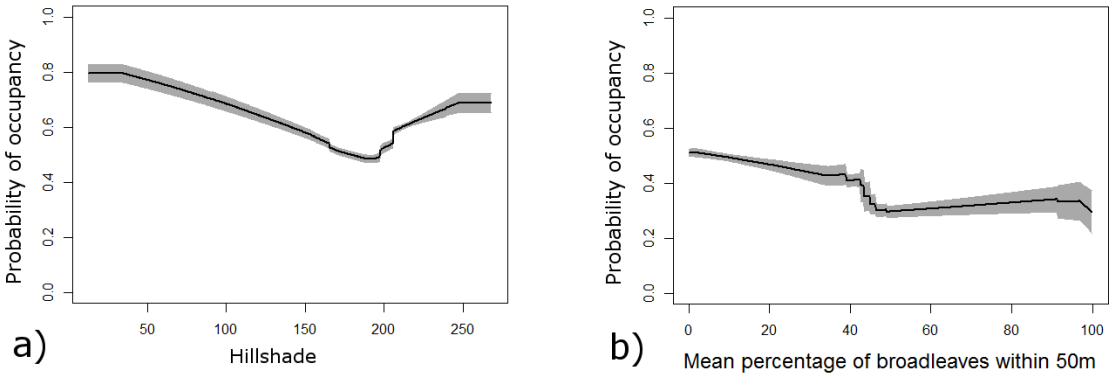

Figure A1.5. The probability of occupancy of *F. lugubris* in relation to **a)** Hillshade (a measure of the shadedness of the landscape) and **b)** mean percentage of broadleaves within 50m. The black lines and grey polygons are the mean and standard deviation of 5 models respectively

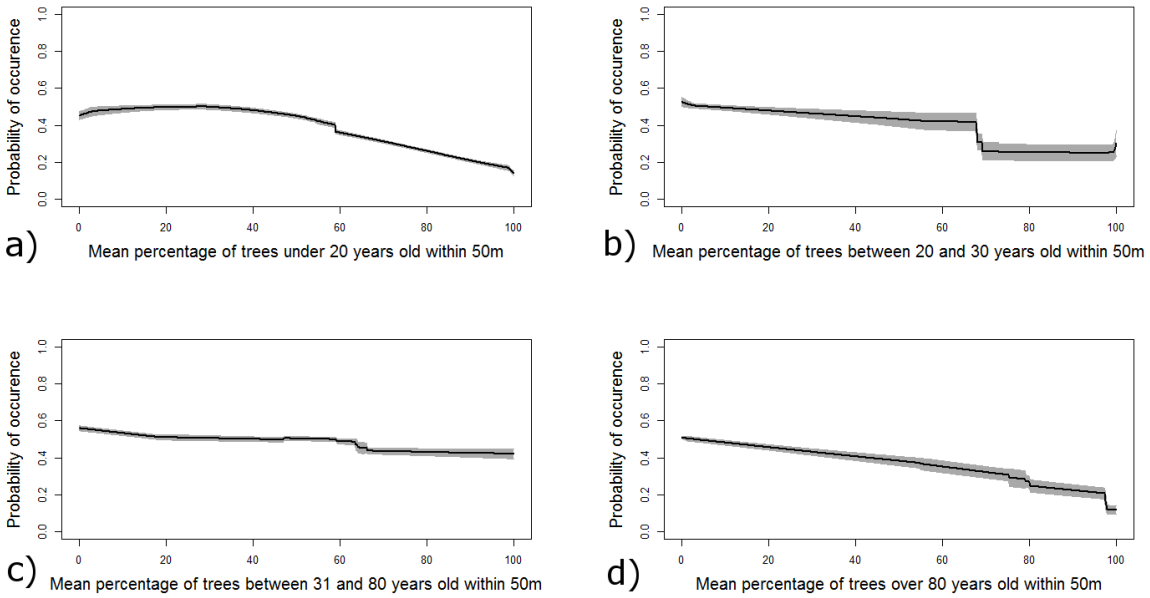

Figure A1.6. The relationships between the probability of occupancy of *F. lugubris* and the percentage of **a)** trees under 20 years old within 50m, **b)** trees 20-30 years old within 50m, **c)** trees 31-80 years old within 50m, **d)** trees over 80 years old within 50m. The black lines and grey polygons are the mean and standard deviation of 5 models respectively

Appendix 2: Supplementary materials for Chapter 3

Table A2.1. Correlations between the different spatial scales of each variable where spatial scale was varied

Variable	Percentage of trees under 20 years old											
Percentage of trees under 20 years old	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.03	0.03	0.04	0.04	0.05	0.06	0.06	0.06	0.07	0.07
	50m		1.00	0.97	0.92	0.86	0.81	0.76	0.71	0.67	0.64	0.61
	100 m			1.00	0.98	0.94	0.89	0.84	0.79	0.75	0.71	0.68
	150 m				1.00	0.98	0.95	0.91	0.86	0.82	0.78	0.75
	200 m					1.00	0.99	0.96	0.92	0.88	0.84	0.81
	250 m						1.00	0.99	0.96	0.93	0.90	0.87
	300 m							1.00	0.99	0.97	0.94	0.91
	350 m								1.00	0.99	0.97	0.95
	400 m									1.00	0.99	0.98
	450 m										1.00	0.99
	500 m											1.00

