Bumblebees in prime landscapes with special reference to the Aran Island bumblebee (hymenoptera : apidae)

BUMBLEBEES IN PRIME LANDSCAPES WITH SPECIAL REFERENCE TO THE ARAN ISLAND BUMBLEBEE (HYMENOPTERA: APIDAE)

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Abstract

The Burren region (inclusive of the Aran Islands) in western Ireland is an example of a prime landscapes that hosts internationally rare bumblebee species, such as *Bombus muscorum*. For the conservation and survival of bumblebees it is important to know nest-site and spring forage plant preferences. Hence nest habitat choices of spring bumblebee queens in the Burren region was investigated by observing their nest-site seeking behaviour. In spring significant nest-site seeking behaviour associations were found for *B. sylvarum*, with preferences for calcareous grassland habitat and scrub-boundaries. The foraging preferences of bumblebee queens in spring were also recorded with *B. sylvarum* and *B. ruderarius* foraging most frequently from *Vicia cracca* and *Lotus corniculatus*, respectively. Significant interspecies foraging differences were found between bumblebee species recorded in this study. A melanic colour variety of *B. muscorum* is found in the Aran Islands, and similar varieties are known from several other islands off the British Isles. Considerable debate has taken place over the last 70 years concerning their taxonomic status. The phylogenetics and genetic differentiation of melanic colour morphs within *B. muscorum* were examined using DNA barcoding. On dried museum and recently caught alcohol-preserved specimens a novel technique involving a modification of the Qiagen DNeasy PBS DNA extraction protocol for insects was developed to extract DNA from the museum specimens. The CO1 barcoding region, cytochrome B and ITS region were all examined. The results can be used to agrue that melanism in *B. muscorum* has no underlying phylogenetic significance (e.g. remnants of a Lusitanian distribution or edge of geographic range effect), and the presence of melanic forms on islands is due to convergence. Cumulatively the information gathered through this atypical study of bumblebees in prime landscapes contributes to bumblebee conservation, genetic analysis and taxonomy. More research on insects in prime landscapes is advocated.

Declaration

This thesis is presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

"It is entirely my own work, and has not been submitted to any other university or higher institution, or for any academic award in this university. Where use has been made of other people's work, it has been fully acknowledged and referenced accordingly"

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Aislinn Deenihan

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List of Abbreviations

B.	*Bombus*
A	Adenine
agg.	aggregate
B	of a blonde colour variety
B.mus	*Bombus muscorum*
Bare	limestone pavement
BOLD	Barcode of Life Data System
Bom_all	*Bombus muscorum allenellus*
Bom_hort	*Bombus hortorum*
Bom_jon	*Bombus jonellus*
Bom_lap	*Bombus lapidarius*
Bom_luc	*Bombus lucorum* agg.
Bom_pas	*Bombus pascuorum*
Bom_rud	*Bombus ruderarius*
Bom_syl	*Bombus sylvarum*
Bom_terr	*Bombus terrestris*
bp	base pairs
C	cytosine
Cal	calcareous grassland
CAP	Common Agricultural Policy
CBOL	Consortium for the Barcoding of Life
CCA	canonical correspondence analysis
CO1	cytochrome *c* oxidase 1
Coll.	collection
cSAC	candidate special area of conservation
DCA	detrended correspondence analysis
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
EU	European Union
G	guanine
Hedge	hedgerow boundary
Imp	improved grassland
Is.	island
ITS	internal transcribed spacer
IUCN	International Union for Conservation of Nature
M	of a melanic colour variety
mtDNA	mitochondrial deoxyribonucleic acid
NaOAC	sodium acetate
NGOs	non governmental organisations
NS	not significant

P	probability of obtaining a test statistic
PBS	phosphate buffered saline
PCR	polymerase chain reaction
rcf	relative centrifugal force
rpm	revolutions per minute
s. str.	sensu stricto
Scrub	scrub boundary
SNP	single nucleotide polymorphism
T	thymine
U	unit
V	volt
var.	variety
Wall	stone wall boundary

Chapter 1 Introduction

1.1 Bumblebees in Prime Landscapes

Insect conservation is important for humanity's wellbeing through the provision of ecosystem services such as pollination, which insects such as bumblebees are involved in providing. Bumblebees are in widespread decline with some species experiencing greater declines than others (Williams and Osborne, 2009). Consequently, there have been calls for more studies on the ecological niches of the rare species to help understand the basis of this loss of biodiversity (Goulson et al., 2005; Fitzpatrick et al., 2007). However, for ecological studies to be completed, clear species identifications have to be in place. Prime landscapes can provide invaluable data required for species conservation. The Burren region (inclusive of the Aran Islands) in western Ireland is a prime landscape that supports bumblebees that are uncommon and rare in Ireland, such as *Bombus sylvarum* and *B. muscorum* (Santorum and Breen, 2005; Fitzpatrick and Murray, 2006) and elsewhere in Europe (Darvill et al., 2006; Goulson, 2010). Despite extensive research being carried out on bumblebee decline, little information has been gathered on the ecological conditions that maintain the rarer bumblebee species. The spring ecological niches of nest-site seeking and foraging of bumblebee queens in the Burren region were investigated during this study.

Within the Burren region there are two colour varieties of one of Ireland's rare bumblebees, *B. muscorum* (Fitzpatrick et al., 2006b). One of these colour varieties has a pale yellow colouration (referred to as "blonde" from here), and is known as *B. muscorum* var. *pallidus* (Evans, 1901). The other colour variety has melanic colouration. The melanic colour variety of this species in Ireland is found only on the Aran Islands, and is referred to as *B. muscorum* var. *allenellus* (Stelfox, 1933). However, there are similar colour morphs of the species on islands off Britain and in Northern Norway (Stelfox, 1933; Richards, 1935; Løken, 1973). The phylogenetics and genetic variation within these colour morphs were unclear

(Williams, 2010a). Hence, the phylogenetic and genetic differentiation of the colour morphs was examined using the DNA barcoding technique.

1.1.1 Introduction to Bumblebees

Bees are the most important and specialist insect pollinating group (Danforth et al., 2006). Bumblebees are a member of this group and there has been evidence of their decline throughout the world (Williams and Osborne, 2009).

1.1.2 Bumblebee Classification

Bees belong to the insect order Hymenoptera and suborder Aculeata (bees, wasps and ants) (Michener, 1974). Bees, like ants, evolved from wasps (Michener, 1974). There are seven families of bee: Megachilidae, Apidae, Colletidae, Stenotritidae, Andrenidae, Halictidae, and Melittidae (Michener, 1974). There are in excess of 16 000 bee species in the world (Michener, 1974). Bumblebees belong to the genus *Bombus*, and bear pollen carrying structures (corbiculae) (Michener, 1974). Worldwide, there are approximately 250 species (Williams, 1998) and 15 recognised subgenera of bumblebees in the world (Williams et al., 2008). The 15 subgenera of *Bombus* have been proposed from papers by Cameron et al. (2007), Williams (1998) and Richards (1968). The sub-generic classification put forth by Williams et al. (2008) lists the following sub-genera: *Mendacibombus*, *Bombias*, *Kallobombus*, *Orientalibombus*, *Subterraneobombus*, *Megabombus*, *Thoracobombus*, *Psithyrus*, *Pyrobombus*, *Alpinobombus*, *Bombus sensu stricto*, *Alpigenobombus*, *Melanobombus*, *Sibiricobombus*, and *Cullumanobombus*.

1.1.3 Bumblebee Distribution

Bumblebees are found throughout the world. Most bumblebee species are found in the northern hemisphere in temperate regions and are most abundant in alpine and high level grassland (Williams, 1985c). A small number of bumblebee species are found in southeast Asia and in the tropics in central and southern America (Williams, 1998). New Zealand also hosts certain introduced bumblebee species (Williams, 1998). Bumblebees present in North America and Europe are believed to be the most studied, but the richest bumblebee assemblages are believed to be present in central Asia (Benton, 2006). Within this worldwide distribution there are specific colour patterns for the different species, and even within a species there can be colour variations (Plowright and Owen, 1980; Williams, 2007). To address the evolution of this diversity a robust knowledge of the phylogeny of the species needs to be available. This project provides evidence on this subject based on investigation of the colour varieties of *B. muscorum* in the British Isles.

1.1.4 Bumblebee Identification

Bumblebee identification is possible through several different techniques. Bumblebees are easily identified by the non-scientist through their characteristic buzzing sound and “furry” appearance. However, on deeper inspection, bumblebee identification is more complex due to the number of different species and different castes present. There are three bumblebee castes (males, females = queens, workers), which can have different sizes and hair colour patterns (Sladen, 1912; Alford, 1975). To identify a species, using colour patterns or male/female genitalia, the sex and caste must be known, as colour differences can occur between the sexes in bumblebees even amongst members of the same species (Sladen, 1912; Alford, 1975). The sex of a bumblebee can be determined by counting the number of antennae. Male bumblebees have 13 segments in their antennae and female bumblebees have 12 segments in their antennae (Alford, 1975). The sex of a bumblebee can also be inferred from the shape of the rear of the abdomen (Benton, 2006). Male bumblebees have a rounded shape to the rear of the abdomen while female bumblebees have a more angular pointed shape to the abdomen (Benton, 2006). After the sex of a bumblebee is determined it should be categorised according to caste to aid identification. The three main castes in bumblebees are the queen, the workers, and the males (Sladen, 1912; Alford, 1975). The castes have the following characteristics:

1. The queen is the reproductive female of the colony. The queen is responsible for establishing the nest in spring, laying eggs, and most importantly, ensuring the successful production of daughter queens and males. The queen is the largest individual of the three castes. Queens are easily identifiable for all species in early spring as they are the only caste present at this time.
2. Worker bumblebees are also females and usually infertile. The worker bumblebees' main tasks are to collect food (pollen and nectar), tend to the brood (while the queen lays eggs) and to maintain the nest. Worker bumblebees can be found during late spring and up until early autumn. Worker bumblebees are smaller than queen bumblebees though large workers can be confused with queens of the same species. Worker bumblebees can have hair colour patterns that differ from the queen and male bumblebees of the same species.
3. The male bumblebees appear late in the season and their main purpose is to fertilise the young queens. The male bumblebees are usually the smallest of the three castes and usually do not appear until early to mid summer. Male bumblebees often have a different hair colouration from the worker and queen bumblebees in the same species.

Once the sex and caste are determined, it is then possible to use identification keys to identify the species.

Bumblebee species can be identified based on their hair colour patterns and special structural appendages, internally through examination of male and female genitalia and genetically using DNA barcoding. Identification using hair colour characteristics is one of the quickest and most easily used characteristics for identifying bumblebees. Guides provided by authors such as Prŷs-Jones and Corbet (1991) and Benton (2006) provide thorough identification keys and more specialist keys are also available (e.g. Løken, 1973; Alford, 1975). Special external structures can exist between species that aid in identification. For example, there is a small spine on the leg of *B. ruderarius* that is not present on the leg of *B. lapidarius*, which allows for these species to be distinguished even though they have similar colour patterns (Alford, 1975). Examination of male and female genitalia, (in particular those of the male) can also be used to distinguish the different species and keys are provided by Alford (1975), Williams (1995) and Benton (2006). However, when there are colour varieties within a species, such as melanism, and there are no accompanying structural or genitalia differences, it can cause confusion especially when the genetics behind the colour variety are not understood. DNA barcoding (Hebert et al., 2003) is a new molecular analysis technique that has the potential to overcome this problem. DNA barcoding is explained in section 1.4 (below). This project investigated the status of the melanic colour variety of *B. muscorum* from the British Isles using DNA barcoding.

1.1.5 Bumblebee Lifecycle

Bumblebees are social species that live in colonies and whose life is determined by the seasons. Bumblebees are eusocial species. Eusociality can be defined as a level of social structure in which there is extensive division of labour and co-operation between the various members of a colony, and where the elite females produce the offspring. In bumblebees, the elite females are the queens, with one reproducing queen per colony and the other less elite females in the colony (the workers) co-operate to maintain and provide for the colony. Male bumblebees contribute to this eusociality through fertilisation. This eusociality means the dominant individuals in the lifecycle of a bumblebee colony, and in effect the most important individuals, are the mated, reproducing and newly emerged virgin queens. The lifecycle of a bumblebee colony follows the lifecycle of its queens. In temperate and sub-arctic areas, bumblebee colonies rarely last more than a year, with the colony dying out (corresponding with the death and/ or removal of the old queen) in autumn and the young queens being the sole members surviving the winter (Sladen, 1912; Michener, 1974; Alford, 1975). Lifecycles in tropical regions are different as colonies can last several years due to climate differences (Michener, 1974). The annual lifecycle of the bumblebee described below is common to all bumblebee species in temperate regions of the world, though there can be variations between species. There are five main stages to a colony cycle of the bumblebee: queen overwintering; queen foraging; establishment of nest by queen; colony development and mating of virgin queens (Alford, 1975).

Queen overwintering is where a new daughter queen finds a safe sheltered area to hibernate for the winter months until more suitable weather and available forage becomes available in spring (Alford, 1975). The overwintering queens need to have successfully mated and to have developed large fat reserves. A successfully overwintered queen will then emerge in spring to begin queen foraging. Lye et al. (2009) postulated that the next two stages in the lifecycle of a bumblebee are the most critical time for bumblebees. These stages are queen foraging and nest establishment. If either of these stages is unsuccessful, no colony will be founded. Newly emerged overwintered bumblebee queens need to feed actively and opportunistically to replenish their fat reserves that have been greatly depleted over the winter months. If they do not replenish these fat reserves they may not survive, or have the capacity to establish and provision a nest (Alford, 1975). When bumblebee queens are establishing a nest they exhibit nest-site seeking behaviour. A nest-site seeking queen has been defined as a queen exhibiting low flight close to ground, flying in a zigzag fashion and those observed crawling on the ground (Svensson et al., 2000). There are, however different foraging and nest-site preferences among bumblebee species (Sladen, 1912; Alford, 1975), and these will be discussed in further sections in the introduction.

After a suitable nest-site is found the queen initiates building and provisioning the nest. Bumblebee queens no longer forage for themselves and instead begin foraging for the future colony (Sladen, 1912; Alford, 1975). All bumblebee nests consist of cells for the growing larvae, storage pots for nectar, pollen for the larva and other structures of wax mixed with pollen which the queen must establish and provision at the start by herself (Michener, 1974). Once the queen has prepared the nest she lays her first batch of eggs which she broods through their development from egg to larva to full grown bumblebee. The first batch of young bumblebees are worker bumblebees. Once workers are produced, the queen curtails foraging for pollen and nectar, and maintaining the nest. The new workers start to forage for themselves, the colony and provision the nest. The use of pheromones by the queen suppresses egg laying by the workers, and ensures that, in the short term, only she lays eggs (Alford, 1975).

The next stage of the bumblebee lifecycle is colony development and this continues from nest initiation with more bumblebee workers being produced by the queen and thus more foraging and collection of pollen and nectar that can be used to provision the nest (Alford, 1975). At some point during this stage the colony switches from producing just worker bumblebees to producing young male and queen bumblebees. Once male bumblebees have fully developed they leave the nest and are believed not to return (Alford, 1975). In contrast the virgin queen bumblebee actively forages for herself to augment her fat supplies for her prospective "overwintering" and she returns to the nest after these foraging bouts. However virgin queens also eventually leave. The bumblebee colony continues to produce new bumblebees until conflict arises in the bumblebee nest resulting in its demise (Michener, 1974; Alford, 1975). This conflict often occurs due to worker bumblebees laying their own eggs which results in aggression from the queen bumblebee and other workers (Michener, 1974; Alford, 1975). The queen is often attacked, killed or evicted by the worker bumblebees as they all start egg laying, or she may also die of old age. Parasitism of the colony may also occur at this time (Alford, 1975; Goulson, 2010). The colony eventually collapses due to the loss of the queen, order and control within the nest (Michener, 1974; Alford, 1975).

The final stage in the bumblebee cycle is mating of the new virgin bumblebee queens (often referred to as gynes) with male bumblebees (Alford, 1975). Male bumblebees gather in small clusters along field boundaries and other landmarks, and even patrol in search of a gyne to fertilise. When male bumblebees see a prospective gyne to fertilise they attach themselves to her body until copulation has been completed. Multiple males mating with one gyne have been reported (Benton, 2006). Once the queen has been fertilised, she continues to build up her fat supplies through foraging and eventually chooses a location for overwintering. Male bumblebees continue to forage for themselves and search for gynes and eventually die as the winter sets in (Alford, 1975).

1.1.6 The Nesting Behaviour of Bumblebees

Bumblebees have particular nesting behaviours that differ from some of the other bee families (Michener, 1974). However, this nesting behaviour, irrespective of the species, has some universal commonalities such as small population size, and nest-site seeking behaviour (Benton, 2006). Bumblebees have a colony size which is usually less than 500 (Alford, 1975). All bumblebee queens exhibit nest-site seeking behaviour when searching for a suitable nesting site. Another similarity that is inherent for all bumblebee nests is that they are extremely difficult to find, with different methods being employed by researchers to find and quantify nests such as training a sniffer dog (Waters et al., 2010), placement of artificial nest boxes (Norgaard Holm, 1966; Richards, 1973, 1978; MacFarlane et al., 1983; Fussell and Corbet, 1992) and observing nest-site seeking behaviour (Svensson et al., 2000; Kells and Goulson, 2003; Lye et al., 2009).

On a large spatial scale the availability of nesting sites can determine the structure of a pollinator community (Steffan-Dewenter et al., 2001), in particular bumblebees. This is because the nest choice preference among bumblebees is particular to each species (Sladen, 1912; Free and Butler, 1959; Harder, 1986; Fussell and Corbet, 1992). The position, structure, and total population often differ according to species, resulting in different habitat and thus landscape preferences. The position of a bumblebee nest can be above or below ground and nests of bumblebees can be made from dead grasses, leaves, fine roots, mosses or established in disused small mammal nests (Alford, 1975). For example, bumblebee species of the subgenus *Thoracobombus* nest above ground. The species *B. pascuorum*, a member of this subgenus, nests above ground in grass and nests of this species can be found during hay cuttings in the summer months (Fig. 1).

In contrast, bumblebee species such as *B. terrestris* and *B. lapidarius* establish nests underground, most often in disused mammal nests (Sladen, 1912). Despite the small size of bumblebee nests, the population of bumblebee colonies can also be species dependent. For example, *B. muscorum* nests typically have a population of 20-40 workers, while *B. terrestris* nests can have a population of several hundred at their peak (Benton, 2006). It has been suggested that availability of nesting sites may play a role in the increased rarity of some bumblebees in relation to the ubiquity of others (Goulson, 2003; Lye et al., 2009).

Fig. 1 - A nest of *B. pascuorum* (sub-genus *Thoracobombus*). This nest was located on the ground and found after hay cutting on the Aran Islands (Inis Oirr, Aran Islands, 2008, Cathal Reid)

Observations on nest-site seeking behaviour were made as part of this study as the method to quantify the suitability of habitats within a prime landscape for the different bumblebee species. Previous research has primarily focused on nest-site seeking behaviour and nest locations in intensive agricultural landscapes (Svensson et al., 2000; Lye et al., 2009), agricultural landscapes where agri-environmental and wildlife friendly farming schemes are in place (Kells and Goulson, 2003; Lye et al., 2009) and highly humanised environments such as gardens and parks (McFrederick and LeBuhn, 2006; Osborne et al., 2008). There has been much data collected on the ubiquitous bumblebee species through these studies but there is still a dearth of knowledge on the nest-site seeking behaviour, nest locations and foraging requirements of queens of the rare bumblebee species.

1.1.7 Pollination and Bees

As previously noted bees are the most important and specialist insect pollinating group (Danforth et al., 2006). The economic worth of pollinator services was valued at €153 billion worldwide in 2005 (Gallai et al., 2009). In Ireland, pollinator services provided by bees have been valued at €85 million euro per annum (Bullock et al., 2008). Bumblebees' high value as pollinators for both wild and cultivated plants was highlighted in the report by the Committee on the Status of Pollinators in North America (2007).

1.1.8 Bumblebee Foraging

Bumblebees are important foraging insects as they are abundant, have longer tongues than most other insects for obtaining nectar (and thus pollinate flowers with longer corollas), will forage in inclement weather and, unlike some species such as honeybees, bumblebees sometimes forage exclusively for pollen (Benton, 2006). Varying corolla lengths provide opportunities for rewards from plants which are not accessible to other insect species due to incompatible tongue lengths (Proctor and Yeo, 1979). A bumblebee is foraging when it is gathering and searching for pollen and/or nectar from a flowering plant. The ability of bumblebees to forage optimally is a major determinant of the success or the failure of a bumblebee colony (Heinrich, 1979). The foraging ability of a bumblebee species is governed by biological, ecological and environmental traits (Heinrich, 1979). One of the most important features responsible for foraging differences between the different bumblebee species is the length of the bumblebee tongue (proboscis) (Heinrich, 1979), and it has been suggested that foraging differences could be responsible for the increased rarity of some species in relation to the ubiquity of other bumblebee species (Goulson and Darvill, 2004; Goulson et al., 2005).

Bumblebees and other flower nectar foraging insects use their tongue to investigate and suck nectar from flowers. The morphological feature of tongue length is important in relation to pollination syndromes, as tongue lengths of bumblebees can vary between species, as does the corolla length of plants (Heinrich, 1979). The differences in the foraging efficiency of different bumblebee species relating to their tongue lengths has been widely reported (Brian, 1952; Teräs, 1976; Heinrich, 1976a, 1976b; Ranta and Lundberg, 1980; Ranta and Vepsäläinen, 1981; Teräs, 1985). It is necessary to know plant species associations with individual bumblebee species in order to maintain habitats suitable for bumblebee forage as the behaviour of pollinators is influenced by the total and relative number of flowering species (Stephens and Krebs, 1986).

One of the critical times for survival and success of a bumblebee colony is when the spring bumblebee queen emerges from overwintering and must rapidly replenish her fat supplies so she can survive and establish a nest (Lye et al., 2009). An ample amount of suitable forage must be available for her at this time. Since this is one of the critical times for survival of a queen and availability of suitable forage has been reported as a factor in the increased rarity of certain bumblebee species (Goulson and Darvill, 2004; Goulson et al., 2005), it is pertinent that information on the foraging of spring bumblebee queens is obtained. The foraging of rare spring bumblebee queens in a prime landscape was investigated in this study.

1.1.9 Melanism and Bumblebees

Melanism is a phenomenon within the animal kingdom wherein there is a darkening of pigmentation not normally present in a species or group of organisms. The term melanism derives its name from the common animal pigment melanin, which is responsible for the darkening of pigmentation. Animal taxa in which melanism has been reported include: microorganisms, insects, arachnids, crustaceans, molluscs, birds, and mammals (Majerus, 1998). Melanism has long been used to investigate evolutionary change (Tutt, 1891). Melanism has been widely reported and investigated in the order Lepidoptera and many of the evolutionary changes responsible for the darkening of pigmentation within this family have been identified (Majerus, 1998). Natural selection is one of the main evolutionary forces responsible for melanism in the order Lepidoptera (Majerus, 1998). Melanism is due to adaptive processes or genetic mutations (Majerus, 1998). An adaptive melanistic colour pattern may evolve due to factors such as defence, the energetic cost of colour production, and previous selective pressures to which the species has been exposed. Examples of adaptive melanism include industrial melanism where melanism is controlled by anthropogenic change in the environment (Majerus, 1998). However melanism does not arise from a single evolutionary factor thus complicating the study of the evolution of melanic character in a species. There is little understanding of the genetics underlying the expression of the melanistic colour trait. There are only a few instances where the genetic link has been established for the occurrence of melanism within a species (Majerus, 1998; Majerus and Mundy, 2003). For example, Majerus and Mundy (2003) reported on mutations in the melanocortin-1-receptor gene which are responsible for the different coat colours of rock pocket mice (Mammalia). However the link between genotype and phenotypical expression for the majority of melanic occurrences within species has not been established.

Colour patterns within bumblebee species can be highly variable (Radoszkowski, 1884; Vogt, 1909) and darkening of colouration or melanism is recognised as occurring within bumblebee species (e.g. Plowright and Owen 1980; Williams, 2007). Melanism has been reported within species such as *B. terrestris* (Rasmont, 1982), *B. trifasciatus* (Williams, 1991), *B. cryptarum* (Bertsch et al., 2005), *B. campestris* (Alford, 1975) and *B. muscorum* (e.g. Stelfox 1933; Richards 1935; Løken, 1973). This melanic colour trait has been known to cause difficulty in identifying a species such as in the melanic form of *B. cryptarum* in Germany (Bertsch et al., 2005). However, the genetics of the two colour varieties were investigated and the two colour varieties were found to be one species. Melanism in this instance aids in the identification of this species from the other cryptic species of similar colouration, *B. lucorum* and *B. magnus* (Bertsch et al., 2005). Once the genetics behind a colour variety are investigated, clarity can be obtained that allows for conservation measures if necessary. Hence, the genetics of the colour varieties of *B. muscorum* in the British Isles were investigated as part of this study.

1.1.10 Bumblebees in Ireland

There has been an increasing awareness of bumblebees in Ireland in recent years as evidenced by the increasing number of reports and papers being published (e.g. Santorum and Breen 2005; Fitzpatrick et al., 2006b, Murray et al. 2008). There are 18 bumblebee species found in Ireland (Table 1) (Fitzpatrick et al., 2006b). Threatened bumblebee species have been identified and classified, and an International Union for Conservation of Nature (IUCN) regional red list for the island of Ireland has been produced (Table 1) (Fitzpatrick et al., 2006b). The growing awareness of bumblebees in Ireland has also led to increasing information on the distribution of bumblebees in Ireland and escalating concerns about the conservation of certain species (Santorum and Breen, 2005; Fitzpatrick et al., 2006b; Fitzpatrick et al., 2007).

Certain Irish bumblebee species are declining at a greater rate than others (Fitzpatrick et al., 2006b; Fitzpatrick et al., 2007). For example, *B. muscorum* was once considered common in Ireland, is now declining (Fitzpatrick et al., 2006a), while bumblebee species such as *B. terrestris*, *B. lucorum*, and *B. pascuorum* have remained common with stable populations in Ireland (Santorum and Breen, 2005). The rarest bumblebee species in Ireland are the species with late emerging spring queens and they have experienced a decline in distribution in Ireland in the last 20 years (Fitzpatrick et al., 2007). Bumblebees of the sub-genus *Thoracobombus* are late emerging species and are found in Ireland. Bumblebees of this sub-genus are considered the most threatened in Ireland (Fitzpatrick et al., 2007). In Ireland, bumblebee species of this sub-genus are *B. pascuorum, B. muscorum, B. ruderarius,* and *B. sylvarum*. Of these four, three are endangered or very vulnerable (*B. muscorum, B. ruderarius,* and *B. sylvarum)* while the other (*B. pascuorum*) is one of the most common bumblebees in Ireland.

The decline of bumblebees is Ireland is believed to be driven by agricultural intensification (Santorum and Breen, 2005). The widespread replacement of hay with silage has been suggested as a major contributory factor in the decline of the late emerging bumblebees (Fitzpatrick et al., 2007). Other factors that have been suggested as contributing to the decline of bumblebees in Ireland include habitat loss, habitat fragmentation and climate change (Fitzpatrick et al., 2007).

Table 1 - Species and sub-genus of Bumblebee species in Ireland (derived from Fitzpatrick et al. 2006b and Williams et al. 2008)

Species	Sub-Genus	IUCN Red List Classification
B. cryptarum (Fabricius)	*Bombus s. str.*	Data deficient
B. lucorum (Linnaeus)	*Bombus s. str.*	Least concern
B. terrestris (Linnaeus)	*Bombus s. str.*	Least concern
B. hortorum (Linnaeus)	*Megabombus*	Least concern
B. lapidarius (Linnaeus)	*Melanobombus*	Near threatened
B. bartutellus (Kirby)	*Psithyrus*	Endangered
B. bohemicus Seidl	*Psithyrus*	Near threatened
B. campestris (Panzer)	*Psithyrus*	Vulnerable
B. rupestris (Fabricius)	*Psithyrus*	Endangered
B. sylvestris (Lepeletier)	*Psithyrus*	Least concern
B. jonellus (Kirby)	*Pyrobombus*	Least concern
B. monticola Smith	*Pyrobombus*	Least concern
B. pratorum (Linnaeus)	*Pyrobombus*	Least concern
B. magnus Vogt	*Bombus s. str.*	Data deficient
B. distinguendus Morawitz	*Subterraneobombus*	Endangered
B. muscorum (Linnaeus)	*Thoracobombus*	Near threatened
B. pascuorum (Scopoli)	*Thoracobombus*	Least concern
*B. ruderarius (*Muller)	*Thoracobombus*	Vulnerable
B. sylvarum (Linnaeus)	*Thoracobombus*	Endangered

1.1.11 *Bombus muscorum*

Bombus muscorum is a species in the sub-genus *Thoracobombus* whose distribution extends across the palaearctic region. It was first described by Linneaus in 1758 (Alford, 1975).The common name of *B. muscorum* is the “moss carder bee”, which it is derived from the way it builds its nest above ground using materials such as moss (Sladen, 1912; Alford, 1975). *Bombus muscorum* was previously considered widespread but not common in Britain (Sladen, 1912) but it has decreased to such an extent in Britain that it is now the subject of an English Nature Species Recovery Programme. In Ireland *B. muscorum* was considered more common than in Britain (Sladen, 1912), however, it is now considered near threatened (Fitzpatrick et al., 2006b).

Within *B. muscorum* there are different colour forms most notably that of the blonde and melanic types, and within these two groups there are variations. Between Britain and Ireland there are six different varieties (Table 2).

Table 2 - The forms and varieties of *B. muscorum* in the British Isles following Alford (1975) and Baker (1996)

Form	Location	Variety	Author	Distribution
Blonde	Mainland	*sladeni*	Vogt	The south of the English mainland, European continent
	Mainland & islands	*pallidus*	Evans	Irish Mainland, northern England, several offshore islands
Intermediate	Islands	*orcadensis*	Richards	Orkney Islands
	Islands	*scyllonius*	Richards	Scilly Islands, Channel Islands
Melanic	Islands	*agricolae*	Baker	Shetland Islands, several of the Hebride Islands
	Islands	*allenellus*	Stelfox	Aran Islands

Blonde (Fig. 2), melanic (Fig. 3) and intermediate types differ from each other in the amount and distribution of black hairs. The presence of black hairs on the legs and ventral surfaces are used to differentiate the melanic forms from the blonde. The melanic island form of *B. muscorum* was first reported in the Shetlands by White (1851) and described as *B. smithianus*. However, an error was made by White that led to *B. pascuorum* being used as the type specimen of *B. smithianus* (Alford, 1975). The Irish melanic form of *B. muscorum*, the Aran Island bumblebee, *B. muscorum* var. *allenellus*, was first described by Stelfox (1933). Stelfox (1933) reported a series of investigations on the species conducted by Mr. C. Winckworth Allen after whom species is named. The Irish melanic form is similar to, but generally darker than, the other melanic forms (Fig. 3). In Ireland the melanic version of *B. muscorum* is restricted to the Aran Islands and the need for its conservation was noted by Fitzpatrick et al. (2006b). The collective term to describe all the melanic varieties of *B. muscorum* is *B. muscorum* var. *smithianus* of various authors.

There has been debate regarding the taxonomic status of the different melanic colour varieties of *B. muscorum.* Authors such as Richards (1935), Rasmont and Adamski (1995) have regarded the melanic colour morphs as a separate species. However, others such as Løken (1973), Alford (1975), and Baker (1996) reported no clear morphometric difference between the different melanic and non-melanic varieties. Previous genetic work has been performed using microsatellites to establish the relationship between the blonde, intermediate and melanic forms (Darvill et al., 2006; Judge, 2007). Both Darvill et al., (2006) and Judge (2007) used microsatellites to study the gene flow between sampled populations of *B. muscorum.* Darvill et al., (2006) examined populations in Britain that included three varieties of *B. muscorum*. These were *B. muscorum* var. *pallidus*, *B. muscorum* var. *sladeni* and the melanic colour morph *B. muscorum* var. *smithianus.* Judge (2007) examined *B. muscorum* populations in Britain and Ireland which included the four varieties, *B. muscorum* var. *allenellus* (Aran Islands)*, B. muscorum* var. *pallidus* (Irish mainland), *B. muscorum* var. *agricolae* (Scotland), and *B. muscorum* var. *scyllonius* (Scilly Islands). Neither study yielded significant genetic differences between the colour morphs that would warrant special species status for the colour morphs (Darvill et al., 2006; Judge, 2007). However Darvill et al., (2006) reported significant inbreeding within the isolated island *B. muscorum* populations in Britain. These studies highlighted the need for studies on *B. muscorum* to continue and Darvill et al., (2006) called for further studies to examine the mitochondrial DNA (mtDNA) of the colour morphs. For appropriate conservation strategies to be developed for this group of bees it is imperative that the taxonomic and phylogenetic status of the *B. muscorum* varieties is clarified. As part of this study, the genetic variability of the different colour varieties of *B. muscorum*, was investigated through examination of mtDNA, and a nuclear gene called the internal transcribed spacer region (ITS). The internal transcribed spacer region is a type of ribosomal RNA (rRNA). These genetic areas were examined in *B. muscorum* samples retrieved from 15 different locations in the British Isles.

Fig. 2 – The mainland form (blonde) bumblebee, *B. muscorum* var. *pallidus* (Clare Island, Co. Mayo, 2005, John Breen)

Fig. 3 - The Aran Islands bumblebee, *B. muscorum* var. *allenellus* foraging on *Lotus corniculatus* (Inis Oirr, Co. Galway, 2006, John Breen)

1.1.12 *Bombus lucorum* agg. and *Bombus terrestris*

In Ireland, *B. lucorum* agg. is the collective name given to a group of cryptic bumblebee species. In spring *B. lucorum* agg. is the collective name given to queens of *B. lucorum, B. magnus,* and *B. cryptarum.* During summer, *B. terrestris* is included in the *B. lucorum* agg. group, as bumblebee workers belonging to *B. terrestris* cannot be accurately distinguished from *B. lucorum, B. magnus* and *B. cryptarum*. Murray et al., (2008) discovered that it was impossible to accurately distinguish these species in Ireland without the use of the Polymerase Chain Reaction (PCR) technique. Murray et al., (2008) set the precedent for the use of the collective term *B. lucorum* agg. in Ireland.

1.1.13 Bumblebees in Decline

A decrease in bumblebee abundance, and localised species extinctions, has been reported throughout the world: Britain (Goulson, 2010); Britain and the Netherlands (Biesmeijer et al., 2006); Western and Central Europe (Kosior et al., 2007); Brazil (Martins and Melo, 2009) and North America (Committee on the status of Pollinators in North America, 2007). In Ireland, bumblebee species of the late emerging species have been found to be declining (Fitzpatrick et al., 2007). This decrease has been attributed to changing land use most notably the intensification of farming, and climatic change affecting the climatic ranges of bumblebees (Williams, 1985b, 1988, 1989).

Some bumblebee species are experiencing greater declines than others (Williams and Osborne, 2009). The mechanisms behind these differential declines remain unclear. Williams (1985a, 1989) suggested that niche differences between bumblebee species, combined with habitat destruction and, potentially, climatic changes, are responsible for the more rapid decline of some species in relation to others. Consequently, there have been calls for more studies on the ecological niches of the rare species to help understand the mechanics behind this loss of biodiversity (Goulson et al., 2005; Fitzpatrick et al., 2007).

1.2 Introduction to Prime Landscapes

A prime landscape is interpreted in this study as a landscape that hosts a wide range of habitats and species, including those that are considered, rare, due to the presence of optimum living conditions (both biotic and physical) in the area.

1.2.1 Importance of Prime Landscapes

Within north-western Europe, prime landscapes can offer a glimpse of what the biodiversity of a region may have resembled pre-agricultural intensification. Prime habitats support high levels of rare species, and a large amount of data may be accumulated which can be applied towards the conservation of such rare species. It is vital that studies on rare species are conducted in such landscapes, as they can provide information on the optimal ecological niches required for their survival. Studies conducted in non-prime landscapes, such as intensive agricultural landscapes, would only provide information on the species surviving in a sub-optimum environment where the species may be experiencing environmental stresses (Samways et al., 2010).

1.2.2 Bumblebees in Prime Landscapes

For a landscape to be considered prime for bumblebees it must provide a suitable environment to support the ecology of both rare and common species of bumblebees. Three important factors in bumblebee ecology are habitat, nesting and foraging (Benton, 2006), and a prime landscape for bumblebees must provide an environment that can support these niches. Prime habitats for bumblebee foraging and nesting are landscapes with unimproved flower rich grasslands (Williams, 1988). The changes in agricultural practices worldwide, particularly land “improvement” through spreading of artificial fertilisers, have particularly threatened bumblebees (Kwak et al., 1998; Goulson et al., 2005; Carvell et al., 2006). Some bumblebee species are more threatened than others due to variations in foraging and nesting preferences (Richards, 1978; Svensson et al., 2000; Kells and Goulson, 2003). Karst landscapes support unimproved flower rich grasslands (calcareous grasslands) which are known to be a prime habitat for bumblebees (Williams, 1988). The Burren region is such a landscape and hosts rare insect species such as the bumblebee species *B. sylvarum*, *B. distinguendus* and *B. muscorum,* all of which are near threatened, threatened, or endangered in the British Isles (Darvill et al., 2006; Fitzpatrick et al., 2006b; Goulson, 2010).

1.2.3 The Burren Region

The Burren region is defined in this study as the limestone mainland region of north Co. Clare and the Aran Islands. The Burren region and Aran Islands are in Western Ireland (Fig. 4). The Burren region and Aran Islands are renowned internationally for their exceptionally high biodiversity (e.g. Nelson 1999; Dunford 2002; O'Rourke 2005). The Burren mainland region spans approximately 24km north to south and approximately 40 km east to west of north Co. Clare (RPS Cairns, 1994). The Aran Islands consist of three islands (Inis Mor, Inis Meain and Inis Oirr) situated to the West of Ireland. The climate is of the Atlantic type (Lousley, 1969). Geographically the Burren region and Aran Islands are known as karst (Warren and O'Connell, 1993). It is this karst landscape that facilitates these landscapes prime habitats, which in turn support the region's special array of flora and fauna.

The main threat to the conservation of the landscape and biodiversity of the Burren region is intensive agriculture (Dunford and Feehan, 2001; Dunford, 2002; O'Rourke, 2005). Ironically, the special biology of the Burren region was initially shaped by agriculture (Dunford, 2002; O'Rourke, 2005). Evidence from paleaoecological studies show that agricultural activity has taken place in the Burren region since Neolithic times (Dunford, 2002). If it were not for this history of extensive agriculture, the high level of biodiversity that is currently found in the Burren region would not exist (Dunford, 2002). However, the Burren region has become increasingly fragmented in the last 30 years due to changing agricultural practices, with increasing areas of improved grassland and scrub habitat (Dunford, 2002; Parr et al., 2007).