Variable	Percentage of trees 20-30 years old											
Percentage of trees 20-30 years old	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.04	0.05	0.06	0.06	0.07	0.08	0.08	0.09	0.10	0.10
	50m		1.00	0.97	0.92	0.86	0.81	0.75	0.70	0.66	0.62	0.58
	100 m			1.00	0.98	0.94	0.89	0.83	0.78	0.73	0.69	0.65
	150 m				1.00	0.98	0.95	0.90	0.85	0.80	0.76	0.72
	200 m					1.00	0.99	0.95	0.91	0.87	0.83	0.79
	250 m						1.00	0.99	0.96	0.92	0.89	0.85
	300 m							1.00	0.99	0.97	0.93	0.90
	350 m								1.00	0.99	0.97	0.94
	400 m									1.00	0.99	0.98
	450 m										1.00	0.99
	500 m											1.00

Variable	Percentage of trees 31-80 years old											
Percentage of trees 31-80 years old	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.07	0.07
	50m		1.00	0.98	0.94	0.90	0.86	0.83	0.80	0.77	0.75	0.72
	100 m			1.00	0.98	0.96	0.92	0.89	0.86	0.83	0.81	0.78

	150 m				1.00	0.99	0.97	0.94	0.91	0.88	0.86	0.84
	200 m					1.00	0.99	0.97	0.95	0.93	0.90	0.88
	250 m						1.00	0.99	0.98	0.96	0.94	0.92
	300 m							1.00	0.99	0.98	0.97	0.95
	350 m								1.00	1.00	0.99	0.97
	400 m									1.00	1.00	0.99
	450 m										1.00	1.00
	500 m											1.00

Variable	**Percentage of trees over 80 years old**											
Percentage of trees over 80 years old	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.02	0.03	0.03	0.04	0.05	0.05	0.05	0.06	0.06	0.06
	50m		1.00	0.95	0.88	0.80	0.74	0.68	0.63	0.59	0.56	0.53
	100 m			1.00	0.97	0.91	0.84	0.78	0.73	0.69	0.65	0.62
	150 m				1.00	0.98	0.93	0.87	0.82	0.78	0.74	0.71
	200 m					1.00	0.98	0.94	0.90	0.85	0.81	0.78
	250 m						1.00	0.99	0.95	0.92	0.88	0.85
	300 m							1.00	0.99	0.96	0.93	0.90
	350 m								1.00	0.99	0.97	0.94
	400 m									1.00	0.99	0.98
	450 m										1.00	0.99
	500 m											1.00

Variable	**Mean Hillshade**											
Mean Hillshade	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	-0.02	-0.02	-0.02	-0.02	-0.03	-0.03	-0.03	-0.03	-0.04	-0.04
	50m		1.00	0.96	0.89	0.83	0.78	0.72	0.68	0.64	0.60	0.57
	100 m			1.00	0.97	0.92	0.87	0.82	0.77	0.72	0.68	0.65
	150 m				1.00	0.98	0.94	0.89	0.85	0.80	0.76	0.72
	200 m					1.00	0.98	0.95	0.91	0.87	0.82	0.78
	250 m						1.00	0.99	0.96	0.92	0.88	0.84
	300 m							1.00	0.99	0.96	0.93	0.90
	350 m								1.00	0.99	0.97	0.94
	400 m									1.00	0.99	0.97

	450 m										1.00	0.99
	500 m											1.00

Variable	Percentage of open ground											
Percentage of open ground	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.08	0.09	0.09	0.10	0.10	0.10	0.11	0.11	0.11	0.11
	50m		1.00	0.98	0.95	0.92	0.89	0.87	0.85	0.83	0.82	0.81
	100 m			1.00	0.99	0.97	0.94	0.92	0.90	0.89	0.87	0.86
	150 m				1.00	0.99	0.98	0.96	0.94	0.93	0.91	0.90
	200 m					1.00	0.99	0.98	0.97	0.95	0.94	0.93
	250 m						1.00	1.00	0.99	0.98	0.96	0.95
	300 m							1.00	1.00	0.99	0.98	0.97
	350 m								1.00	1.00	0.99	0.98
	400 m									1.00	1.00	0.99
	450 m										1.00	1.00
	500 m											1.00

Variable	Percentage of broadleaved trees											
Percentage of broadleaved trees	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	50m		1.00	0.95	0.87	0.79	0.73	0.68	0.64	0.61	0.58	0.56
	100 m			1.00	0.97	0.91	0.84	0.79	0.74	0.71	0.68	0.65
	150 m				1.00	0.98	0.93	0.88	0.83	0.80	0.76	0.73
	200 m					1.00	0.98	0.95	0.91	0.87	0.84	0.81
	250 m						1.00	0.99	0.96	0.93	0.89	0.86
	300 m							1.00	0.99	0.97	0.94	0.91
	350 m								1.00	0.99	0.97	0.95
	400 m									1.00	0.99	0.98
	450 m										1.00	0.99
	500 m											1.00

Variable	Percentage of coniferous trees											
Percentage of coniferous trees	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.10	0.10	0.11	0.11	0.12	0.12	0.12	0.12	0.12	0.12
	50m		1.00	0.98	0.95	0.92	0.90	0.87	0.85	0.84	0.82	0.81

	100 m			1.00	0.99	0.97	0.95	0.92	0.90	0.89	0.87	0.86
	150 m				1.00	0.99	0.98	0.96	0.94	0.93	0.91	0.90
	200 m					1.00	0.99	0.98	0.97	0.95	0.94	0.93
	250 m						1.00	1.00	0.99	0.98	0.96	0.95
	300 m							1.00	1.00	0.99	0.98	0.97
	350 m								1.00	1.00	0.99	0.98
	400 m									1.00	1.00	0.99
	450 m										1.00	1.00
	500 m											1.00

Variable	**Mean slope (degrees from horizontal)**											
Mean slope (degrees from horizontal)	Scale	10 m	50 m	100 m	150 m	200 m	250 m	300 m	350 m	400 m	450 m	500 m
	10m	1.00	0.00	0.00	-0.01	-0.01	-0.01	-0.01	-0.01	-0.01	-0.01	-0.01
	50m		1.00	0.96	0.90	0.85	0.80	0.77	0.73	0.71	0.68	0.66
	100 m			1.00	0.98	0.93	0.89	0.85	0.82	0.79	0.76	0.74
	150 m				1.00	0.98	0.95	0.92	0.89	0.86	0.83	0.81
	200 m					1.00	0.99	0.96	0.94	0.91	0.88	0.86
	250 m						1.00	0.99	0.97	0.95	0.93	0.90
	300 m							1.00	0.99	0.98	0.96	0.94
	350 m								1.00	0.99	0.98	0.96
	400 m									1.00	1.00	0.98
	450 m										1.00	1.00
	500 m											1.00

Table A2.2. All variables with a correlation coefficient of a greater magnitude than 0.7 and their corresponding univariate model AUC avlues

Variable 1	**AUC 1**	**Variable 2**	**AUC 2**	**Correlation**
Percentage conifers within 100m	0.67	Percentage open ground within 200m	0.64	-0.84
Percentage conifers within 100m	0.67	Percentage of trees 31-80 years old within 200m	0.62	0.7
Percentage conifers within 10m	0.54	Percentage open ground within 10m	0.58	-0.76

Table A2.3. Variations in regularisation parameter and their corresponding performance in terms of AIC

Regularisation parameter	AIC score
1	11976.21
2	11982.13
5	12025.05
10	12086.49
20	12160.69

Table A2.4. Multivariate model selection using AIC

Model selection level 1	AIC score
Full	11995.0
No percentage of trees 20-30 years old within 200m	11991.6
No percentage of trees over 80 years old within 200m	11997.3
No percentage of trees under 20 years old within 200m	11983.7
No distance to forest cover in 1854	12054.0
No mean hillshade within 450m	12010.8
No percentage broadleaves within 200m	11972.5
No percentage of conifers within 100m	12041.1
No mean slope within 200m	11979.3
No primary tree genus	11990.1
No percentage of trees 20-30 years old within 10m	11981.5
No percentage of trees 31-80 years old within 10m	11972.1
No percentage of trees over 80 years old within 10m	11993.5
No percentage of trees under 20 years old within 10m	11971.7
No hillshade (10m)	11989.9
No percentage broadleaves within 10m	11975.5
No percentage open ground within 10m	12017.7
No slope (10m)	12004.5

Model selection level 2	AIC score
Full	11978.2
No percentage of trees 20-30 years old within 200m	11991.9
No percentage of trees over 80 years old within 200m	11999.2
No percentage of trees under 20 years old within 200m	11961.4
No distance to forest cover in 1854	12053.4
No mean hillshade within 450m	11992.9
No percentage broadleaves within 200m	11980.8
No percentage of conifers within 100m	12055.0
No mean slope within 200m	11989.2
No primary tree genus	11983.1
No percentage of trees 20-30 years old within 10m	11977.8
No percentage of trees 31-80 years old within 10m	11975.2

No percentage of trees over 80 years old within 10m	11977.3
No hillshade (10m)	11976.7
No percentage broadleaves within 10m	11973.4
No percentage open ground within 10m	12005.4
No slope (10m)	12021.9

Model selection level 3	**AIC score**
Full	11984.6
No percentage of trees 20-30 years old within 200m	11988.5
No percentage of trees over 80 years old within 200m	12003.5
No distance to forest cover in 1854	12051.4
No mean hillshade within 450m	11988.0
No percentage broadleaves within 200m	11973.1
No percentage of conifers within 100m	12049.4
No mean slope within 200m	11974.1
No primary tree genus	11977.1
No percentage of trees 20-30 years old within 10m	11984.3
No percentage of trees 31-80 years old within 10m	11981.8
No percentage of trees over 80 years old within 10m	11964.1
No hillshade (10m)	11993.4
No percentage broadleaves within 10m	11979.1
No percentage open ground within 10m	12008.3
No slope (10m)	12011.1

Model selection level 4	**AIC score**
Full	11986.6
No percentage of trees 20-30 years old within 200m	11985.2
No percentage of trees over 80 years old within 200m	11988.1
No distance to forest cover in 1854	12063.9
No mean hillshade within 450m	11990.0
No percentage broadleaves within 200m	11964.7
No percentage of conifers within 100m	12025.7
No mean slope within 200m	11977.6
No primary tree genus	11981.9
No percentage of trees 20-30 years old within 10m	11965.1
No percentage of trees 31-80 years old within 10m	11956.9
No hillshade (10m)	11977.2
No percentage broadleaves within 10m	11991.7
No percentage open ground within 10m	11990.2
No slope (10m)	12006.2

Model selection level 5	AIC score
Full	11954.1
No percentage of trees 20-30 years old within 200m	11989.8
No percentage of trees over 80 years old within 200m	11983.6
No distance to forest cover in 1854	12039.9
No mean hillshade within 450m	11990.1
No percentage broadleaves within 200m	11960.9
No percentage of conifers within 100m	12035.2
No mean slope within 200m	11977.2
No primary tree genus	11961.6
No percentage of trees 20-30 years old within 10m	11976.4
No hillshade (10m)	11978.5
No percentage broadleaves within 10m	11968.3
No percentage open ground within 10m	12005.2
No slope (10m)	12005.1

Table A2.5. The genera that make up the 'other conifers' and 'other broadleaves' variables. Each of the genera grouped into these variables contain less than five sub compartments within the study landscape.