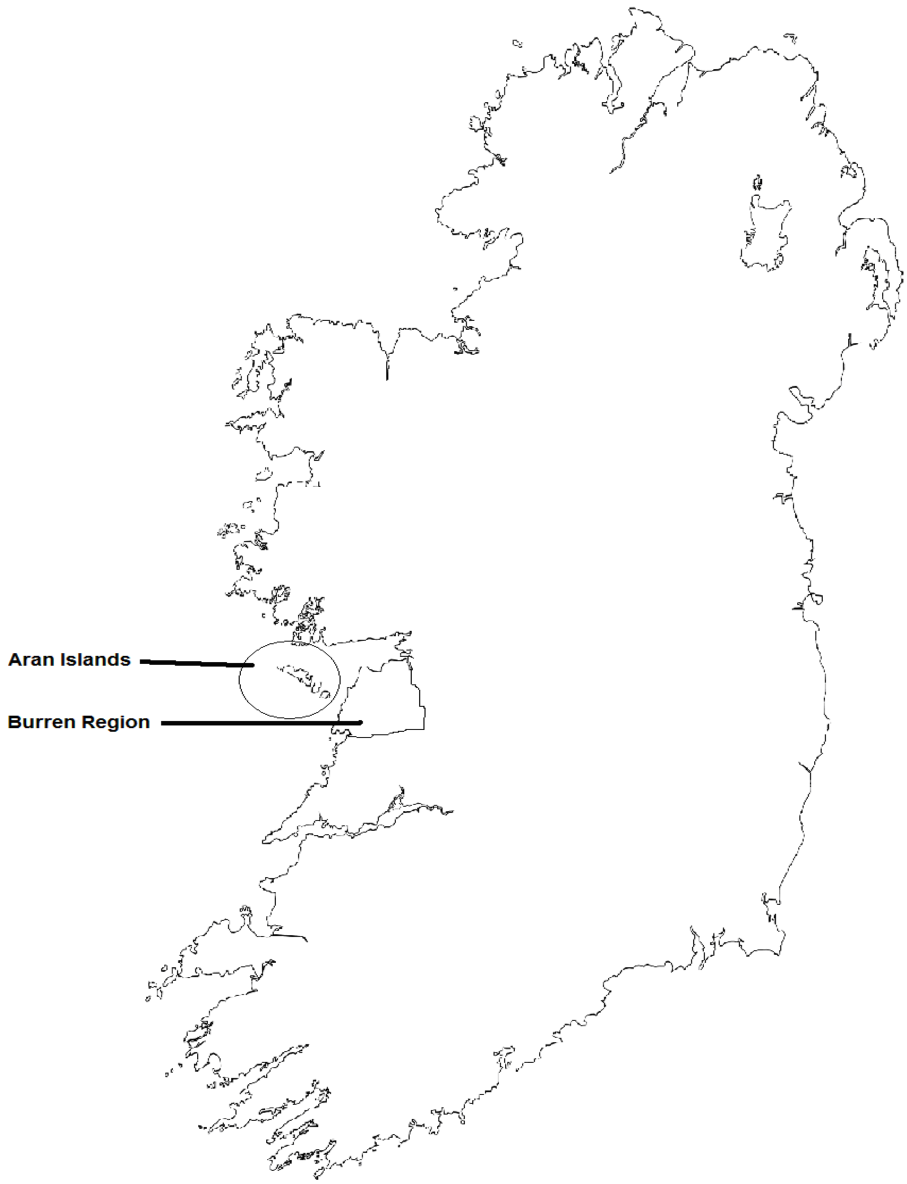

Fig. 4 - Map of Ireland with Aran Islands and mainland Burren region

Features of agricultural systems in the Burren region that have developed since 1973 (when Ireland joined the European Union) include: an increase in stocking density, increased mechanisation, larger farm size, routine application of chemical fertilisers and a change in cattle breeds (Dunford and Feehan, 2001; Dunford, 2002; O'Rourke, 2005). Similar changes in agriculture did not affect the Aran Islands, as the rugged terrain on the Islands constrains the use of modern farm equipment (O'Rourke, 2005). In contrast, the biodiversity of the Aran Islands is under threat from other factors, including land abandonment and excessive construction of holiday homes (personal observation). Extensive agricultural systems need to be practised and maintained in the mainland Burren region and Aran Islands to maintain these unique Irish landscapes (Dunford, 2002; O'Rourke, 2005).

1.2.4 Prime Habitats within the Burren Region

It is the prime habitats within the Burren region which support its exceptional biodiversity (Dunford, 2002; O'Rourke, 2006). Prime habitats within the Burren region include: unimproved calcareous grassland, turloughs, dunes and limestone pavement. Unimproved calcareous grassland and limestone pavement are the two prime habitats which were included in the present study. These habitats are listed as priority habitats designated under the EU habitat directive 1992. These two habitat types are also the prime habitat types which cover the greatest amount of area in the Burren region (Parr et al., 2007).

Unimproved calcareous grassland is one of the main habitats in the Burren region that supports the region’s biodiversity (e.g. Warren and O’Connell, 1993; Dunford, 2002; Moles et al., 2005). These unimproved calcareous grasslands contain niches with particular environmental and biological characteristics that support a high plant species richness and diversity (Rodwell, 1991). In the Burren region, there are often more than 40 species of higher plants per square metre in calcareous grassland (Dunford and Feehan, 2001). The unimproved calcareous grassland of the Burren region is threatened by land improvement and scrub encroachment due to land abandonment (Dunford, 2002; ERA-Maptec et al., 2006). Furthermore, Deenihan et al., (2009) suggested that undergrazing and overgrazing may also be threats to unimproved calcareous grassland in the Burren.

Limestone pavement habitat can be described as an area of fissured limestone bedrock that may be level, terraced or gently sloping. Limestone pavement habitat has the potential for high plant species diversity and is listed as a priority habitat in Ireland (Fossitt, 2000). During the 1980s in Ireland, large areas of limestone pavement habitat were destroyed in the Burren region by land reclamation (Drew and Magee, 1994). Currently, limestone pavement in the Burren region is threatened by unauthorised removal, scrub encroachment (ERA-Maptec et al., 2006) and, locally, by ill-advised tourist activities such as the construction of mock dolmens (Dunford, 2002).

1.3 Molecular analysis of insects

Studies on the genetics of insects provide information that increases the understanding of insect evolution, ecology and conservation (Thompson et al., 2007). Molecular analysis is the key tool used for genetic investigations. Molecular analysis is the use of molecular tools for genetic analysis. Molecular analysis of insects can be used for taxonomic identification, phylogenetics, population structure and identifying the genetic basis of certain behavioural traits (Thompson et al., 2007; Samways et al., 2010). Molecular methods can be used to gather information on population structure particularly through the use of historic specimens. A detailed background of molecular analysis and molecular ecology is given in the textbook by Campbell and Reece (2008; chapters 20-24).

1.3.1 Molecular Analysis of Bumblebees

Genetic analysis can contribute to bee conservation through the estimation of parameters such as population size (numbers of colonies), inbreeding, and species genetic variability which allow for informed management decisions (Zayed, 2009). Genetic analysis can be important for the conservation of bumblebees as the genetic structure and sociality of bumblebees can make them vulnerable to inbreeding (Zayed, 2009). Bumblebees, as do all bees, show haploid-diploidy which means that female bumblebees (queen and workers) have two sets of chromosomes (diploid) and male bumblebees one (haploid). This type of ploidy, in combination with the eusocial nature of bumblebees as insects, makes them vulnerable to population inbreeding, but it does not imply directly that bumblebees are more at risk of extinction (Zayed, 2009). The loss of genetic variation within a bee population is the main factor driving extinction (Zayed, 2009); the main causes of reduction in genetic variation arise from habitat fragmentation and loss (Whitlock and Barton, 1997).

Bumblebee genetic analysis has been greatly aided by results derived from genetic studies on the honeybee and other insects. One of these results was the mapping of the mtDNA of the honeybee (Crozier and Crozier, 1993) which has aided in taxonomic (Ellis et al., 2005; Murray et al., 2008) and population studies of bumblebees (Widmer et al., 1998). Additionally protocols developed for general insect DNA extraction have also been applied to bumblebees. Examples include: the PBS technique listed in the Qiagen DNeasy Blood and Tissue handbook (Anonymous, 2006a), and Junqueira (2002) have been used to extract DNA from historical samples of bumblebees (Dr. Tomás Murray, Martin-Luther-University Halle-Wittenberg, personal communication). All of these developments have greatly enhanced our understanding of bumblebee genetics. However, the universality of these techniques might not be suitable for specimens killed or preserved using particular techniques (Dr. Jim Carolan, National University of Ireland, Maynooth, personal communication). In this project, DNA extraction techniques designed for historical insect specimens failed and other techniques had to be specifically developed.

There have been many investigations into the genetics of bumblebees. However, the majority of published papers on bumblebee genetics have concentrated on the common species such as *B. terrestris* (Estoup et al., 1995; Widmer et al., 1998), *B. lucorum* agg. (Bertsch et al., 2005; Murray et al., 2008), and *B. pascuorum* (Pirounakis et al., 1998; Widmer and Schmid-Hempel, 1999). This is most likely due to the ease in obtaining the necessary sample sizes of these species for molecular analysis. Recent advances in DNA extraction technology have allowed for non-lethal sampling of bumblebees. This has greatly aided the study of the rarer species such as *B. sylvarum* and *B. muscorum* (Goulson et al., 2007). Molecular studies on the rarer bumblebees are important because they determine the level of genetic variation and inbreeding, and help resolve phylogenetic relationships. Such data are vital for the conservation management of a species (Thompson et al., 2007). To understand the level of genetic variation of any species, but particularly of rare insect species, it is important to have historical

DNA records (Thompson et al., 2007). Drawing on techniques developed for DNA extraction from museum specimens of bumblebees, this study examined the haplotypes present in bumblebee samples from the 1970s.

1.4 Introduction to DNA Barcoding

DNA barcoding is the characterisation of a species using a fixed short sequence of DNA that is standardised for the genome of that organism type (CBOL, 2010b). DNA barcoding is regarded as a master taxonomic key that will enable accurate identification of a species without a detailed taxonomic knowledge of the species family (Hebert et al., 2003). The term “DNA barcoding” was coined for this technique, as it uses a fixed standardised region of DNA as a marker which allows for rapid and accurate species identification. This is similar to the barcodes on products in supermarkets that are used for rapid identification and subsequent billing at supermarket check outs (Hebert et al., 2003). The term “DNA Barcoding” was originally proposed by Paul Hebert of the University of Guelph, Canada (Hebert et al., 2003; CBOL, 2010b). The Consortium for the Barcoding of Life (CBOL) was the global initiative established to implement the technique.

1.4.1 Background of DNA Barcoding

DNA barcoding is a new molecular technique and term which has emerged in the last seven years to aid in species identification. Accurate and fast taxonomic identification is important in today’s world of rapidly declining biodiversity, as it enables the enaction of conservation measures if necessary. The technique was developed as a solution to problems that were occurring with taxonomic identification (Hebert et al., 2003). Problems that occur with taxonomic species identification include: phenotypic and genetic variability of the characters used for species recognition, morphologically cryptic species, and problems with identifying species at different life cycle stages as most morphological keys only provide identification information for one life cycle stage (Hebert et al., 2003). Another large problem with species identification is the requirement for specialist taxonomists. Taxonomists are only specialists in their chosen area and it has been recognised that each taxonomist can on average accurately identify 0.01% of the

10-15 million species on the planet (Hammond, 1992; Hawksworth and Kalin-Arroyo, 1995). Despite modern improvements in identification keys such as computerised interactive keys, a new method was required to overcome these obstacles and DNA barcoding was developed as a response (Hebert et al., 2003).

DNA barcoding was first introduced in a landmark paper by Hebert et al., (2003). Prior to this paper, other authors such as Kurtzman (1994) and Wilson (1995) had proposed that genetic diversity among organisms could be used as a genomic approach to taxon identification (Kurtzman, 1994; Wilson, 1995). However, Hebert et al. (2003) were the first to publish this method as a standardised technique, and were the first to propose a standardised gene area for animal identification, the mitochondrial gene cytochrome *c* oxidase 1 (CO1). Hebert et al. (2003), demonstrated how the CO1 region in the higher animal taxa can be used to identify species and to assign genetically analysed taxa to the relevant phylum and order. Hebert et al. (2003) also demonstrated how the technique could be used to create a model CO1 profile, based on single individual samples from 200 lepidopterans and how it could be used as a DNA barcoding key to identify subsequent unknown individuals. Subsequent papers have highlighted the usefulness of this technique (e.g. Hebert and Gregory, 2005; Hajibabaei et al., 2007; Valentini et al., 2009).

1.4.2 Classification of DNA Barcoding Regions

DNA barcoding involves the analysis of a short sequence of DNA, relative to a known genomic profile. The gene region that is tentatively established for animals is encoded within the mitochondrial cytochrome *c* oxidase 1 (CO1) and composed of c.650 bp. This region is called the “Folmer region”. The “Folmer region” is located at the 5’ end of the mitochondrial (CO1) subunit. This region is bordered by conserved sequence regions which allows for high confidence in species identification. The CO1 region was chosen for animals for a number of reasons including: the low intraspecies and high interspecies sequence variability of the CO1 region (Hebert et al., 2003); the robustness of universal primers for this region (high percentage of 5’ ends recovered) (Folmer et al., 1994; Simmons and

Weller, 2001); and the CO1 region has a greater phylogenetic signal than any other gene in the mitochondria (Hebert et al., 2003). Examples of animal groups where DNA barcoding has been used include: birds (e.g. Hebert et al., 2004); bees (e.g. Kim et al., 2009); and marine turtles (e.g. Naro-Maciel et al., 2009).

1.4.3 Utility of DNA Barcoding

DNA barcoding was developed to overcome the challenges faced by morphological and genetic keys in species identification (Hebert et al., 2003). DNA barcoding has overcome many of these challenges and has opened up new avenues of scientific research, not originally envisaged when the technique was originally proposed. The major advantage of DNA barcoding over other taxonomic methods for species identification is the lack of subjectivity, and the ability of such DNA sequences to be re-analysed in the future, in accordance with the development of other genetic taxonomic techniques. DNA barcoding allows for non-specialists to identify a specimen. DNA barcoding can allow for assignment of new species, discovery of new variation within a previously presumed single species and aid in the documentation of biodiversity in poorly sampled regions in the world. Species identification helps protect endangered species, taxonomic research, sustainable harvesting of natural resources, exploration of marine biodiversity, control of disease vectors and agricultural pests, and monitoring of environmental quality (Valentini et al., 2009).

1.4.4 The DNA Barcoding Initiative and Repository

DNA barcoding as a technique was established to provide a global standard for species identification (Hebert et al., 2003). To promote and utilise the full potential of this method effectively, a global initiative was required and this was recognised at the “Taxonomy, DNA and the Barcode of Life Conference” in 2003 (Stoeckle et al., 2003). The CBOL was the resulting initiative that was created. The CBOL was established in 2004 and has a membership of over 200 organisations, from 50 different countries (CBOL, 2010a). Member groups include: natural history museums; biodiversity and conservation organisations;

non-governmental organisations (NGOs); and zoos (CBOL, 2010a). The CBOL promotes barcoding through working groups, workshops, conferences, outreach, and training (CBOL, 2010a). The CBOL assists in the rapid compilation of CO1 barcodes through repositories and to do this the CBOL had to initiate the establishment of a repository for such records (Ratnasingham and Hebert, 2007).

A major component of the DNA barcoding concept is that the CO1 barcodes obtained for the various species should be collated in a database, that will serve as a global bio-identification system for animals (Hebert et al., 2003). The CBOL entered into talks with Genomic repositories prior to establishing a barcoding repository. A database which contained information on the genetic diversity of species was already present prior to the DNA barcoding project, called Genbank. However, Genbank is a database for records of all genetic sequences, whereas a repository was needed for the DNA barcoding project that would serve as a repository for records of a single gene for bioidentification (Hebert et al., 2003; Ratnasingham and Hebert, 2007). Such a database was established and it was named the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert, 2007). BOLD aids in the acquisition, analysis, distribution, storage and publication of DNA barcode sequences and is free to researchers (Ratnasingham and Hebert, 2007). New technological developments have the potential to increase the analysis and input rate of barcodes. This aids the rapidity in identification of species (Ratnasingham and Hebert, 2007).

1.4.5 Functionality of DNA Barcoding

There are four main components to DNA barcoding projects, the specimens, the laboratory analysis, the database, and the data analysis. The specimens are the core part of the data analysis. Specimens are readily available through museums, biological banks (such as seed banks) and individuals' private collections. The laboratory analysis thus follows barcoding protocols to obtain DNA sequences from these specimens. DNA extraction and specimen preservation techniques are the critical parts of these barcoding protocols in the laboratory. If no DNA is preserved nothing can be extracted or sequenced. Ivanova et al. (2010) provide guidelines on how to preserve, extract, and sequence DNA for DNA barcoding projects. However these are general guidelines, and are not species specific.

If DNA extraction and specimen preservation are the critical parts of DNA barcoding projects it can be said that from the information derived from sequences, the database component is the next most important part. It is essential that the information derived from the sequences in relation to each species is made available so that taxonomists can label unknown species. The two main barcode databases are 1) The International Nucleotide Sequence Database Collaborative and 2) BOLD (CBOL, 2010b). The data analysis is the final component of a barcoding project and it works by correlating sequenced specimens with the nearest matching references in a database. Such information is compiled in a "bottom-up" approach and it is expected that a large encyclopaedia of information will be available for analysis and potentially free access (CBOL, 2010b).

1.5 Aims

Based on the above review, the following aims of this thesis are

1. To investigate the nest site-seeking preferences of bumblebee queens in prime habitats
2. To resolve the phylogenetic relationship between the colour morphs of *B. muscorum*
3. To develop a technique for extracting DNA from museum specimens of bumblebees

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Chapter 2 Bumblebee Nest-site Seeking and Foraging in Prime Landscapes

2.1 Abstract

Insect conservation is important for humanity's wellbeing through the provision of ecosystem services such as pollination, which insects such as bumblebees are involved in providing. Bumblebees are declining worldwide with some species experiencing greater decline and more localised extinctions than others. Despite extensive research being carried out on bumblebee decline little information has been gathered on the ecological conditions that maintain the rarer bumblebee species. This study examined the nest-site seeking behaviour, nesting locations, and foraging of spring bumblebee queens in the prime landscape of the Burren region (inclusive of the Aran Islands) in Western Ireland. Prime habitats support high levels of rare species. This study recorded a bumblebee species assemblage containing a number of internationally and nationally rare species at high levels. *Bombus sylvarum* was the fourth most common species recorded exhibiting nest-site seeking behaviour, yet it is recognised as one of the most endangered bumblebee species in Britain and Ireland. Other internationally and nationally rare species observed during this study include *B. muscorum,* and *B. distinguendus.* Significant nest-site seeking associations were found for *B. pascuorum, B. sylvarum,* and *B. ruderarius* for calcareous grassland habitat and stonewall- and scrub-boundaries. *Bombus muscorum* var. *allenellus* and *B. pascuorum* nests were located during this study enabling a preliminary comparison with results from nest-site seeking behaviour. Significant interspecies foraging differences were found between bumblebee species recorded in this study. More research in prime habitats and more support, acknowledgement and rewards for the farmers participating in agri-friendly practises in these regions is advocated.