Other Broadleaves	Other Conifers
Alnus	*Abies*
Salix	*Chamaecyparis*
Aesculus	*Thuja*
Populus	Mixed conifers
Nothofagus	Other conifers
Sorbus	-
Castanea	-
Prunus	-
Other Broadleaves	-
Mixed Broadleaves	-

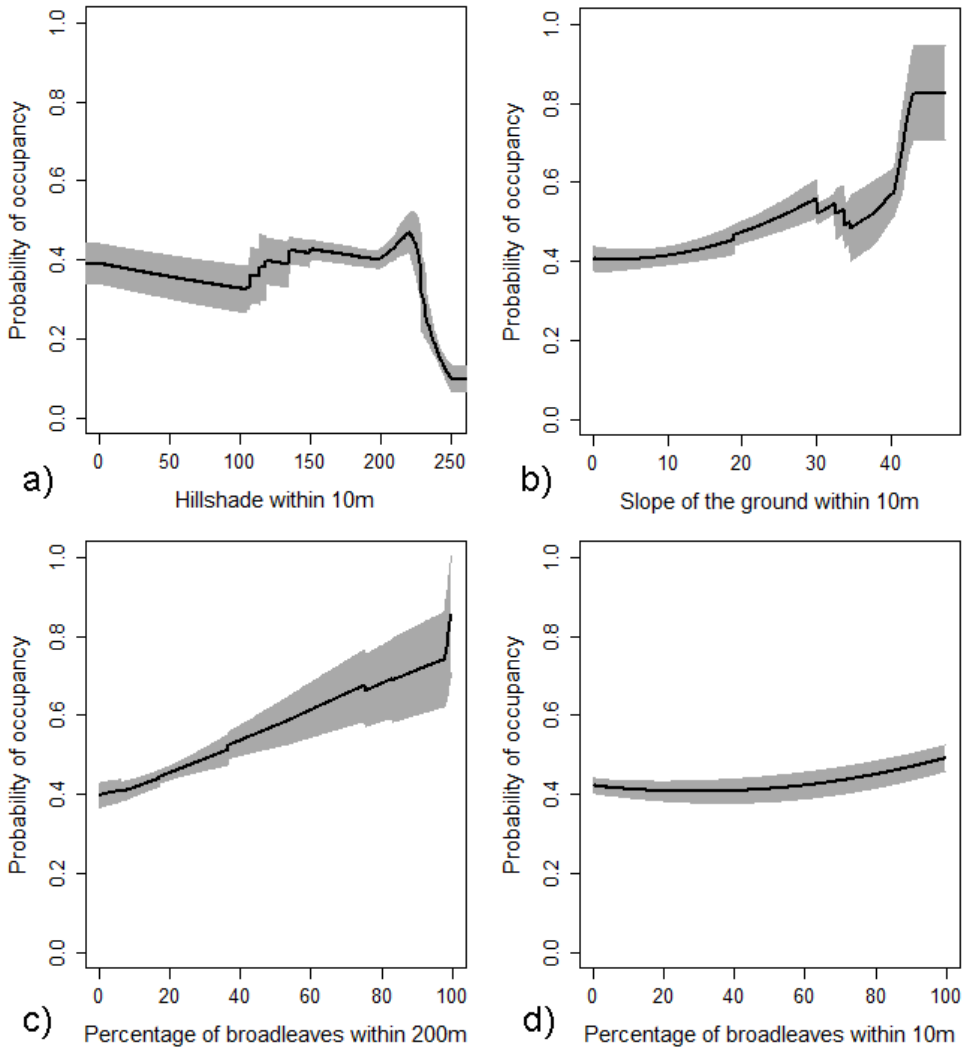

Figure A2.1. The relationships between the probability of occupancy of *F. lugubris* and the four least important variables to the model: **a)** hillshade within 10m, **b)** slope of the ground within 10m, **c)** percentage of broadleaves within 200m and **d)** percentage of broadleaves within 10m