2.2 Introduction

Insects contribute to the functionality of almost every terrestrial and freshwater ecosystem in the world. The conservation of insects is essential to maintain ecosystem services such as climate regulation and pollination upon which the human race depends for its survival (Kremen and Chaplin-Kramer, 2007; Samways et al., 2010). However, insects are becoming extinct more rapidly than at any other time in history (Samways et al., 2010). This acceleration can be directly attributed to anthropogenic activities that are resulting in worldwide environmental change. These environmental changes include habitat loss and fragmentation, invasive alien species, climate change, pollution and over-harvesting resulting in worldwide landscape change (Samways et al., 2010). Worldwide, agricultural intensification and expansion is the greatest driver of environmental change resulting in biodiversity loss and habitat homogeneity (Robinson and Sutherland, 2002; Tilman et al., 2002). A loss in habitat heterogeneity in landscape results in a decrease in insect diversity and abundance (Samways et al., 2010). The simplification of invertebrate communities in a landscape results in an increase in pest outbreaks and limitation of ecosystem services (Tscharntke et al., 2007).

Agricultural landscapes and ecosystems are often considered poor areas for biodiversity conservation (Tscharntke et al., 2007). However, insect conservation is not always threatened by agriculture. In western Europe, extensive farming practices such as low intensity grazing can create species-rich habitats. Many species depend on extensive agricultural systems for their survival in a region (Dunford, 2002; Tscharntke et al., 2007). Nevertheless, agricultural intensification and expansion is the biggest driver of biodiversity loss in the world (Tilman et al., 2001; Robinson and Sutherland, 2002). Agriculture has been modifying the earth’s landscape for the last 4 000 years, with the most concentrated period of land transformation and intensification taking place in the mid-twentieth century (Robinson and Sutherland, 2002). Agricultural subsidies in the early years of the European Union encouraged this rapid acceleration in the intensification of land by having production linked subsidies. The rapid acceleration in the agricultural intensification of land in a country upon joining the EU has been noted in Britain and Ireland (Dunford, 2002; Robinson and Sutherland, 2002). Reformation of the Common Agricultural Policy (CAP) in

Europe is now attempting to militate against the early effects of agricultural policy in the EU. Subsidies are now almost exclusively decoupled from production (Schmid et al., 2007). Supporting measures are now being directed to conserving semi-natural landscapes with a high biodiversity (Anonymous, 2006b). These landscapes are often referred to as prime landscapes.

Prime landscapes can provide invaluable data required for species conservation. Within north-western Europe, prime landscapes and can offer a glimpse of what the biodiversity of a region may have resembled pre-agricultural intensification. The Burren region is such a prime landscape in western Ireland. The Burren region is defined in this study as the limestone region of north County Clare, and the Aran Islands (derived from Praeger (1939)). It is an area known internationally for its exceptionally high biodiversity. Extensive agriculture is practised in many parts of the region. The Burren region has become fragmented in the last 30 years due to changing agricultural practices, with increasing areas of improved grassland and scrub habitat and decreasing calcareous grassland habitat (Dunford, 2002; Parr et al., 2007). The European Union financially assisted the Irish Government in setting up the BurrenLife project in 2004 to develop a sustainable agricultural model for the region to help promote its conservation (Anonymous, 2005). The Burren region hosts rare insect species such as the bumblebee species, *B. sylvarum*, *B. distinguendus* and *B. muscorum,* all of which are rare in Europe (Darvill et al., 2006; Fitzpatrick et al., 2006b; Kosior et al., 2007; Goulson et al., 2008; Goulson, 2010). Karst landscapes support unimproved flower rich grasslands (calcareous grasslands) which are known as a prime habitat for bumblebees (Williams, 1988; Edwards, 1998).

Bumblebees are considered an insect taxon of high conservation importance as they contribute to the ecosystem service of pollination. Localised species extinctions and a decline in abundance of bumblebees have been reported in many parts of the world (Peters, 1972; Williams, 1982; Biesmeijer et al., 2006; Committee on the status of Pollinators in North America, 2007; Fitzpatrick et al., 2007; Kosior et al., 2007; Martins and Melo, 2009; Goulson, 2010). The main drivers of bumblebee decline are thought to be climate change and changing agricultural policy and practises (Williams, 1985b; Williams, 1986a; Williams, 1988; Goulson et al., 2005; Carvell et al., 2006), however there are different reasons for bumblebee decline depending on the location in the world (Williams and Osborne, 2009). Introduction of exotic bumblebees (Matsumura et al., 2004; Inoue et al., 2008), spread of pathogens (Colla et al., 2006; Otterstatter and Thomson, 2008), use of pesticides and herbicides (Williams, 1986b) and urbanisation (Williams, 1986b) are all thought to be factors for bumblebee decline in different parts of the world.

In comparison to the more common bumblebee species, rare bumblebee species are known to be declining at a faster rate and suffering more from localised extinctions (Williams and Osborne, 2009). While the mechanisms behind these differential declines remain unclear, a series of papers (Williams, (1985a, 1989)) suggested that niche differences between bumblebee species, combined with habitat destruction and, potentially, climatic changes, are responsible for the more rapid decline of some species in relation to others. Consequently, there have been calls for more studies on the ecological niches of the rare species to help understand the mechanics behind this loss of biodiversity (Goulson et al., 2005; Fitzpatrick et al., 2007).

Springtime habitat niches are of high ecological importance for the propagation and survival of annual insects, including bumblebee species. In spring, the sole procreating unit of the nest, the queen bumblebee, emerges from hibernation. To survive and reproduce successfully a spring bumblebee queen must rapidly replenish her fat supplies and successfully establish a nest in the space of a few weeks (Alford, 1975). However there are different nesting choice (Sladen, 1912; Free and Butler, 1959; Harder, 1986; Fussell and Corbet, 1992) and foraging preferences between the bumblebee species (Brian, 1952; Teräs, 1976; Heinrich, 1976a, 1976b; Ranta and Lundberg, 1980; Ranta and Vepsäläinen, 1981; Teräs, 1985). Consequently for a landscape to be considered prime for bumblebees, it must provide a diverse range of habitats with a wide range of suitable nesting locations and foraging types. However, the springtime habitat niches that bumblebee queens of the rare species require for nesting and foraging are virtually unknown.

Nesting locations of bumblebee queens are notoriously difficult to find in large numbers. Attempts to quantify the nesting locations of bumblebees such as training a sniffer dog (Waters et al., 2010), placement of artificial nest boxes (Norgaard Holm, 1966; Richards, 1973, 1978; MacFarlane et al., 1983; Fussell and Corbet, 1992), and observation of workers movements during the summer months (Aislinn Deenihan, personal observation) have provided limited results. Observation of bumblebee queens exhibiting nest-site seeking behaviour has been used most recently to quantify the nesting locations of bumblebees (Svensson et al., 2000; Kells and Goulson, 2003; Lye et al., 2009). Previous research has primarily focused on nest-site seeking behaviour and nest locations in intensive agricultural landscapes (Svensson et al., 2000; Lye et al., 2009), agricultural landscapes where agri-environmental and wildlife friendly farming schemes are in place (Kells and Goulson, 2003; Lye et al., 2009) and highly humanised environments such as gardens and parks (McFrederick and LeBuhn, 2006; Osborne et al., 2008). There has been much data collected on the ubiquitous bumblebee species through these studies but there is still a dearth of knowledge on the nest-site seeking behaviour, nest locations and foraging requirements of queens of the rare bumblebee species. Prime landscapes can provide the location to elucidate this information.

This study aims to

- determine the nest-seeking preferences of bumblebees in a prime landscape
- investigate if there is a correlation between habitat features and bumblebee abundance and species number in a prime landscape.
- investigate the foraging of bumblebee queens in late spring/early summer in a prime landscape

2.3 Materials and Methods

2.3.1 Overview of Methods

Nest-site seeking behaviour and bumblebee queen foraging was investigated using the prime landscape of the Burren region as a case study. A prime landscape is interpreted in this study as an area with habitat(s) that host a wide range of species including those that are considered rare due to the provision of apt living conditions (both biotic and physical) in the area. A nest-site seeking queen has been defined as a queen exhibiting low flight close to ground, flying in a zigzag fashion and those observed crawling on the ground. Bumblebee queen nest-site seeking behaviour primarily occurs along field boundaries and open areas (Svensson et al., 2000). Foraging bumblebee queens were defined as those collecting nectar and/or pollen from flowering plants. The cryptic species group *B. lucorum*, *B. magnus* and *B. cryptarum* were all recorded as the species *Bombus lucorum* agg. following research by Murray et al. (2008). The species and caste type of each bumblebee observed was identified following Prŷs-Jones and Corbet (1991). The taxonomy for *B. muscorum* was detailed according to the two colour morphs found in the region. Within the Burren region there is a melanic colour variety of *B. muscorum* known as the Aran Island bumblebee, *B. muscorum* var. *allenellus*. The melanic morph is restricted to the Aran Islands in Ireland. The need for the conservation of *B. muscorum* var. *allenellus* was noted by Fitzpatrick et al. (2006). The colour variety that is recorded in the rest of Ireland is known as *B. muscorum* var. *pallidus.* The taxonomy was described in this way to allow for examination of the nest-site seeking preferences between the two colour varieties.

A combination of standing at one point and a quadrat method was used to observe nest-site seeking behaviour (following Carvell, 2002). This method was adopted because it allows a greater area to be covered and it does not require straight line transects which are problematic in the Burren region. The Burren region is highly divided among hundreds of small farmers with field sizes of 10m x 20m present on one of the Aran Islands (Aislinn Deenihan, personal observation). The chosen methodological combination allowed observation of species nest site-seeking behaviour along field margins and open areas. This method was also used to investigate foraging behaviour. The statistical tests that could be applied were limited due to the low number of records for certain species of bumblebee exhibiting nest-site seeking and foraging. Absence of scrub and improved grassland habitat type on the Aran Islands prevented overall comparison of habitat and boundary types within the Burren region. However, the methods used allowed the number of nest seeking bumblebees to be quantified for each habitat type and associated boundary type, and data on foraging bumblebee queens to be collated.

2.3.2 Study Sites

The largest habitat types in the Burren region are calcareous grassland, improved grassland and limestone pavement (Parr et al., 2007). Bumblebee queens exhibiting nest-site seeking behaviour and foraging were recorded in these habitats with the different boundary types: scrub, hedgerow and stonewall as defined by Fossitt (2000) and delineated by Parr et al. (2007).

2.3.3 Sampling

Sampling took place in the mainland Burren region and Aran Islands (Fig. 5). Thirty-six 10m x 10m quadrats were established in the mainland Burren region of the County Clare mainland (Fig. 6). Twelve were established in each of the main habitat types in the mainland Burren region: calcareous grassland, improved grassland and limestone pavement. Each quadrat was established with one of the different boundary types: scrub, hedgerow and stonewall – four quadrats with each boundary type. Eighteen similar quadrats were established on each of the two of the three Aran Islands, (Inis Óirr and Inis Meáin) with boundary types of Hedgerow and Stonewalls (Improved Grassland habitat and Scrub boundary types do not occur on these two Aran Islands) (Fig. 7). The quadrats were chosen randomly using habitat maps and visual observation in the study sites. The quadrats were chosen using habitat maps and visual observation in the study sites. Approximately twice as many quadrats were chosen initially as desired. There was an excess of potential quadrats at the preliminary stage of this study as land access negotiations with landowners were still taking at this stage. The final quadrats were chosen by picking numbers from a hat, with each number representing a quadrat with access permission. Quadrats were separated by from 200 m to 20 km, with the shortest distances being, of necessity, on the islands.

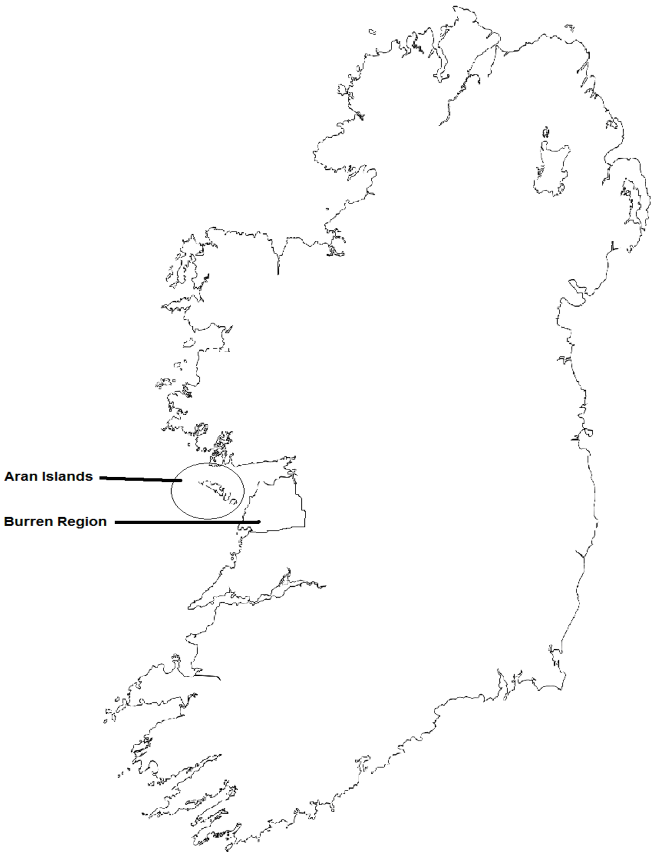


Fig. 5 – Outline of Ireland showing the location of the mainland Burren region and Aran Islands

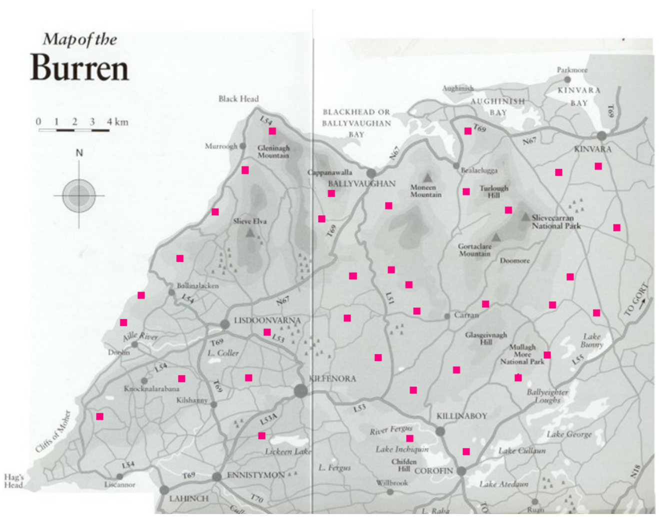


Fig. 6 – Map of the mainland Burren region (Clare County Library, 2007) with the location of each quadrat indicated by a red square

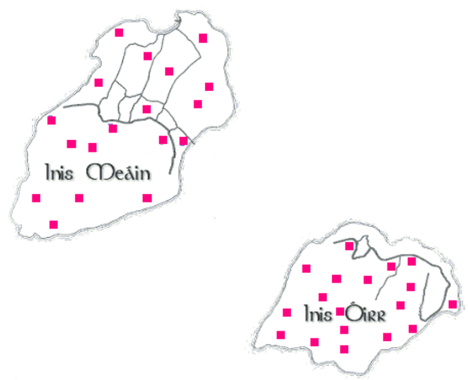


Fig. 7 – Outline map of the two Aran Islands sampled, Inis Oirr and Inis Meain, with the location of each quadrat indicated by a red square

Bumblebee species differ in their emergence time (Alford, 1975). To facilitate the inclusion of the largest number of bumblebee species exhibiting nest-site seeking behaviour, the study took place from mid-spring to early summer, between the dates of April 18th to June 18th in the years 2008 and 2009. The start date was determined by the onset of appropriate weather conditions and the study was limited to this early and mid-season to minimise the effects of the bumblebee parasite *Sphaerularia bombi*. The parasite affects the queen's orientation and nest seeking behaviour causing them to dig and nest in unusual places, and nest seek throughout the summer until death (Lundberg and Svensson, 1975).

Certain climatic and time conditions were adhered to when collecting data for the study. Field sites were only visited when temperatures and wind speed ranged from 6-24°C and 0-4m/s respectively. No observations were taken during precipitation and when wind speed was over 4m/s. The time of inspections varied randomly between sites and in random orders during the day between 08:30 and 19:30. Each study site was visited a maximum of twice a week and not on consecutive days. This sampling strategy was adopted for two reasons. Firstly, to avoid oversampling nest-site seeking queens who visit favourable nesting locations multiple times before establishing a nest. Secondly, to avoid over sampling certain species, as different species of bumblebee nest-site seek at different times due to differences in emergence times from overwintering. Bumblebee queens exhibiting nest site-seeking behaviour were counted and identified in each quadrat on six occasions for 15 min each, for a total of 1.5 h of observation. All bumblebee queens foraging on the study quadrats during this time were also recorded. Quadrats were not taken to quantify the available forage as the highly heterogeneous nature of the Burren landscape prevents an even distribution of forage and bumblebee species even within the same habitat type. Additionally because of the length of the study (approximately three months) there would be different flower species predominant at different times further skewing any analysis.

2.3.4 Bumblebee Nest Locations

Bumblebee nests located during the study had the species and the habitat and boundary types present recorded. Additionally the following vegetation characteristics surrounding the nest were recorded following Carvell (2002); vegetation structure, vegetation height, depth of ground moss, and percentage of bare rock present.

2.3.5 Analysis

The data were analyzed using Microsoft Excel ®, SPSS ver. 16, Canoco ver. 4.5. To investigate the effect of habitat and boundary type on bumblebee nest site-seeking queens, the records for the total number of bumblebee queen species at each site were converted into presence/ absence data. Cross tabulation and chi-squared tests were then performed in SPSS ver. 16 on the results for habitat type and then boundary type. Canoco ver. 4.5 was used to further analyse the data. Following preliminary assessment of the data using a detrended correspondence analysis (DCA), a canonical correspondence analysis (CCA) was performed on the data. The habitat / environmental variables were input as dummy (0, 1) variables and combined to provide 12 environmental variables. Canonical correspondence analysis (CCA) was employed because a preliminary analysis using DCA of the species data indicated that the method was appropriate (length of gradient = 3.913). The habitat / environmental variables were input as dummy (0, 1) variables and combined within Canoco ver 4.5 to provide 9 environmental variables (calcareous grassland * hedge; calc_grass * wall; calc_grass * scrub; similarly for improved grassland and bare rock). In CCA, the Canoco ver 4.5 forward selection procedure was used to select variables, with acceptance of entry at $P<0.05$. Once no further variables were available for selection (i.e. all remaining had P for entry >0.05), the procedure was re-run including only the selected variables which were entered simultaneously. The statistical significance of the model was tested using Monte Carlo permutation tests under a reduced model for the canonical axis (1000 permutations) and environmental variable-axis relationships. Automatic forward selection was used on the environmental variables. Only bumblebee species where more than 10 queens were observed exhibiting nest site-seeking behaviour were evaluated. A co-occurrence test was run in EcoSim ver. 5.0 (Gotelli and Entsminger, 2000) to test for significance of association between bumblebee species and plant species visited. Cross-tabulation was performed in SPSS ver. 16 to determine the observed and expected counts between each of the bumblebee species recorded foraging and the associated flowering plants, and to determine if chi-squared or G- tests should be performed. .

2.3 Results

2.3.1 Number of Bumblebee Queen Species Observed and Number of Bumblebees Recorded Nest-Site Seeking

In total, 108 field hours were spent observing the quadrats while investigating the nest-site seeking behaviour and foraging of bumblebee queens. Twelve species of bumblebee queen were observed and the date of their first emergence was noted (Table 3). *B. sylvarum* was recorded throughout the Burren mainland region on the three habitat types; calcareous grassland, limestone pavement and improved grassland. In 2008 and 2009, *B. lucorum* agg., *B. jonellus*, *B. pascuorum*, *B. pratorum* and *B. terrestris* were all observed in the Burren region prior to the initiation of the study. Species considered uncommon in north western Europe that were observed during the study included *B. distinguendus*, *B. muscorum pallidus*, *B. muscorum allenellus*, *B. campestris*, *B. bohemicus* and *B. sylvarum*. All species bar *B. bohemicus* were observed in both study years.