Appendix 3. Figure A3.1

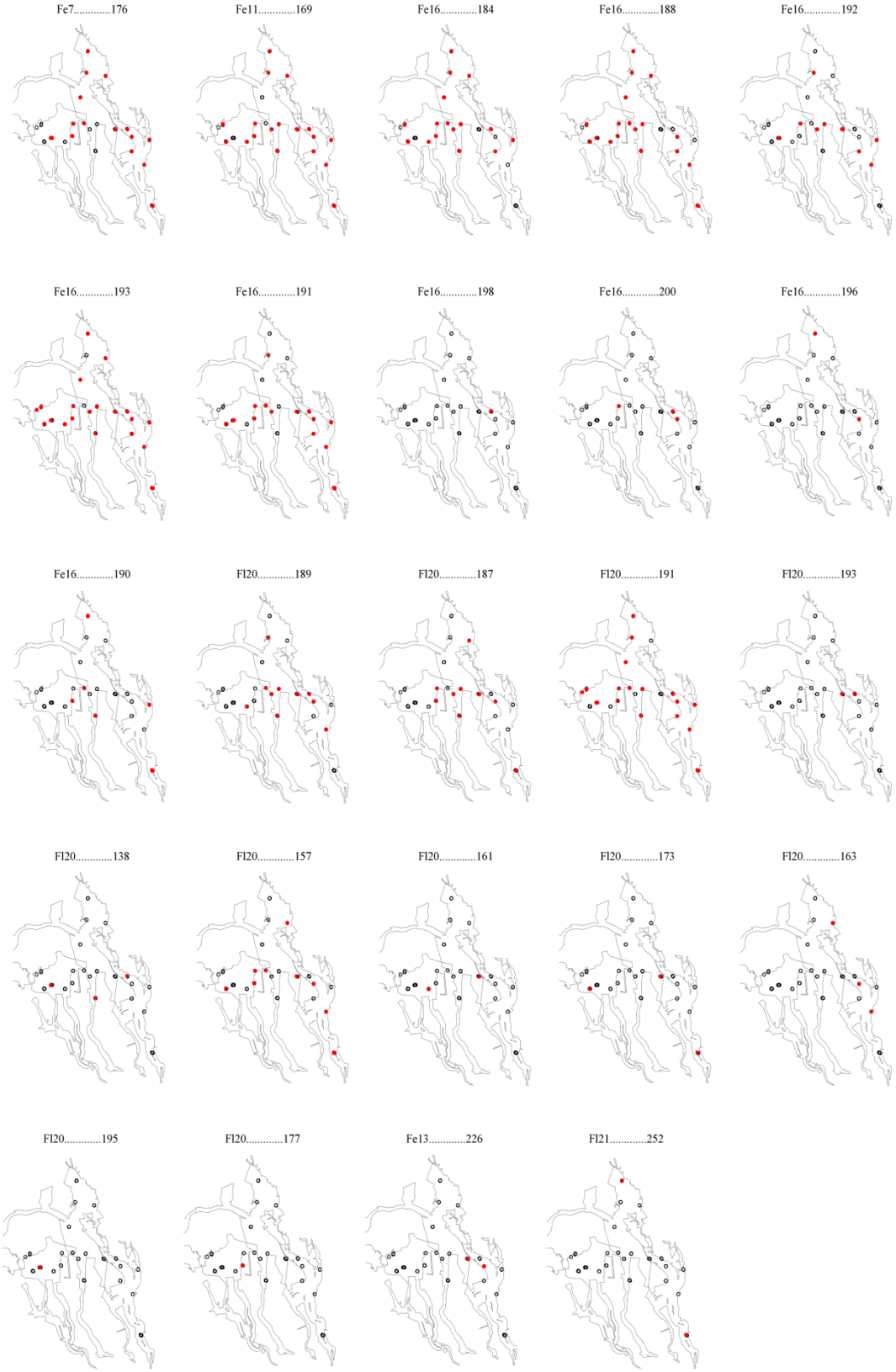

Figure A3.1. The distributions of all alleles present in less than 5% of data, whose spatial distribution did not support the SPCA groupings, or who showed too low abundance to be predictive. Red points show the presence of the allele, hollow points are absence.

Appendix 4: Supplementary materials for Chapter 6

The effect of nest size

Hypotheticaly, nest size could be a predictor of trail presence, if, for example, nests under a certain under a certain size nests cannot maintain trails if nests over a certain size there have no need for them. We found no evidence of this. Mound volume, which correlates with worker population (Chen & Robinson 2013), did not differ between the base, connected and unconnected nests (LMM, df=2, χ=0.95, P=0.62). There was no significant difference between the minimum volume of connected and unconnected nest pairs (LMM, df=1,3, χ=1.00,P=0.32). There was also no significant difference in the maximum nest volume of connected or unconnected nest pairs (LMM, df=1,3, χ=0.08, P=0.78). There was no significant variation in size difference between nests within the connected or unconnected nest pair (LMM,df=1,3, χ=0.007,P=0.93). There was also no significant difference in the combined volume of the connected nest pair and the unconnected nest pair (LMM, df=1,3, χ=1.61,P=0.21)

Table A4.1. Distances between different nest pairs within the triplet set up. For an explanation of the nest terminology see Fig. 6.1

	Distance between nests (m)		
Triplet	**Base-Connected**	**Base-Unconnected**	**Connected-Unconnected**
1	13.62	11.34	14.02
2	2.46	29.21	31.66
3	2.95	7.71	7.15
4	18.58	29.5	47.83
5	3.56	5.05	4.99
6	26.62	35.2	57.34
7	3.3	9.96	22.85
8	6.37	7.8	13.41
9	7.63	14.47	16.02
10	2.26	11.93	14.09
11	4.23	8.33	12.5
12	7.44	8.12	15.45
13	26.92	32.32	59
14	4.3	13.9	10.82
15	3.85	19.63	16.76
16	2.72	5.68	8.37
17	1.32	10.76	11.89
18	13.94	26.42	26.98
19	22.97	17.88	28.98
20	3.16	11.77	14.15
Mean	8.9	15.8	21.7

Aggression bioassays

We conducted preliminary aggression bioassays to assess whether they would be a useful tool for differentiating between cooperating nests and those that are in competition. We collected approximately 100 ants from each of five nests, three from one of the experimental triplets used in this study, one nest approximately 4km away from within the same continuous population and one approximately 15km away from a population separated from the study population by over 10km of wood ant-free habitat. Ants were taken from the field and housed with their nest-mates for 48 hours in the laboratory with ad libitum sucrose solution and protein.