The combined number of bumblebees recorded nest-site seeking for the study period in 2008 and 2009 was 222, representing nine species (Fig. 8). Most of these observations occurred in 2008 (69.4%). The cuckoo bumblebees *B. bohemicus* and *B. campestris* were also observed nest seeking but were not recorded as they ultimately depend on the nest seeking preferences of their host species. *Bombus pascuorum* was recorded nest seeking most often (37.8%), followed by the *B. lucorum* agg. (19.8%), *B. lapidarius* (15.3%), *B. sylvarum* (10.4%), *B. ruderarius* (4.9%), *B. muscorum allenellus* (4.1%), *B. terrestris* (3.2%), *B. jonellus* (2.3%) and *B. hortorum* (2.3%).

Table 3 - Observed bumblebee queen species and date of first observation time

* where the term prior emergence is used in lieu of a date, it indicates the species was observed nest-site seeking in the Burren region in preliminary field work prior to the initiation of the study.

Bumblebee Species	**Date Of First Observation**	
	2008	**2009**
B. bohemicus	May 1st	Not observed
B. campestris	May 1st	May 2nd
B. hortorum	May 15th	May 10th
B. jonellus	Prior emergence	Prior emergence
B. lapidarius	Prior emergence	Prior emergence
B. lucorum agg.	Prior emergence	Prior emergence
B. muscorum var. *allenellus*	April 24th	May 1st
B. muscorum pallidus	April 24th	May 16th
B. pascuorum	Prior emergence	Prior emergence
B. pratorum	Prior emergence	Prior emergence
B. sylvarum	May 10th	May 16th
B. terrestris	Prior emergence	Prior emergence

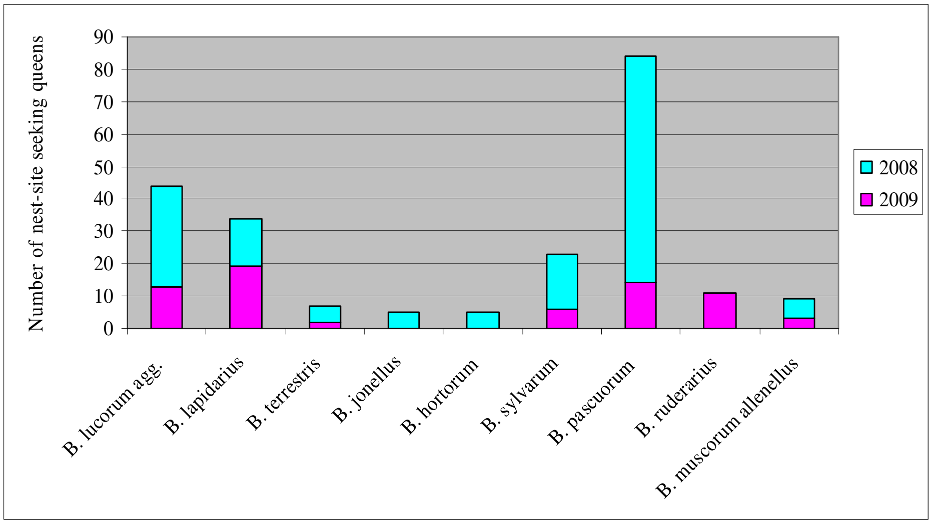


Fig. 8 - Number of nest-seeking bumblebees observed for each species

2.3.2 Observed Bumblebee Queen Preferences and Associations with Habitat and Boundary Types.

Bombus sylvarum, *B. ruderarius* and *B. pascuorum* showed significant preferences for habitat type (Table 4). No significant relationships were recorded with boundary type (Table 5). Further analysis carried out in Canoco ver. 4.5 showed, in more detail, the relationships between the combined habitat and boundary type and bumblebee species. The overall CCA ordination was significant (F = 1.617; p = 0.015). The results of the ordination are in Table 6 and Fig. 9. The ordination diagram (Fig. 9) suggests that the nest-site seeking behaviour of *B. sylvarum*, *B. ruderarius* and *B. pascuorum* is associated with calcareous grassland stonewalls and scrub type boundaries. *Bombus lucorum* agg. was most associated with improved grassland with a hedgerow boundary, and limestone pavement with a stone wall boundary. *B. lapidarius* was most associated with limestone pavement with a hedgerow boundary, and improved grassland with a scrub boundary. In an effort to separate the effects of the island and mainland, an identical analysis was carried out on island-only and mainland-only data. The island-only analysis was not significant (F-ratio = 1.18; p = 0.3077). Likewise, the mainland-only analysis was not significant (F-ratio = 2.363; p = 0.7203).

In order to compare the nesting behaviour patterns on the islands versus the mainland, a CCA analysis was then carried out which was confined to those habitats which occurred on both islands and mainland. Hence, observations made in improved grasslands and on scrub boundaries were excluded as these did not occur in the island samples. This left a database of 96 rows for the analysis. The CCA analysis was carried out, as before, by first selecting significant variables using forward selection. Only island_calcareous_wall and island_calcareous_hedge were significant. The ordination is shown in Fig. 10. Total inertia was 3.168; F-ratio was 4.114 and p = 0.002.) *Bombus terrestris* was the only species that showed an association and this was with Calcareous grassland with a hedgerow boundary.

Table 4 -Chi-squared results from presence absence tests using cross tabulation for habitat type (NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = *)**

Bumblebee Species	**Chi-Squared**	**Df**	**Significance**
B. hortorum	4.05	2	NS
B. jonellus	0.47	2	NS
B. lapidarius	6.76	2	NS
B. lucorum agg.	5.30	2	NS
B. muscorum var. *allenellus*	4.40	2	NS
B. pascuorum	17.92	2	**
B. ruderarius	8.97	2	*
B. sylvarum	8.14	2	*
B. terrestris	2.98	2	NS

Table 5 - Chi-squared results from presence absence tests using cross tabulation for boundary type (NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = *)**

Bumblebee Species	Chi-Squared	Df	Significance
B. hortorum	0.02	2	NS
B. jonellus	2.97	2	NS
B. lapidarius	4.27	2	NS
B. lucorum agg.	3.29	2	NS
B. muscorum var. *allenellus*	1.821	2	NS
B. pascuorum	1.623	2	NS
B. ruderarius	0.216	2	NS
B. sylvarum	0.705	2	NS
B. terrestris	9.57	2	NS

Table 6 - Details of CCA relating habitat variables and bumble bee nest-site seeking behaviour.

*N/A = not applicable

Axes	1	2	3	Total Inertia
Eigenvalues	0.230	0.143	0.104	3.722
Species specific environmental correlations	0.684	0.552	0.444	N/A
Cumulative percentage of variance				
Of species data	6.2	10.0	12.8	N/A
Of species-environmental relation	35.9	58.2	74.5	N/A

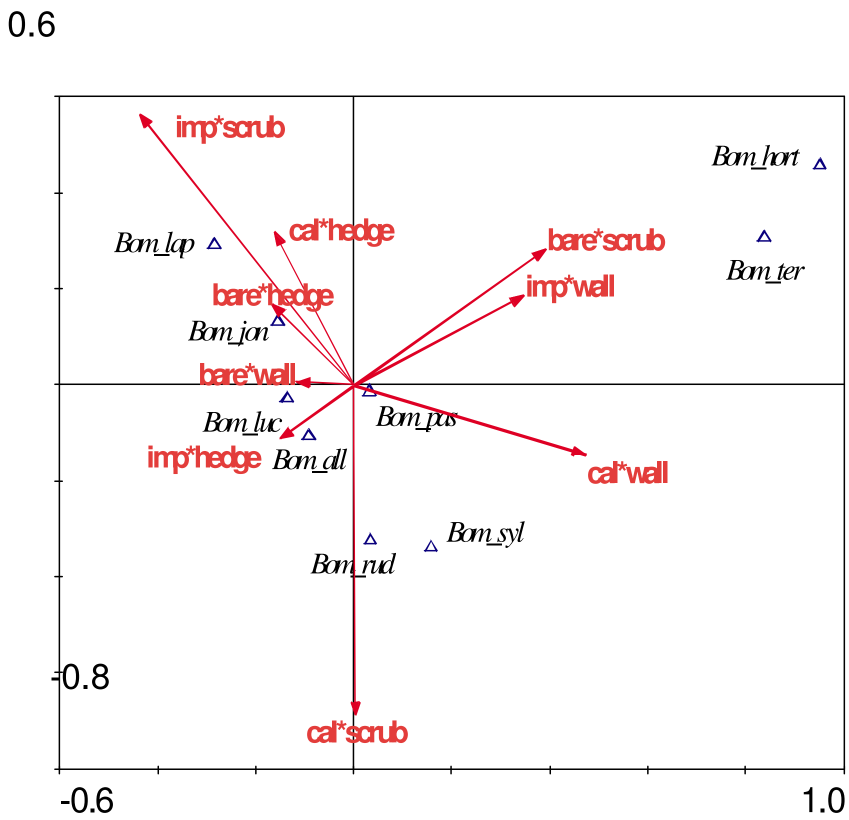


Fig. 9 - CCA ordination of bumblebee nest seeking behaviour in different habitat types * boundary types. Shorthand notation: Bare =Limestone pavement, Cal = Calcareous Grassland, Imp = Improved GrasslandHedge =Hedgerow boundary, Scrub = scrub boundary, Wall = Stone wall boundary,Bom_jon = *Bombus jonellus*, Bom_syl = *Bombus sylvarum*, Bom_rud = *Bombus ruderarius*, Bom_all = *Bombus muscorum* var. *allenellus*, Bom_luc = *Bombus lucorum* agg., Bom_lap = *Bombus lapidarius*, Bom_hort = *Bombus hortorum*, Bom_terr = *Bombus terrestris*, Bom_pas = *Bombus pascuorum.*

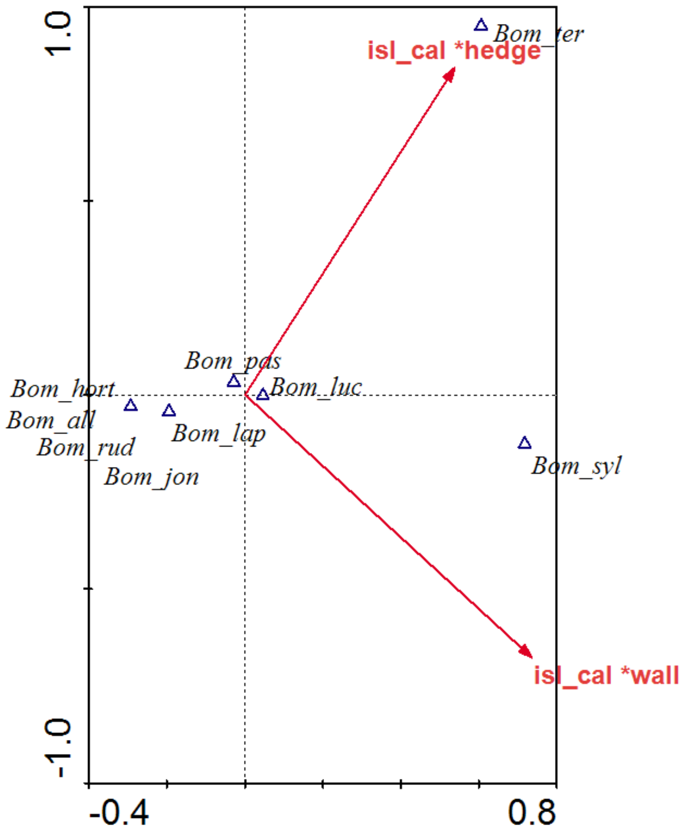


Fig. 10 - CCA ordination of bumblebee nest seeking behaviour in different habitat types * boundary types. Shorthand notation: Isl = Island, Cal = Calcareous Grassland, Hedge =Hedgerow boundary, Wall = Stone wall boundary, Bom_jon = *Bombus jonellus*, Bom_syl = *Bombus sylvarum*, Bom_rud = *Bombus ruderarius*, Bom_all = *Bombus muscorum* var. *allenellus*, Bom_luc = *Bombus lucorum* agg., Bom_lap = *Bombus lapidarius*, Bom_hort = *Bombus hortorum*, Bom_terr = *Bombus terrestris*, Bom_pas = *Bombus pascuorum*.

2.3.3 Bumblebee Queen Foraging and Foraging Analysis

Bumblebee queen foraging was recorded 265 times by 10 different bumblebee species. Observations of bumblebee queens foraging were recorded at 15 different flowering plants representing eight different flower families (Table 7-9). *Lotus* *corniculatus*, *Vicia cracca* and other members of the Fabaceae family were the most utilised forage and plant family (Fig. 11). All bumblebee species observed fed from *Lotus corniculatus*, while *B. lapidarius* and *B. ruderarius* were recorded foraging most often from this plant. *Vicia cracca* visits consisted of the greatest proportion of records for *B. pascuorum* and *B. sylvarum*. Only *B. lucorum* agg. and *B. muscorum* var. *allenellus* were recorded visiting *Taraxacum* spp. *B. lapidarius*, *B. pascuorum* and *B. ruderarius* were recorded foraging from *Trifolium pratense* and *B. sylvarum* was the only bumblebee queen species observed foraging from *Ajuga reptans*.

EcoSim ver. 5.0 (Gotelli and Entsminger, 2000) was used to estimate the co-occurrence between bee species and flowers visited. The default options of Ecosim were used (and give these). The co-occurrence index was 3.022 (with upper tail $p = 0.968$ and lower $p = 0.034$) which shows that there is a significant relationship between the bees/flowers visited. Cross tabulation was used to further explore appropriate tests to investigate the individual relationships. A cross tabulation statistical test was computed in SPSS ver. 16 to investigate the feasibility of performing a chi-squared test on the foraging preferences of the bumblebee species recorded. Cross tabulation results found that more than one-fifth of the expected counts were less than five (Table 10). These cross tabulation results invalidated the use of chi-squared tests on this data. G-tests in SPSS ver. 16 were deemed to be the next most appropriate statistical test.

G-tests were used to determine if there were differences in preferences in foraging between the different species of bumblebee queen, and if so to determine interspecies differences. Only species that had more than 10 records of foraging were included in the analysis. G-test results showed differences in foraging preferences between the species ($p<0.001$) (Tables 11-16). Interspecies G-test analysis showed that bumblebee species *B. lucorum, B. pascuorum B. sylvarum,* had significantly different interspecies foraging preferences, while the other three species only showed significant differences among the species. The species *B. muscorum* var. *allenellus* did not significantly differ in foraging preferences from *B. lapidarius* and *B. ruderarius*. Additionally *B. lapidarius* and *B.ruderarius* did not differ significantly in foraging preferences.

Table 7 - Number of bumblebee species observed feeding per flowering plant - Fabaceae Family

	Flowering Plant Species **Fabaceae**				
Bumblebee species	*Lotus corniculatus*	*Anthyllis vulneraria*	*Vicia cracca*	*Trifolium pratense*	*Lathyrus pratensis*
B. campestris	0	0	2	0	0
B. hortorum	1	0	1	1	0
B. lapidarius	12	0	0	4	0
B. lucorum	2	0	0	0	0
B. muscorum var. *allenellus*	19	0	1	4	0
B. pascuorum	23	4	58	20	5
B. pratorum	0	0	0	0	0
B. ruderarius	21	0	0	2	0
B. sylvarum	5	0	17	0	0
B. terrestris	0	0	0	0	0
Total	83	4	79	31	5

Table 8 - Number of bumblebee species observed feeding per flowering plant - Lamiaceae, Primuleae, Asteraeae and Orchideae families

	Flowering Plant Species				
	Lamiaceae	**Primuleae**		**Asteraceae**	**Orchideae**
Bumblebee species	*Ajuga reptans*	*Primula veris*	*Primula vulgaris*	*Taraxacum* spp.	*Orchis mascula*
B. campestris	0	0	0	0	0
B. hortorum	0	0	0	0	0
B. lapidarius	0	0	0	0	0
B. lucorum	0	0	0	24	3
B. muscorum var. *allenellus*	0	0	1	7	0
B. pascuorum	0	5	8	1	0
B. pratorum	0	0	0	1	0
B. ruderarius	0	0	0	0	0
B. sylvarum	12	0	0	0	0
B. terrestris	0	0	0	2	0
Total	12	5	9	35	3

Table 9 -Number of bumblebee species observed feeding per flowering plant- Geraniceae, Violaceae and Rosaceae families

	Flowering Plant Species				
	Geraniceae		**Violaceae**	**Rosaceae**	
Bumblebee species	*Geranium robertianum*	*Geranium sanguineum*	*Viola* spp.	*Prunus spinosa*	*Geum rivale*
B. campestris	0	0	0	0	0
B. hortorum	0	0	0	0	0
B. lapidarius	0	0	0	0	0
B. lucorum	3	1	0	2	2
B. muscorum var. *allenellus*	0	1	0	0	0
B. pascuorum	0	0	1	0	0
B. pratorum	0	0	0	0	0
B. ruderarius	0	0	0	0	0
B. sylvarum	0	0	0	0	0
B. terrestris	0	0	0	0	0
Total	3	2	1	2	2

Table 10 – Cross tabulation results on the foraging preferences of the bumblebee queens observed in the quadrats, showing more than one fifth of the expected counts to be less than 5

		Bombus lapidarius	*Bombus lucorum* agg.	*Bombus pascuorum*	*Bombus muscorum* var. *allenellus*	*Bombus ruderarius*	*Bombus sylvarum*	Total
Lotus corniculus	Count	15	2	23	16	19	5	80
	Expected Count	5.9	10.5	36.4	9.8	6.9	10.5	80.0
Vicia cracca	Count	0	0	62	1	0	15	78
	Expected Count	5.8	10.2	35.5	9.6	6.7	10.2	78.0
Trifolium pratense	Count	3	0	21	4	2	0	30
	Expected Count	2.2	3.9	13.6	3.7	2.6	3.9	30.0
Taraxacum spp.	Count	0	25	1	8	0	0	34
	Expected Count	2.5	4.5	15.5	4.2	2.9	4.5	34.0
Prinos spinosa	Count	0	2	0	0	0	0	2
	Expected Count	.1	.3	.9	.2	.2	.3	2.0
Geranium sanguineum	Count	0	1	0	1	0	0	2
	Expected Count	.1	.3	.9	.2	.2	.3	2.0
Primula vulgaris	Count	0	2	0	0	0	0	2
	Expected Count	.1	.3	.9	.2	.2	.3	2.0
Lathyrus pratensis	Count	0	0	4	0	0	0	4
	Expected Count	.3	.5	1.8	.5	.3	.5	4.0
Ajuja reptans	Count	0	0	0	0	0	12	12
	Expected Count	.9	1.6	5.5	1.5	1.0	1.6	12.0
Total	Count	18	32	111	30	21	32	244
	Expected Count	18.0	32.0	111.0	30.0	21.0	32.0	244.0

Table 11 - G-test results for *B. lapidarius* interspecies foraging differences

Bumblebee Species	**Df**	**Significance**
B. lucorum agg.	4	***
B. muscorum var. *allenellus*	4	NS
B. pascuorum	4	***
B. ruderarius	1	NS
B. sylvarum	3	***

(NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = ***)

Table 12 - G-test results for *B. lucorum* agg. interspecies foraging differences

Bumblebee Species	**Df**	**Significance**
B. lapidarius	4	***
B. muscorum var. *allenellus*	6	***
B. pascuorum	7	***
B. ruderarius	5	***
B. sylvarum	6	***

(NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = ***)

Table 13 - G-test results for *B. muscorum allenellus* interspecies foraging differences

Bumblebee Species	**Df**	**Significance**
B. lucorum agg	4	***
B. lapidarius	6	NS
B. pascuorum	5	***
B. ruderarius	4	NS
B. sylvarum	5	***

(NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = ***)

Table 14 - G-test results for *B. pascuorum* interspecies foraging differences

Bumblebee Species	**Df**	**Significance**
B. lapidarius	4	***
B. lucorum agg.	7	***
B. muscorum var. *allenellus*	5	***
B. ruderarius	4	***
B. sylvarum	5	***

(NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = ***)

Table 15 - G-test results for *B. ruderarius* interspecies foraging differences

Bumblebee Species	**Df**	**Significance**
B. lapidarius	1	NS
B. lucorum agg.	5	***
B. muscorum var. *allenellus*	4	NS
B. pascuorum	4	***
B. sylvarum	3	***

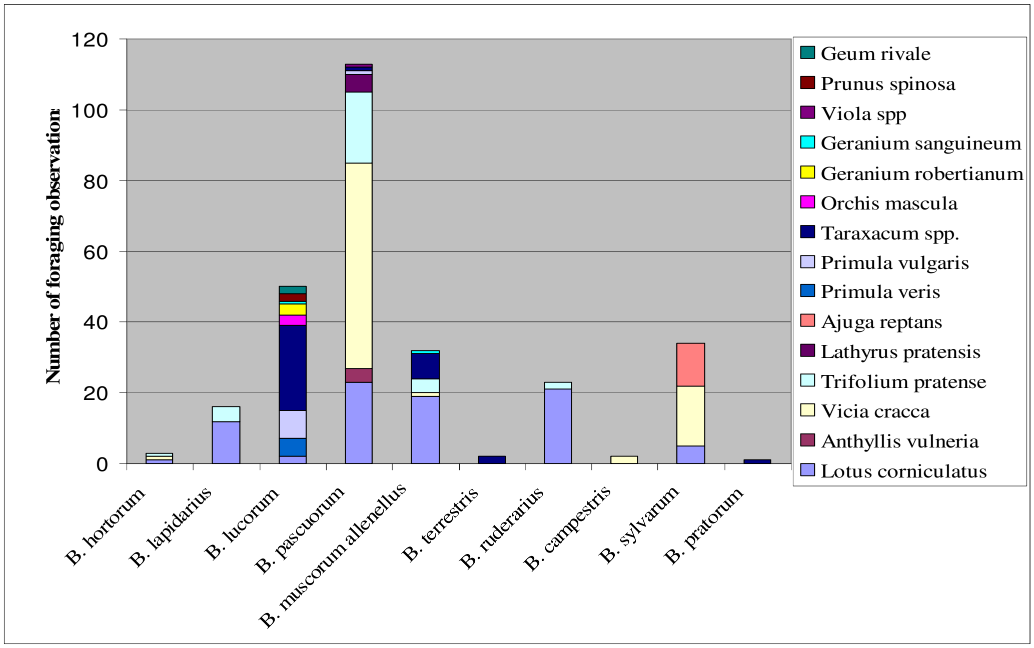
(NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = ***)

Table 16 - G-test results for *B. sylvarum* interspecies foraging differences

Bumblebee Species	**Df**	**Significance**
B. lapidarius	3	***
B. lucorum agg.	6	***
B. muscorum var. *allenellus*	5	***
B. pascuorum	5	***
B. ruderarius	3	***

(NS= not significant, P< 0.05= *, P<0.01= **, P<0.001 = ***)

Fig. 11 - Abundance of bumblebee species foraging at each plant species



2.3.4 Located Bumblebee Nests

Eight nests of *B. muscorum* var. *allenellus* and three of *B. pascuorum* were found at the end and directly after the study period. Of the *B. muscorum* var. *allenellus* nests, four of the these nests were located by hay cutters on the Aran Islands, two were found through observation of foraging *B. muscorum* var. *allenellus* workers, and two others were found by observing the cuckoo bumblebee *B. campestris* exhibiting nest-site seeking behaviour. All of the *B. muscorum* var. *allenellus* nests located were found in calcareous grassland habitat with a stone wall boundary. Two *B. pascuorum* nests were located through observation of workers in calcareous grassland with a hedgerow boundary and the other during hay cutting in calcareous grassland with a stone wall boundary. All the nests located were typical of the species of sub-genus *Thoracobombus*, being made of moss and located above ground. All nests were located on the northern sides of the nearest boundary. Vegetation characteristics (following Carvell, 2002) surrounding the nests were recorded, and are displayed in Table 17.

Table 17 -Vegetation characteristics surrounding located bumblebee nests

(Vegetation structure was scored on a scale of 1-4 where 0 = very open, sunlight penetrating more than 70% of the ground layer and where 4 = dense, sunlight reaching less than 10% of the ground layer (Carvell, 2002). Vegetation height was ranked according to height intervals, where 1 = less than 25cm, 2 = 25cm – 50cm, 3 = 50cm- 100cm, and 4 = >100cm)

Bumblebee Species	**Nest Number**	**Vegetation Height**	**Depth Of Moss/Grass Litter(cm)**	**Vegetation Structure**	**% Bare Rock**
B. muscorum var. *allenellus*	1	4	1.5	3	0
B. muscorum var. *allenellus*	2	4	1.4	3	0
B. muscorum var. *allenellus*	3	4	1.3	3	0
B. muscorum var. *allenellus*	4	4	1.2	3	0
B. muscorum var. *allenellus*	5	4	1.4	3	0
B. muscorum var. *allenellus*	6	4	1.4	3	0
B. muscorum var. *allenellus*	7	4	1.5	3	0
B. muscorum var. *allenellus*	8	4	1.5	3	0
B. pascuorum	9	4	1.3	3	0
B. pascuorum	10	3	1.4	3	0
B. pascuorum	11	4	1.4	3	0

2.4 Discussion

The basic ecological requirements of queens of rare bumblebee species are mostly unknown. Here it is shown that the nest-site seeking behaviours of rare queens differ from those of common, more easily observed species. This suggests that conservation action for rare species must be underpinned by species specific ecological studies. For example, the nest-site seeking behaviour of the rare bumblebee species *B. sylvarum* and *B. ruderarius,* and the ubiquitous bumblebee species *B. lucorum* agg. and *B. pascuorum* was recorded. Statistical analysis showed that the two rare species had a preference for exhibiting nest-site seeking behaviour in calcareous grassland type habitat with a scrub type boundary while the common species *B. lucorum* agg had a preference for nest-site seeking in improved grassland habitat with a hedgerow habitat, and *B. pascuorum* had a preference for calcareous grassland with a stonewall boundary. These results suggest that it may be unsound to extrapolate ecological information derived from the nest-site seeking behaviour of common species to rare species.

Many conservation studies have placed an emphasis on obtaining and analysing data on the foraging of rare bumblebees (e.g. Carvell, 2002 ; Goulson et al., 2005; Fitzpatrick et al., 2007), as the availability of forage is known to be a factor in the decline of bumblebees (Goulson and Darvill, 2004; Williams, 2005). However, results from this study suggest that other ecological factors such as availability of nesting sites need to be considered when investigating the increased rarity of one species in relation to another in some areas. For example the endangered bumblebee, *B. sylvarum* was recorded foraging from common plants such as *Ajuga reptans* and *Vicia cracca*, both of which are considered common in Ireland. *B. sylvarum* queens were also recorded foraging on improved grassland in spite of not being recorded nest site-seeking on that habitat type during this study. The decline of calcareous grasslands has had a detrimental effect on the distribution of *B. sylvarum* (Carvell, 2002; Benton, 2006), yet in this study it is not limited to calcareous grassland for foraging. These results suggest that declining *B. sylvarum* populations are not limited by forage, but by other constraints, such as nest-sites and hibernation sites. Alternatively, foraging in improved grasslands may actually be a response to limited forage in the prime habitat. Further research on the ecological niches of *B. sylvarum* is required to distinguish between these explanations.

Observation of nest-site seeking behaviour has become an accepted methodology for quantifying the suitability of habitats for bumblebee queens to nest, due to the extreme difficulty in locating bumblebee nests (Svensson et al., 2000; Kells and Goulson, 2003). Results from this study enabled a tentative examination of this methodology. The discovery of a limited number of nests enabled comparison between preferred locations for bumblebee nesting from observing nest-site seeking behaviour and the actual locations of bumblebee nests. Two of the recorded nest sites of *B. pascuorum* agreed with the CCA results for the nest-site seeking behaviour. However there were another two nests located in calcareous grassland with a hedgerow boundary, and the CCA did not produce an association as high for this result. While a much larger number of bumblebee nests need to be located in an area where nest-site seeking behaviour for the different bumblebee species has been recorded before a definitive answer can be made on the fallibility of observing the nest-site seeking behaviour of bumblebees, our results suggest that the behavioural method has some validity. Alternative methodologies for locating nests such as observing cuckoo bumblebees nest seeking and other parasites of bumblebee nests might prove successful in prime landscapes. The location of nesting sites is an important aspect of the ecology of rare bumblebee species that needs further investigation but is hampered by the difficulty of studying rare species with limited distributions. As can be seen from this study, the results from the CCA of nest site seeking queens of *B. muscorum* var. *allenellus* were not analysed due to the low number of records and thus could not be correlated with the actual location of bumblebee nest found.

In contrast to previous studies, which concentrated on intensive and semi-natural agricultural systems and common bumble bees (Svensson et al., 2000; Svensson, 2002; Kells and Goulson, 2003; Lye et al., 2009), this study has produced valuable data on the foraging behaviour of both rare and common bumblebee queens in potentially their optimum habitats. Significant interspecies foraging differences were found between bumblebee species recorded in this study. These results could be due to the greater variety and availability of flowering plants in prime landscapes. However, it may also indicate that when a greater variety and larger area of flowering plants are available bumblebee species have different preferences and a greater degree of polylecty. Consequently, these results may provide a unique insight that can be extrapolated to the development of agri-environmental schemes. Clear differences, presumably due to habitat quality, exist between our results on nest-site seeking and queen foraging behaviour and those of previous studies. For example, Lye et al. (2009) described legumes such as *Lotus corniculatus* as of little importance to the early stages of bumblebee colony establishment and growth, while our data show the late emerging species of *B. muscorum* var. *allenellus*, *B. pascuorum*, *B. sylvarum*, and *B. ruderarius* foraging from it extensively. Even queens of the early emerging species group *B. lucorum* agg. were recorded foraging from *Lotus corniculatus*. Fitzpatrick et al. (2007) described the late emerging bumblebee species such as *B. sylvarum* and *B. muscorum* as those experiencing the most decline in Britain and in Ireland and postulated that this decline could be due to changes in land use. In the British Isles, changing land use has resulted in a decrease in the area of calcareous grassland habitat (Rodwell, 1991). Calcareous grasslands support species rich grassland that support legume wildflowers (Rodwell, 1991) and results from this study and others (e.g. Carvell 2002; Santorum and Breen 2005; Goulson et al., 2006) have shown that queens of the rarer species feed in calcareous grassland and from the legume wildflowers supported by it. Thus the aim of promoting legumes in unimproved grassland margins often found in agro-environmental schemes may have a sound basis.

It is the extensive agricultural practises and conservation measures in the Burren region that maintain prime habitats such as calcareous grassland (Dunford, 2002, 2008) and that support these rare bumblebees. Currently, changing agricultural practises such as under and overgrazing, removal of field boundaries and application of fertilisers are threatening the biology of the Burren region (Dunford, 2002). Scrub encroachment is a growing problem in the Burren mainland region (ERA-Maptec et al., 2006; Parr et al., 2007) and is becoming a problem on the Aran Islands due to land abandonment and lack of maintenance (personal observation). The preferences of *B. sylvarum* and *B. ruderarius* for calcareous grassland habitat with a scrub boundary suggests that the species nest-site seeking activities could have a buffering capacity against the scrub encroachment on stone structures currently being reported in the Burren region (ERA-Maptec et al., 2006), as more scrub boundary habitat may become available for them to nest-site seek in. However, if the expansion of scrub is on calcareous grassland habitat, as is being reported in many places in the Burren region (ERA-Maptec et al., 2006; Parr et al., 2007), this buffering capacity will be non-existent as scrub expansion on calcareous grassland could have a potentially devastating impact on these species' survival in this region. Results in this study show that *B. sylvarum* and *B. ruderarius* have a preference for nesting in calcareous grassland habitat and were recorded most often feeding in calcareous grassland habitat, thus if scrub expands on calcareous grassland in the Burren region it will be affecting potential locations for the foraging and nest-site seeking of these species. The Burren region is the only region in Ireland known to sustain significant populations of *B. sylvarum,* as in the rest of Ireland it exists only in isolated pockets (Fitzpatrick et al., 2006b). Thus the survival of this species in the Burren region is essential for its persistence in Ireland.

2.5 Conclusions

Data obtained from this study highlight the importance of prime landscapes for biodiversity conservation. Studies in areas with extensive farming practices can give a snap-shot of what the biodiversity of north western Europe may have been pre- agricultural intensification. Environmentally friendly practices on farms can help a number of bumblebee species, e.g. in Scotland where agro-practices aimed at birds were found to also benefit bumblebee conservation (Redpath et al., 2010). It is the opinion of the author that farmers of regions participating in environmentally friendly agricultural practices need to be continually acknowledged and rewarded for their part in maintaining these landscapes to encourage their future participation. More research on insects within prime landscapes should be conducted so that applicable data can be applied to agri-environmental schemes to help promote insect conservation. Further research on the ecology of the bumblebee species *B. sylvarum* in prime habitats should be carried out as a matter of urgency due to its decreasing populations in Britain and Ireland (Ellis et al., 2006; Fitzpatrick et al., 2006b).

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Chapter 3 A Comparison of DNA Extraction Methods for Barcoding Historic Bumblebee Specimens

3.1 Abstract

Genetic studies of historic insect specimens depend on our ability to extract DNA of sufficient quantity and quality for PCR amplification and sequencing. This study investigated different methods for extracting DNA from museum specimens of bumblebees that ranged from 20 to 38 years old, which were killed and treated using different methods. Two different lysis approaches were tested to extract DNA from the samples. The first lysis approach involved a "non destructive" method used previously to extract DNA from old specimens of beetles. This method resulted in a deterioration of the morphological integrity of the samples through discoloration and was not utilised further. The second lysis approach involved five semi-destructive methods. These methods involved the removal and destruction of the mid-leg from the thorax of the bumblebee specimens. Only one of these methods was successful in repeatedly producing PCR amplified DNA. The successful method was a novel modification of the Qiagen PBS protocol for insects. The modification involved extending the incubation time in the digestion buffer from 25 min to four hours. The quality of the extracted DNA was assayed using PCR with primers for the cytochrome oxidase 1 DNA barcode region of the mitochondrial genome. An internal primer for the CO1 barcode region was designed which permitted successful PCR amplification. The method was successful in extracting DNA from queen and worker caste specimens. The author advocates care in the use of museum specimens for DNA extraction, and stresses the importance of preserving the diagnostic morphological characters of these specimens.

3.2 Introduction

DNA from insect specimens stored in museum and private collections can be an invaluable resource for conservation, phylogenetics and taxonomy (Cano, 1996; Hofreiter et al., 2001; Pääbo et al., 2004; Strange et al., 2009). DNA from museum specimens can be used to estimate the population structure of a species at the time of collection, and when compared with DNA from present day populations, current population health may be estimated, and the need for conservation measures deduced (Cano, 1996; Thompson et al., 2007). DNA sequences from historic insect specimens can be compared within and between species and correlated with geographic data allowing for the phylogeny and phylogeography of species to be elucidated (Pääbo et al., 2004). The author uses the description "historic specimen" to describe specimens collected 10 years before the date of DNA extraction as it was the author's observation that DNA obtained from specimens younger than 10 years generally amplified well when used in PCR. DNA obtained from historic specimens can aid in taxonomic classification through sequencing DNA from near type specimens that can be used as reference (Valentini et al., 2009). Such data from museum samples would be impossible to retrieve if it were not for advances in DNA extraction and DNA amplification techniques such as polymerase chain reaction (PCR) and sequencing technologies (Cano, 1996; Valentini et al., 2009).

The attraction of including historic insect specimens into molecular analysis is made more pertinent by the Barcoding of Life initiative. Under this initiative it is proposed that a c.650 bp region of the cytochrome c oxidase (CO1) mitochondrial gene (the DNA Barcode) is sequenced for all organisms on earth. These barcode sequences and collection and taxonomic records are stored in the DNA Barcode of Life Data system (BOLD, http://www.boldsystems.org) and as of December 2010 there are 841,679 viable barcode sequences thought to represent 82,581species. The principle aim of BOLD is to assist species identification and to make a contribution to the classification and taxonomy of those taxa using DNA sequencing. For the most part BOLD records originate from freshly caught specimens but the inclusion of type, rare or even extinct specimens into barcoding studies holds huge promise. For example, the taxonomic treatment of many taxa will only be resolved satisfactorily if the DNA barcode from historic (particularly type) specimens can be included in a barcode analysis. However before the vast bounties of museums and private collections are made available for barcoding, successful DNA extraction methods suited to the taxa under investigation are required.

DNA retrieved from museum samples is often difficult to extract and are often referred to as ancient DNA (aDNA,) or historic DNA (hsDNA). In this study the DNA extracted from the museum specimens will be referred to as hsDNA and not aDNA as the samples are not older than 38 years. hsDNA is difficult to extract as molecular damage occurs to DNA after death of an organism (Lindahl, 1993). The degradation of DNA in an organism occurs continuously post mortem due to a variety of factors such as enzymatic activity by lysosomal nucleases, DNA degrading activities by bacteria, fungus and insects and common atmospheric elements such as water, heat and oxygen (Pääbo et al., 2004). Due to the degradation of DNA over time, standard DNA extraction techniques generally have a low success rate, as hsDNA is often reduced to a few hundred base pairs making amplification using polymerase chain reaction (PCR) more difficult (Hofreiter et al., 2001).

Different protocols have been developed to extract hsDNA. Most of these protocols involve the enzymatic amplification of shorter sequences such as in mitochondrial DNA (mtDNA) (Cano, 1996) such as the DNA barcode. Primers can also be designed to amplify smaller regions within these short sequences. These are usually known as internal primers. However, many of these protocols destroy the sample or have the undesirable impact of destroying some of the morphological features of a sample (Mandrioli, 2008). The difficulty in utilising museum specimens for DNA extraction is in achieving this balance and whether the information gained justifies modifying and often destroying the samples' morphological integrity.

hsDNA can also be used to provide information on species' phylogenies, population history and phylogeography (Pääbo et al., 2004). However the quality and quantity of hsDNA can restrict the molecular markers available for analysis. For example most phylogenetic studies are based on multiple loci across different sources of DNA (e.g. nuclear and mitochondrial) and population analyses based on microsatellite loci require DNA of high quality and quantity. In most organisms nuclear DNA degrades at a faster rate than mitochondrial DNA. An additional benefit of targeting mtDNA is that it is present in a higher copy number with respect to nuclear DNA. The mitochondrial origin of the CO1 barcoding region and the usefulness of the sequence obtained make the barcode an ideal candidate locus for amplification from museum and historic specimens. The aim of this study was to extract DNA from museum samples of bumblebees to assist in ascertaining information relating to the phylogeny and phylogeography of the bumblebee species *Bombus muscorum* L. This study examined DNA in the CO1 region of the bumblebee sequence with the use of internal primers which have not been reported in DNA analysis for bumblebees.