To perform the assays we took a single ant from the base nest (see Fig. 6.1) and placed it in a Petri dish with an ant from one of the five experimental nests. Tests were blinded by an independent assistant so the observer had no knowledge of where the second ant was from. Ants were allowed to acclimatise for one minute and then were observed for the subsequent five minutes. Interactions were scored on the following 0-3 scale:

0 - Ignore - Physical contact made but no antennation or aggression
1 - Touch - Antennation - Contact between antennae and other ant
2 - Avoid - Ant approaches and either upon contact or just before physical contact retreats from other ant
3 - Aggression - Biting, lunging with flared mandibles, positioning of abdomen ready to fire formic acid

A composite aggression score was then calculated as the mean score of all interactions. This was repeated until single ants from the base nest had been assayed with 10 of each of the five experimental nests. Each ant was used in a maximum of one trial. There was no difference in the composite aggression score between the different treatments (Kruskal-Wallis, df=4, X=2.97, P=0.56, Fig. A4.1). There was also no significant difference between antennation durations between treatments (Kruskal-Wallis, df=4, X=8.24, *P*=0.08, Fig. A4.2). Aggression bioassays were therefore not deemed to be a useful tool for this study

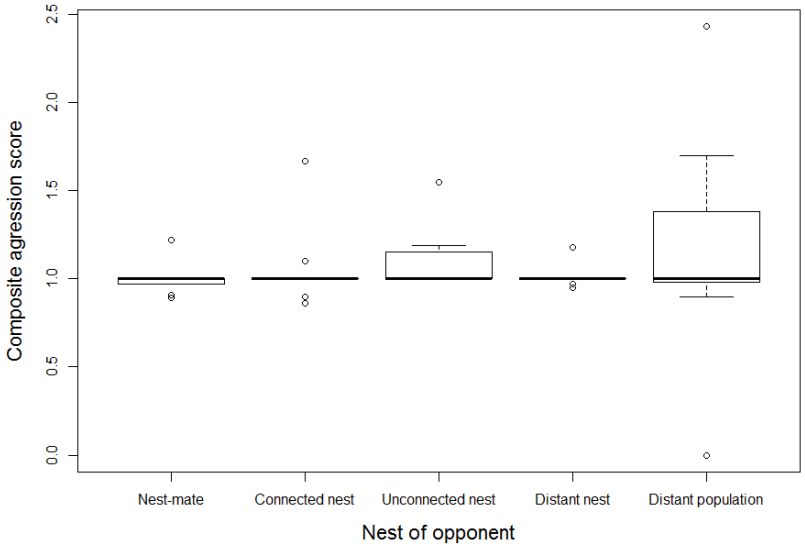

Figure A4.1. The composite aggression score for 1vs 1 interactions between ants of the base nest (Fig. 6.1) and opponents from five different nests.

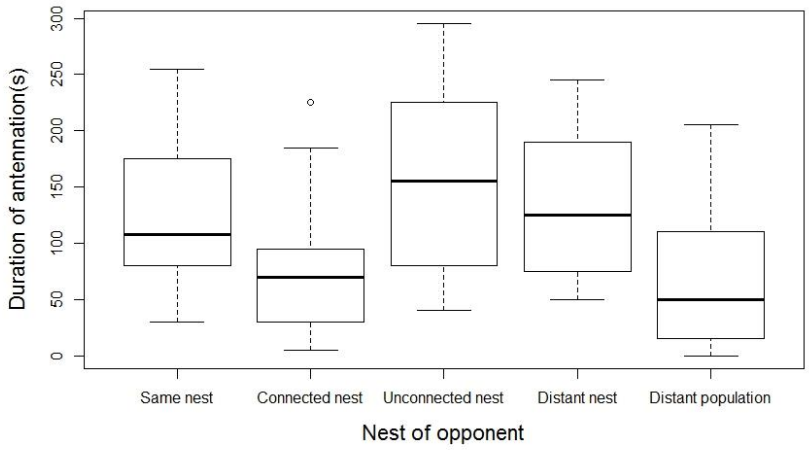

Fig. A4.2. The duration of antennations during 1vs 1 interactions between ants of the base nest and opponents from five different nests.

The effect of forest age

The forests within this population were planted at different time points, which might be expected to affect the inter-nest relatedness patterns we see. In the main analysis as presented in the paper, we did not take into account the age of forest and we concluded that there was no difference in inter-nest relatedness between connected and unconnected nest pairs (GLMM, df=1,3, X=0.12, P=0.73) If we include a variable separating triplets in ancient woodland

(continuous since at least 1600) from the rest of the triplets in the models we still recover the same pattern in inter-nest relatedness: connected nest pairs do not show a significant difference in relatedness (GLMM, df=1,4, X=0.12, P=0.73). The age of forest when split into these two categories also has no significant effect on inter-nest genetic relatedness (GLMM, df=1,4, X<0.001, P=0.98, Fig. A4.3). If we incorporate a variable separating the triplets into three age classes (ancient woodland, forested since at least 1854, and forested more recently than 1854) then there is a significant effect of forest age on inter-nest genetic relatedness (GLMM, df=1,4, X=9.12, P=0.01, Fig. A4.4), however there is still no significant difference inter-nest genetic relatedness between connected and unconnected nest pairs (GLMM, df=1,4, X=0.05, P=0.82). Therefore inter-nest genetic relatedness does vary with the age of forest in which the ants are located, but the pattern of inter-nest genetic relatedness between connected and unconnected nest pairs is unaffected. Further investigation of the effects of forest age on genetic patterns is beyond the scope of this paper.