3.3 Materials and Methods

3.3.1 Specimens

Different methods were used to extract DNA from museum samples of the bumblebee species *B. muscorum, B. lucorum*, *B. magnus* and *B. cryptarum* and *B. terrestris*. The latter four species form a cryptic species complex referred to as *B. lucorum* agg. (Murray et al., 2008). The museum specimens consisted of dried pinned specimens of *B. muscorum* and *B. lucorum* agg. collected throughout Ireland dating from the early 1970s to the 1980s (Prof. John Breen, personal collection). These samples were stored and killed under different conditions. The majority of samples were killed using hydrogen cyanide but some were killed using chloroform and ethyl acetate. It was not known which samples were subjected to the different chemical treatments as they were not labelled with this information at the time of treatment. A number of freshly sampled specimens of *B. muscorum* and *B. lucorum* agg. were collected during the summer of 2009 and stored according to CBOL guidelines. Samples were stored frozen at -80 °C or preserved in 70% ethanol. These were used as DNA extraction and PCR positive controls.

Sequences for the CO1 region of these species are known and available on Genbank. Primers are also available for the CO1 region of bumblebees. This information allowed for internal primers to be designed for the species being examined. The use of internal primers was deemed necessary due to the fragmented nature of hsDNA and the previous harsh treatments the samples were subjected to.

3.3.2 DNA extraction

Two different lysis approaches were tested to extract DNA from the samples. The first approach involved the use of a so called "non destructive" method while the second lysis approach used methods that had been previously described as semi-destructive. The details of each of these methods are listed in Table 10. Each DNA extraction protocol involved the use of eight historic and four control specimens of both *B. muscorum* and *B. lucorum* agg. All DNA extractions were stored at -20 °C until required.

The first lysis approach involved the use of the entire specimen following the protocols utilised by Gilbert et al. (2007) and Thomsen et al. (2009). This method was chosen as it was described in the studies by Gilbert et al. (2007) and Thomsen et al. (2009) as non-destructive to the morphological integrity of the sample. This method yielded PCR amplifiable DNA from up to 100 year old carabid beetles, and it involves submersing the specimen in an extraction/lysis buffer prior to DNA extraction. This method is listed in Table 10 as method i) Gilbert et al. (2007).

The second lysis approach involved using methods that were semi-destructive to the specimens being used as they involved the removal of a leg from the thorax using a forceps from each museum and control specimen. The forceps used for the removal of the legs were cleaned in bleach, distilled water and 70% ethanol prior to removing each leg. Museum leg samples were removed at different times to legs from specimens stored following CBOL guidelines. The forceps were sterilised between each set of extractions using UV light. All legs removed were washed with ethanol and distilled water to remove any surface contaminants. Each leg was placed in a 1.5ml Eppendorf tube, which was then immersed in liquid nitrogen and the leg was then homogenised using a pestle. One leg was used from each specimen for each DNA extraction protocol. Museum specimens were used once to limit the morphological damage to the specimens. Hence each DNA extraction protocol involved the use of a different specimen.

Five different DNA extraction methods and combinations of DNA extraction parameters were tested in an attempt to extract amplifiable DNA from the historical samples. The details for these methods are given in Table 18 and they are listed as method ii) The Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a) iii) Junqueira et al. (2002) iv) Strange et al. (2009) and v) Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a) and vi) Modified Qiagen DNeasy PBS protocol for insects (four hour incubation). This modification involved changing the incubation time from ten min to four h as detailed in Table 10. The Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a) is the method of choice in our laboratory as it proved successful for extracting DNA from samples stored following CBOL guidelines for a wide range of bee species. The Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a) and Junqeira et al. (2002) protocols were chosen as they are specific to insects and the protocol described by Strange et al. (2009) was successful in extracting DNA from museum specimens of bumblebees.

Table 18 - Summary of DNA extraction methods trialled

Method	Name	Digestion Buffers	Incubation Time	Incubation Temperature	DNA Elution
i	Gilbert et al. (2007)	2ml Digestion Buffer: 3mM $CaCl_2$ 2% sodium dodecyl sulphate (SDS) 40mM dithiotreitol (DTT) 250g/ml proteinase K 100mM Tris buffer pH8 100mM NaCl	16-20 h	55°C	Qiagen DNeasy spin column protocol
ii	Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a)	180 µl of Qiagen ® Buffer ATL 20 µl of Proteinase K	4 h	56°C	Qiagen DNeasy spin column protocol
iii	Junqueira et al. (2002)	100 µl of 10% Chelex 100	Overnight 10-12 h	56°C	Samples vortexed for 10 s Incubated in boiling water for 5 min Vortexed for 10 s Centrifuged at 15 000g
iv	Strange et al. (2009)	150 µl of 5% Chelex 100	2.5 h	1 hr @ 55°C 15 min @ 99°C 1 min @ 37°C 15 min @ 99°C	None
v	Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a)	180 µl Qiagen ® Buffer ATL 20 µl Proteinase K	10 min	56°C	Qiagen DNeasy spin column protocol
vi	Modified Qiagen DNeasy PBS protocol for insects (four hour ncubation) (Anonymous, 2006a)	180 µl Qiagen ® Buffer ATL 20 µl Proteinase K	4 h	56°C	Qiagen DNeasy spin column protocol

3.3.3 PCR Amplification and CO1 sequencing

The quality of DNA was assayed using polymerase chain reaction (PCR). Each PCR was performed in a 20 µl reaction volume comprising 1 × PCR Buffer (Invitrogen), 2 µl template, 3mM $MgCl_2$, 200 µM of each dNTP, 0.6 µM of each primer and 0.5 units of *Taq* polymerase (Invitrogen). The CO1 barcoding fragment was amplified using the primers LCO_Hym and Nancy_Short (Magnacca and Brown, 2010). A number of internal primers were designed and are listed in Table 19. During preliminary investigations, all internal primers were used. However, a section (100-200 bp) of the DNA barcoding region was of significant interest due to the occurrence of a single nucleotide polymorphism (SNP) (see chapter 4). Thus the focus of the amplification of DNA with internal primers was primarily with the primer pair LCO_Hym and CO1 IntR1. The PCR conditions were: initial denaturation at 95°C for 3 min followed by 32 cycles of 1 min denaturation at 95°C, 60 s annealing at 48°C and 60 s elongation at 72°C and a final extension for 4 min at 72°C. The products from the PCR reaction were run on a 2% agarose gel at 120v for 30 min in 1 x TAE buffer.

Table 19 - Primer names and sequences used to amplify DNA and hsDNA in the DNA barcoding region

Primer Name	Primer Sequence
Nancy_Short	5' CCCGGTAAAATTAAAATATAAAC-3’
LCO_Hym	5'–TATCAACCAATCATAAAGATATTGG–3'
CO1 IntR1	5’-CGAGGRAAAGCTATATCTGG-3’
CO1 IntF1	5’-GTAATACCATTTATAATYGG-3’
CO1 IntF2	5’-GGWATTTCCTCWATTATYGG-3’
CO1 IntR2	5’-CRATAAAATTTAATGATCC-3’

3.4 Results

The non destructive DNA extraction method described by Gilbert et al. (2007) resulted in the damage of important morphological traits in particular hair characteristics as shown in Fig. 12. The five other methods tested involved the removal of the middle leg from each specimen. All methods in the second lysis approach yielded PCR amplifiable DNA from the freshly sampled specimens that were stored following CBOL guidelines. These DNA samples yielded PCR amplicons using primers for the full length CO1 barcode fragment and CO1 internal regions. The PCR products from each of these DNA extraction types were run on gels.

Only a single method, the modified Qiagen DNeasy protocol for insects (four hour incubation) resulted in amplifiable DNA from the older museum specimens (Table 20-21). However only DNA from bumblebee queen specimens yielded PCR amplifiable DNA and the DNA extracted from workers consistently failed to yield PCR products. PCRs with the LCO_Hym/Nancy_Short primers failed for all but two samples whereas the internal primer pair LCO_Hym/CO1 IntR1 yielded PCR amplicons for 90% of all queen DNA extracts. Figures 13-22 show gel images of four samples (two workers and two queens) from each DNA extraction protocol and species type. Each DNA extraction protocol contains a 100bp ladder and two positive controls for the species and DNA extraction type tested.

Fig. 12 – *Bombus lucorum* agg. sample following DNA extraction using the method of Gilbert et al. (2007) showing morphology before (left) and after (right).

Table 20 - Results for DNA extraction protocols in relation to PCR amplification using the primers Nancy_Short and LCO_Hym.

Protocol	**CBOL Stored *Bombus muscorum***	**Museum *Bombus muscorum***	**CBOL Stored *Bombus lucorum* agg.**	**Museum *Bombus lucorum* agg.**
Gilbert et al. (2007)	ND	ND	0	0
Junqueira et al. (2002)	X	0	X	0
Strange et al. (2009)	X	0	X	0
Qiagen DNeasy Bench protocol for animal tissues ((Anonymous, 2006a)	X	0	X	0
Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a)	X	0	X	0
Modified Qiagen DNeasy PBS protocol for insects (four hour incubation) (Anonymous, 2006a)	X	1	X	1

Where X= DNA amplified for all samples, 0 = no DNA amplified, 1,2,3…. =number of samples DNA was amplified for, and ND= not determined.

Table 21 - Results for DNA extraction protocols in relation to PCR amplification using the primers LCO_Hym and CO1 IntR1

Protocol	**CBOL Stored *Bombus muscorum***	**Museum *Bombus muscorum***	**CBOL Stored *Bombus lucorum* agg.**	**Museum *Bombus lucorum* agg.**
Gilbert et al. (2007)	ND	ND	0	0
Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a)	X	0	X	0
Junqueira et al. (2002)	X	0	X	0
Strange et al. (2009)	X	0	X	0
Qiagen DNeasy PBS protocol for insects	X	0	X	0
Modified Qiagen DNeasy PBS protocol for insects (four hour PBS incubation)	X	8	X	8

Where X= DNA amplified for all samples, 0 = no DNA amplified, 1,2,3…. =number of samples DNA was amplified for, and ND=not determined.

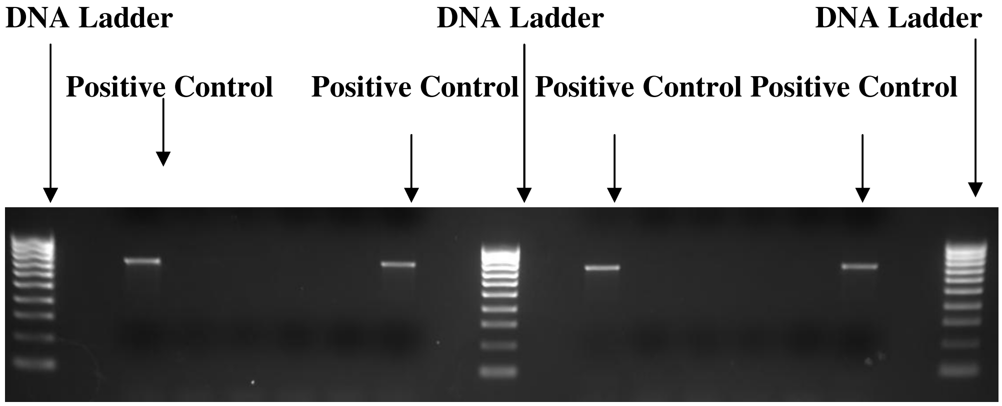

Fig. 13 - Amplified PCR products from *B. lucorum* agg. using the Junqueira et al. (2002) and Strange et al. (2009) DNA extraction protocols, respectively, using the primer pair LCO_Hym and Nancy_Short

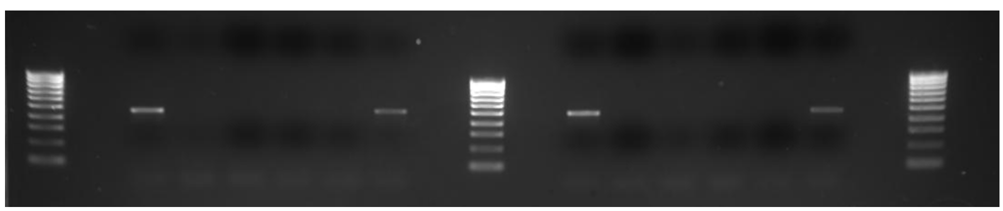

Fig. 14 - Amplified PCR products from *B. lucorum* agg. using the Junqueira et al. (2002) and Strange et al. (2009) DNA extraction protocols, respectively, using the primer pair LCO_Hym and CO1 IntR1.

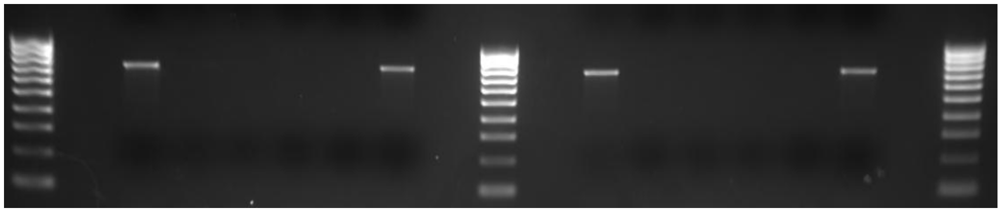

Fig. 15 - Amplified PCR products from *B. muscorum* using the Junqueira et al. (2002) and Strange et al. (2009), DNA extraction protocols, respectively, using the primer pair LCO_Hym and Nancy_Short

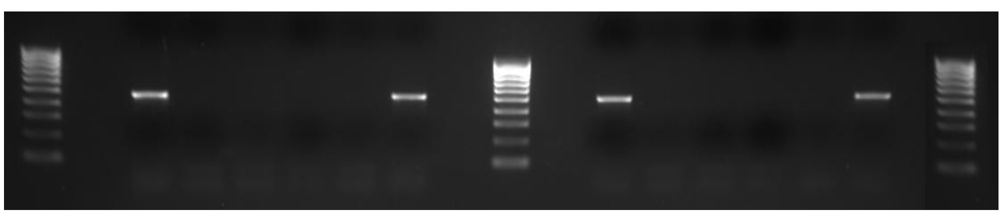

Fig. 16 - Amplified PCR products from *B. muscorum* using the Junqueira et al. (2002), and Strange et al. (2009), DNA extraction protocols, respectively, using the primer pair LCO_Hym and CO1 IntR1

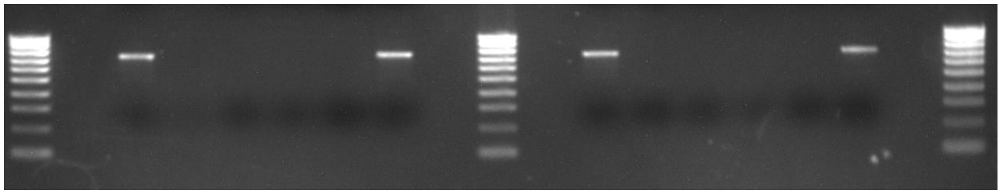

Fig. 17 - Amplified PCR products from *B. lucorum* agg. using the Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a) and the Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a) DNA extraction protocols, respectively, using the primer pair LCO_Hym and Nancy_Short

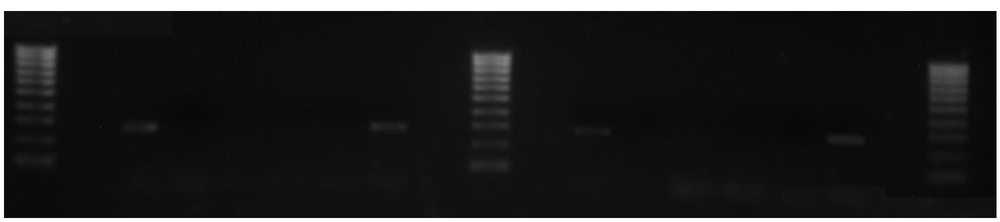

Fig. 18 - Amplified PCR products from *B. lucorum* agg. using the Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a), and the Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a) DNA extraction protocols, respectively, using the primer pair LCO_Hym and CO1 IntR1

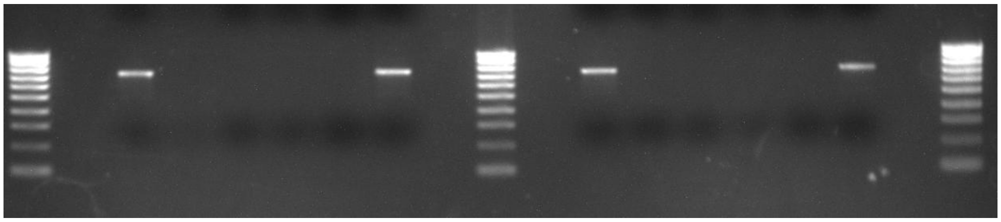

Fig. 19 - Amplified PCR products from *B. muscorum* using the Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a) and the Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a) DNA extraction protocols, respectively, using the primer pair LCO_Hym and Nancy_Short

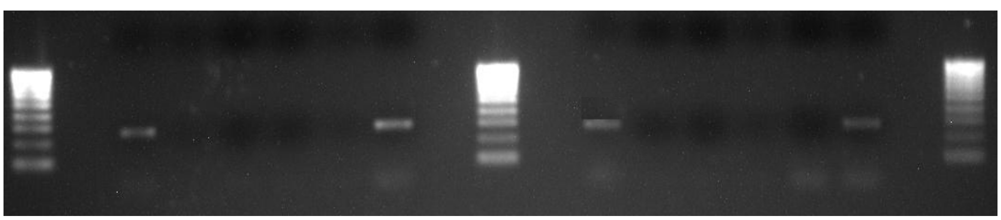

Fig. 20 - Amplified PCR products from *B. muscorum* using the Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a) and the Qiagen DNeasy PBS protocol for insects (Anonymous, 2006a) DNA extraction protocols, respectively, using the primer pair LCO_Hym and CO1 IntR1

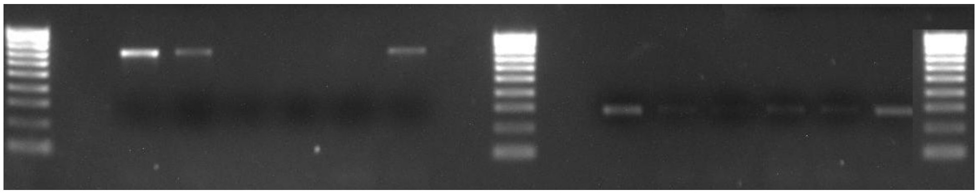

Fig. 21 - Amplified PCR products from *B. lucorum* agg. using the Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a), using the primer pairs LCO_Hym and Nancy_Short and LCO_Hym and CO1 IntR1 respectively. Only one sample was successfully amplified using the LCO_Hym and Nancy_Short primer combination as shown in gel images while all samples were successful using the LCO_Hym and CO1 IntR1 primer pair.

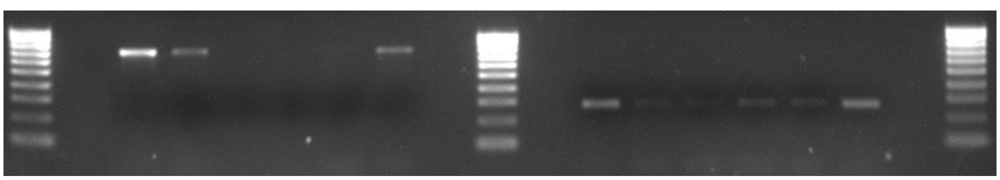

Fig. 22 - Amplified PCR products from *B. muscorum* using the Qiagen DNeasy Bench protocol for animal tissues (Anonymous, 2006a), using the primer pairs LCO_Hym and Nancy_Short and LCO_Hym and CO1 IntR1 respectively. Only one sample was successfully amplified using the LCO_Hym and Nancy_Short primer combination as shown in gel images while all samples were successful using the LCO_Hym and CO1 IntR1 primer pair.