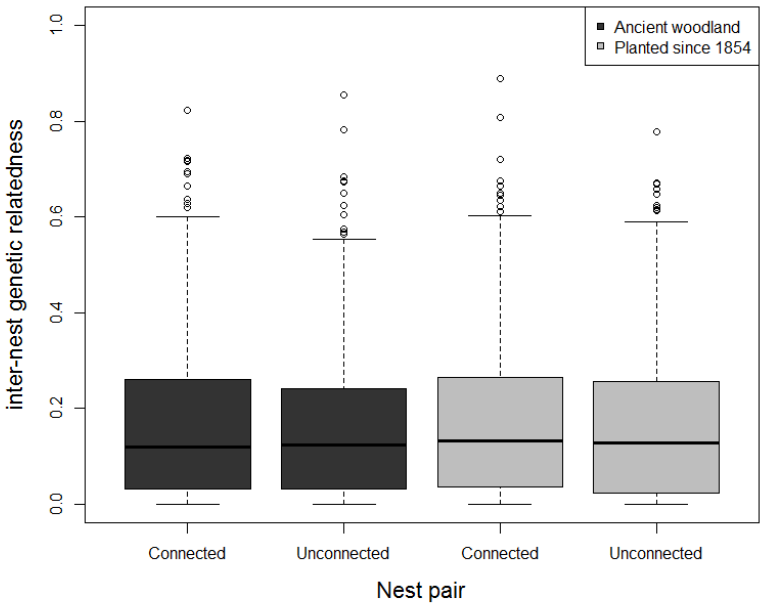

Figure A4.3. Inter-nest genetic relatedness between connected and unconnected nest pairs (B-U), separated into two woodland categories: ancient woodland and woodland planted since 1854. Dark bars are ancient woodland and light bars planted since 1854.

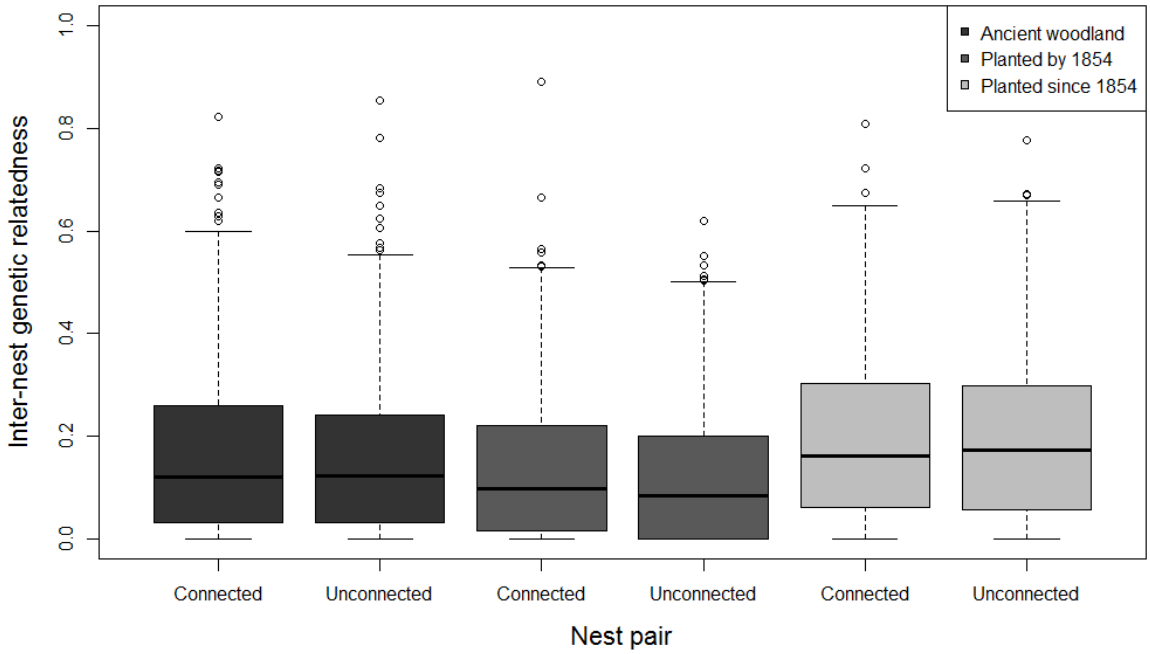

Figure A4.4. Inter-nest genetic relatedness between connected and unconnected nest pairs separately for ancient forest (continuously present since before 1600, dark bars), forest planted by 1854 (mid-grey bars), and forest planted since 1854 (light grey).

Pedersen and Boomsma's measures of genetic delineation of ant colony boundaries

Pedersen and Boomsma (1999) developed three methods for the delineation of ant colonies using genetic data. To provide additional support to the methods we have used in our main text we applied these three further method, namely: G-distance, Neighbour relatedness and Rare genotype sisterhoods. Application of these methods does not change the conclusions of our study

G-distance is a derivative of standard G-statistics (Sokal & Rohlf 1981) designed specifically for colony delineation (Pedersen & Boomsma 1999). It produces a value which is a measure of the heterogeneity of workers sampled from different nests. It is unclear precisely what magnitude of G-distance should be considered sufficient to conclude that nests are not part of the same colony, so values are comparative. Lower values for one nest pair compared to another suggests lower genetic distance and therefore a greater likelihood of being within the same colony. We would expect to find that the connected nest pairs have lower G-distance values than those of unconnected nest pairs if connected nests are genetically more similar.

Neighbour relatedness takes the relatedness of a pair of nests and then compares this to their within-nest relatedness. An output value is then given, which, if greater than 0, suggests the nests should be considered to belong to the same colony and, if lower than 0, should not be considered to belong to the same colony (Pedersen & Boomsma 1999). We would expect that connected nest pairs show more positive values and unconnected nest pairs more negative values if trails denote genetic units.

The high degree of genetic variation within ant nests and strong genetic isolation by distance within many ant populations can mean that it is more useful to look at rare alleles rather than across all available alleles. Rare-genotype-sisterhoods are genotypes that are shared between neighbouring nests, and are sufficiently rare in the population that they are unlikely to be shared by chance (Pedersen & Boomsma 1999). Instead, two neighbouring nests sharing rare genotypes are likely to have descended from a common ancestor and consequently may be more likely to be members of the same colony. We would expect that more connected nest pairs show rare-genotype-sisterhoods than unconnected nest pairs if they are genetically distinct colonies.

G-distance was determined using the hierfstat package of R (Goudet 2005). Neighbour relatedness and rare genotype sisterhoods were calculated according to the methods of Pedersen and Boomsma (1999) in R (R Core Team 2015).

Neighbour relatedness, G-distance and rare-genotyope sisterhoods are designed to be interpreted together (Pedersen & Boomsma 1999). Neighbour relatedness was greater than 0 in 5/20 connected nest pairs, and 6/20 unconnected nest pairs. Those nest pairs with values greater than 0 are suggested to be members of the same genetic colony (Table A4.2). When comparing G-distance values for connected nest pairs with those of unconnected nest pairs belonging to the same triplet, connected nests had lower G-distance values in 12/20 triplets and unconnected nest pairs were lower in 8/20 triplets (Table A4.2). Mean G-distance values for connected nest pairs were not significantly lower than mean G-distance values for unconnected pairs (t test, t=-0.3, df=37, P=0.77). Connected nest pairs shared rare genotype sisterhoods in 14/20 triplets and unconnected nest pairs in 9/20 triplets (Table A4.2). There were more total rare-genotype-sisterhoods found in connected than unconnected nest pairs (31 vs 23). None of the three methods presented here support the hyoothesis that connected nest pairs are more likely to be members of the same colony than unconnected nest pairs. These three methods therefore reinforce our conclusions reported in the meain text, based on inter-nest relatedness and hierarchical F-statistics.

Table A4.2. The results of the three ant-specific colony delineation methods, neighbour relatedness, G-distance and rare-genotype sisterhoods. The far right columns present a summary of the overall conclusions from all methods with one tick per method that suggests connected (C) or unconnected (U) nest pairs are part of the same genetic colony and '–'where a method does not suggest these should be considered within the same colony. Neighbour relatedness and rare genotype sisterhoods may support the connected or unconnected nest pair or both. For G-statistics the pair (connected or unconnected) from each triplet with the higher value is taken as more likely to be within the same colony. As a result there is one tick possible per method per column.

	Neighbour relatedness		G-statistics		Number of rare genotype sisterhoods		All	
Triplet	**Connected**	**Unconnected**	**Connected**	**Unconnected**	**Connected**	**Unconnected**	**C**	**U**
1	-0.015	0.007	49.3	51.7	2	0	- ✓✓	✓- -
2	0.002	0.007	55.2	54.4	4	1	✓- ✓	✓✓✓
3	0.001	-0.005	61.9	72.7	1	0	✓✓✓	- - -
4	-0.003	-0.045	48.9	58.2	0	0	- ✓-	- - -
5	-0.018	0.018	76.5	46.9	0	0	- - -	✓✓-
6	-0.011	-0.010	49.1	50.7	2	0	- ✓✓	- - -
7	0.017	0.006	34.8	44.7	3	2	✓✓✓	✓- ✓
8	-0.010	-0.016	45.3	51.2	2	5	- ✓✓	- - ✓
9	-0.002	0.002	51.6	55.2	0	1	- ✓-	✓- ✓
10	0.037	0.019	18.8	32.7	7	2	✓✓✓	✓- ✓
11	-0.026	-0.052	49.4	70.3	4	4	- ✓✓	✓- ✓
12	-0.021	-0.023	61.3	66.8	0	0	- ✓-	- - -
13	0.000	-0.010	49.5	39.8	1	0	- - ✓	- ✓-
14	-0.025	-0.018	49.6	43.8	1	3	- - ✓	- ✓✓
15	-0.005	-0.010	68.3	67.4	0	0	- - -	- ✓-
16	-0.016	-0.006	63.9	54.0	1	4	- - ✓	- ✓✓
17	-0.029	-0.021	48.3	45.7	1	1	- - ✓	- ✓✓
18	-0.002	-0.019	74.9	62.5	1	0	- - ✓	- ✓-
19	-0.036	-0.057	66.0	74.3	1	0	- ✓✓	- - -
20	-0.014	-0.026	54.8	57.9	0	0	- ✓-	- - -

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