The sequences obtained for successfully amplified fragments were aligned against CO1 barcode sequences from referenced accessions of *B. muscorum* and *B. lucorum* agg. To verify this result the DNA of a further eight samples (four *B. lucorum* and four *B. muscorum*) were extracted successfully using the modified Qiagen DNeasy protocol for insects (four hour incubation) and the LCO_Hym and CO1 IntR1 primer pair (Fig. 23).

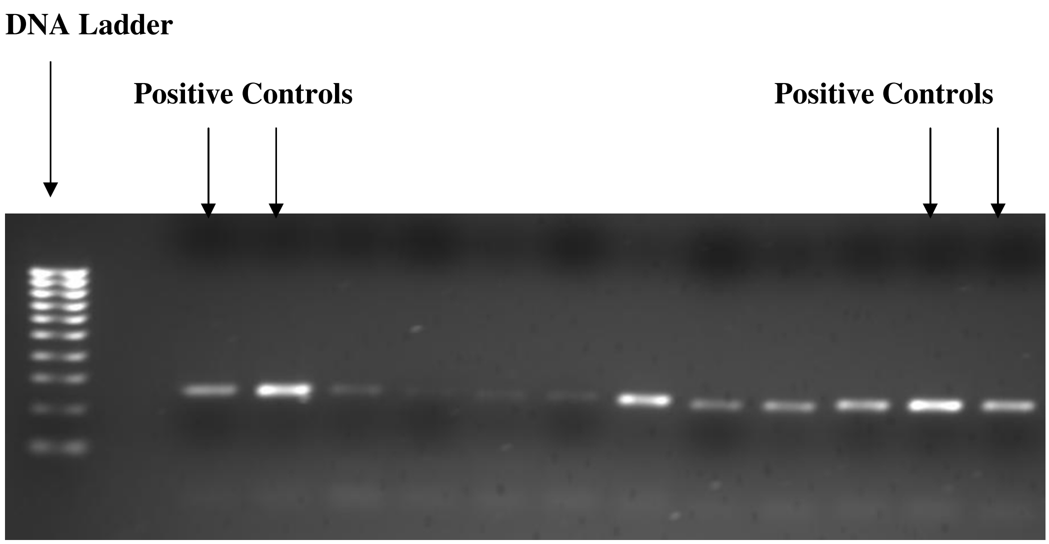

Fig. 23 - Eight PCR amplified DNA extracts (four from *B. lucorum* agg. and four from *B. muscorum* respectively) from historical specimens of bumblebees using the primers LCO_Hym and CO1 IntR1.

3.5 Discussion/Conclusion

This study tested a number of different DNA extraction methods used routinely on freshly caught and pinned museum insect specimens. The extent to which pinned specimens from museum or private collections can be incorporated into a genetic study depends greatly on the age of specimen, the condition of the specimen after DNA extraction method and also on how the specimen was treated after capture. The method described by Gilbert et al. (2007) and Thomsen et al. (2009) proved unsuitable for extracting DNA from the bumblebee samples. This non-destructive method is perfectly suited to insects lacking "hair" and other delicate characters that may be exposed on the outer exoskeleton. Although Gilbert et al. (2007) and Thomsen et al. (2009) report minimal damage to beetle specimens, there was damage to the morphological integrity of the bumblebee specimens in this study. Hair colour is a key feature in bumblebee identification and although DNA was extracted, these diagnostic characters were compromised, making the taxonomic identification of these specimens impossible. The main problem with this method was the requirement of the sample to be fully immersed in a specimen tube such as a 1.5ml or 2.0ml microcentrifuge tube which contained the lysis buffer. The body of a bumblebee can be extremely robust due to its chitin exoskeleton but it has delicate features such as its wings. The wings of museum bumblebees become dry and brittle over time. The handling and placement of test specimens into sample tubes resulted in damage to the wings, which is undesirable. Morphometric analysis of wings can be used to aid in the identification of bee species and ecotypes (Daly, 1985; Andere et al., 2008). We advise caution to researchers considering this method to extract DNA from museum specimens of bumblebees and other insects where there is significant morphological association with hair colour and where there are prominent delicate features present.

The failure to extract amplifiable DNA using the method outlined by Strange et al. (2009) was unexpected as this method had previously been successful on museum specimens of bumblebees, some of which were 100 years old. The oldest specimen in this study was 38 years old. Although one would expect a lower rate of DNA degradation in younger specimens, the treatment of specimens once collected may have a major effect on DNA preservation. For example, insect specimens are routinely killed with ethyl acetate and hydrogen cyanide depending on taxon and the preference of the collector (Gilbert et al., 2007). These chemical procedures may have resulted in the production or introduction of PCR inhibitory compounds. A previous study on insects that were preserved in ethyl acetate found that the DNA was more degraded and difficult to extract (Dillon et al., 1996). Cyanide is known to have a destructive effect on mithochonrial DNA (Borowitz et al., 1992). In addition specimens may have to go through rigorous treatment before being permitted into a museum to prevent potential biotic threats to the museum collections. Most of the samples used in this study were killed with cyanide and underwent a pesticide treatment regime (50°C, 100% relative humidity, 24 h) when housed temporarily in the museum of the University of Bergen, Norway during 1976. Many of these samples were put through this treatment twice (Prof. John Breen, personal communication).

The different chemical and heating regimes used on the specimens in this study are the most likely reasons for the complete failure of four of the methods to obtain PCR amplifiable DNA. The only method that yielded amplifiable DNA was the modified Qiagen DNeasy PBS protocol for insects. Thus it seems PBS is in some way increasing the success of obtaining amplifiable DNA most likely through the removal of PCR interfering compounds rather than increasing the quantity of DNA. However this would have to be determined experimentally to understand how PBS improves the recovery of amplifiable DNA from pinned specimens. The heating procedures in the museum in the University of Bergen may have increased the rate of fragmentation of the DNA in the samples. Hence, the use of an internal primer was probably essential in amplifying the DNA in the samples. This method may be particularly useful for specimens that have been subjected to harsh chemical treatments during specimen preparation and preservation. DNA degradation in dead tissues is affected not only by how the material is processed and stored but also factors such as the presence of water, heat, oxygen and time since death (Lindahl et al., 1993; Gilbert et al., 2007). DNA degradation generally results in sheared DNA and relatively small length DNA fragments. This reduction in template size was evident in the DNA samples extracted using the Qiagen PBS homogenisation method. PCRs using an internal primer for the CO1 barcode were used successfully. Amplifying the full CO1 barcode using the internal primers requires three separate PCRs. However considering the potentially enrichment of a molecular genetic study including pinned historic specimens, we deem this increase in labour very worthwhile.

The results highlight the difficulties in developing universal protocols and guidelines that can be used on all historic insect collections. It seems the age, rarity, importance and condition of specimens must be considered before a portion of the specimen is removed. Ideally non-destructive sampling methods are preferable but as shown in the present study, currently available methods are not suitable for bumblebees. Differences in killing and storage conditions vary greatly and depend upon the preference of the collector. Additionally, some DNA extraction methods do not seem to be universally applicable. Thus it seems that the choice of DNA extraction method (and, in fact, whether the sample should be compromised in the first place) must be made on a specimen by specimen basis. If museum and historic specimens are to give up their potential molecular genetic information the molecular biologist must have a means of assessing the chances of success before proceeding. However, to generate informed decisions about which methods to use and which types of specimen to target requires the "sacrifice" of the leg of historic specimens. For example we found that very few bumble bee worker specimens yielded amplifiable DNA and we recommend that, where possible, queens are used for the extractions if available to save the potentially futile damage to worker specimens.

The laborious technical challenges represented by extracting amplifiable DNA from old or museum insect specimens is offset by the potential benefits that can result from the inclusion of such samples in molecular analyses. New DNA extraction techniques and genetic methods are opening new avenues of information for science but respect must still be held by those involved in the molecular analysis for a specimen's integrity. The protective policies of museums and collectors around the world will only be relaxed if the benefits of DNA sequencing can be guaranteed. Considering that the modified Qiagen DNeasy PBS based lysis protocol (four hour incubation) for insects yielded PCR amplifiable DNA when other published methods did not indicates that another viable option DNA extraction from historic specimens is available and can be recommended for future investigations.

Recommendations

1. **The method for killing the specimen should be included on the label.**
2. **CBOL guidelines for specimen storage should be followed.**
3. **Homogenising the sample in PBS prior to a four hour incubation in Buffer ATL, and proteinase K should be used.**
4. **Internal primers yielding amplicons of 200 bp in length should be used.**
5. **Queens rather than smaller castes should be targeted as they represent the best type of material to provide amplifiable DNA.**
6. **If specimens are to be pinned and stored at room temperature the mid leg should be removed and stored in 70% ethanol (with appropriate label) to provide DNA if required in the future.**

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Chapter 4 The Phylogenetic Significance of Melanism in *Bombus muscorum* L.

4.1 Abstract

The identification of a species using a fixed short region of the cytochrome c oxidase (CO1) mitochondrial gene has become known as DNA barcoding. Although primarily used for species level identification, DNA barcoding is shedding light on groups of taxa with ambiguous biogeographic and phylogenetic characteristics. The moss carder bumblebee, *Bombus muscorum* has a palaeartic distribution and a number of blonde and melanic varieties exist. Taxonomically, melanic forms have been variously recognised as distinct species, subspecies or varieties. In an effort to resolve these issues we sequenced the CO1 barcode region for 59 specimens from a number of Irish and British mainland and island populations. Six specimens were also sequenced for the cytochrome B and ITS region. Within the CO1 alignment seven variable sites were observed. However, only one single nucleotide polymorphism (SNP) was widely distributed with all specimens having either a C or T at the base pair (bp) position 144 in the CO1 alignment. British specimens were almost exclusively of the C haplotype, whereas the Irish specimens were of the T or C haplotype. Four of the seven polymorphisms were present in two melanic specimens (both specimens had identical sequences) collected on the Inner Hebrides island of Tiree. Sequences were partitioned based on geographic location or melanic status to permit inter- and intra-group comparisons. However the inter- and intra-group genetic distance values obtained using the K2P distance model were extremely low indicating that insufficient polymorphism existed to permit an informative population level or phylogenetic analysis. Considering that previously generated sequence data for a cryptic species aggregate of *Bombus* revealed intra and interspecific estimates of variation of 0.36% and 2.8% respectively, our results (1.1% intraspecific variation) can be used to argue that British and Irish *B. muscorum* (whether melanic or blonde)

are a single species. The majority of the sequence variation is accounted for by the two Tiree sequences which display the same amount of divergence to both melanic and blonde forms. In addition no unique and shared polymorphisms were identified that separate the melanic/blonde forms and our results suggest that melanism in *B. muscorum* has no underlying phylogenetic significance and the presence of melanic forms on islands is due to convergence.

4.2 Introduction

The DNA barcode, a c.650 bp region of the cytochrome c oxidase (CO1) mitochondrial gene has become an invaluable tool for the ecologist and taxonomist alike primarily through species identification and diagnosis. Species identification through DNA barcoding occurs under the assumption that genetic variation between species will be greater than the genetic variation within species (Herbert, 2003). In addition sequence variability within the barcode region can provide taxonomists with valuable characters to support the proposal of new species or to distinguish cryptic species (Hebert et al., 2004a; Hebert et al., 2004b; Hebert and Gregory, 2005; Hajibabaei et al., 2007; Valentini et al., 2009). Reference barcodes for morphologically vouched material can be obtained from databases such as the Barcoding of Life Data System (BOLD, www.barcodinglife.org) that can facilitate the identification process. Although this measure of divergence (the barcode gap) and the means to determine species boundaries remains a contentious and problematic area, it is evident that DNA barcoding can successfully provide insight into similarity among taxa that can supplement taxonomic circumscriptions based on morphological data.

DNA barcoding results in the identification of genetically divergent groups which can then be used as a framework to examine morphological variation, which can lead to more efficient species diagnosis and determination of species boundaries. The framework of relationship provided by DNA barcoding permits the evaluation of the morphological characters that have been used traditionally to define particular taxa. Molecular sequence data can often lead to the discovery of homoplasious characters (phenotypic similiarity among different characters of species that do not accurately reflect co-ancestry) that have contributed to erroneous and non-phylogenetic taxonomic classifications. As such DNA barcoding has increasingly been viewed as a taxomonic tool rather than an attempt to replace more conventional taxonomic practices.

Within the Apoidea, DNA barcoding has recently been applied to a number of groups including solitary bees (Magnacca and Brown, 2010) and bumblebees (Kim et al., 2009). These studies have utilised DNA barcoding successfully for the delineation of species. DNA barcoding has also been used to characterise the bees of Nova Scotia, Canada (Sheffield et al., 2009), where the presence of two new species was revealed, highlighting the utility of the technique for discovering cryptic or novel species. Over the coming decades the DNA barcoding resources for bees will be greatly expanded through the Bee Barcode of Life Initiative (Bee-BOL), a collaboration between numerous international groups to barcode all of the estimated 20 000 bees of the world.

Presented in this study is an appraisal of the taxonomic status of the moss carder bee *Bombus muscorum* using the CO1 barcode region. *Bombus muscorum* is distributed across the palaearctic region (Fig. 24) and a number of blonde and melanic varieties of the species exist (Williams, 2010a). The species has a distribution ranging from east Russia (Proshchalykin, 2004), Ireland (Stelfox, 1927; Fitzpatrick and Murray, 2006), Northern Europe and Scandanavia (Løken, 1973) and other European countries such as Hungary (Sarospataki et al., 2005), and France (Iserbyt et al., 2008). In many countries the species is recognised as being rare such as in Ireland (Fitzpatrick et al., 2006b), Britain (Goulson, 2010), and central Europe (Kosior et al., 2007).

One of the main difficulties with the taxonomy of *B. muscorum* relates to the existence of melanic varieties within the species. Melanism is a phenomenon whereby there is a darkening of pigmentation not normally present in a species (Majerus, 1998). Animal taxa in which melanism has been reported include microorganisms, molluscs, arachnids, crustaceans, insects, birds and mammals (Majerus, 1998). Melanism has long been used to investigate evolutionary change (Tutt, 1891). A melanistic colour pattern may evolve due to factors such as defence, the energetic cost of colour production, and selective pressures to which a species has been exposed. However melanism does not arise from a single evolutionary factor thus complicating the study of the evolution of the melanic character in a species.

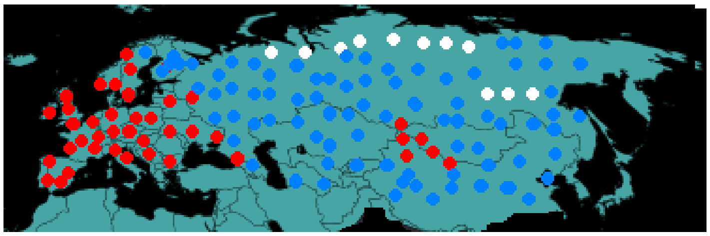

Fig. 24 - Palaearctic distribution of *Bombus muscorum* based on records assembled by Paul H Williams (PHW), Natural History Museum, London (Williams, 2010b). Red circles indicate specimens identified by PHW, blue circles recorded in the literature and white circles their expected distribution.

The study of melanism is further complicated by a lack of understanding of the genetic mechanisms underlying its expression. There are only few instances where the genetic link has been established for the occurrence of melanism within a species (Majerus, 1998; Majerus and Mundy, 2003). For example, Majerus and Mundy (2003) reported mutations in the melanocortin-1-receptor gene which are responsible for the different coat colours of rock pocket mice (Mammalia). However the link between genotype and phenotypic expression for the majority of melanic occurrences within species has not been established. Another problem that arises from a poor understanding of the genetics of melanism is species misidentification, as melanism is a trait that can create confusion around the colour features that are used to identify a species.

The bumblebee, *B. muscorum,* is a species wherein the melanic colour morphs have created considerable debate regarding the taxonomy of the species. Authors such as Richards (1935), and Rasmont and Adamski (1995) have regarded the melanic colour morphs as separate species. However Løken (1973), in Scandinavia, and other authors such as Alford (1975), in the UK, found no morphometric difference between the different melanic and non-melanic types, but because the genetics behind the melanism has not been fully elucidated, the taxonomic status of *B. muscorum* species remains unclear (Williams, 2010a). *Bombus muscorum* is in the sub-genus *Thoracobombus* and was first described by Linneaus in 1758 (Benton, 2006). Within *B. muscorum* there are different colour varieties, most notably that of the blonde and melanic, and within these two groups there are intermediate variations. Blonde, melanic, and intermediate varieties differ from each other in the amount and distribution of black hairs. The presence of black hairs on the legs and ventral surfaces are used to differentiate the melanic forms from the blonde. Between Britain and Ireland six different varieties are recognised (Table 22 and Fig. 25) (following Alford (1975) and Baker (1996)).

Table 22 - The forms and varieties of *B. muscorum*

Form	Location	Variety	Author	Distribution
Blonde	Mainland	*sladeni*	Vogt	The south of the English mainland, European continent
	Mainland & islands	*pallidus*	Evans	Irish Mainland, northern England, several offshore islands
Intermediate	Islands	*orcadensis*	Richards	Orkney Islands
	Islands	*scyllonius*	Richards	Scilly Islands, Channel Islands
Melanic	Islands	*agricolae*	Baker	Shetland Islands, several of the Hebride Islands
	Islands	*allenellus*	Stelfox	Aran Islands

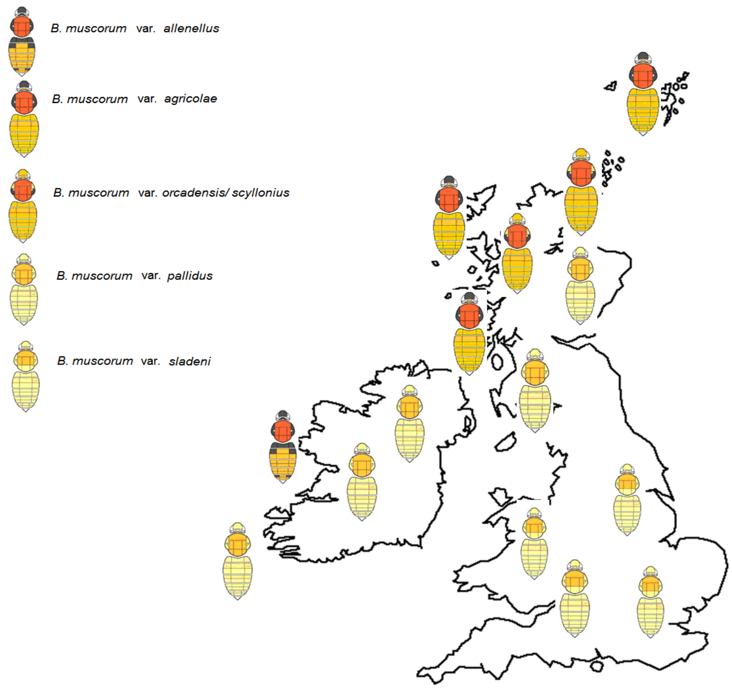

Fig. 25 - Recorded distribution of the varieties of *B. muscorum* for the British Isles (from Judge, (2007), based on colour patterns designed by Williams (2007b))

The melanic island form of *B. muscorum* was first reported from the Shetland Islands by White (1851), who described it as *B. smithianus*. However, an error was made by White that led to *B. pascuorum* being used as the type specimen of *B. smithianus* (Alford, 1975). Melanic forms of *B. muscorum* have also been reported on the Faroe Islands, Northern Norway (Løken, 1973) and Corsica (Rasmont, 1982). Within the Burren region in Ireland, there is a melanic colour morph of *B. muscorum* known as the Aran Island bumblebee. The Irish melanic form of *B. muscorum*, the Aran Island bumblebee, *B. muscorum* var. *allenellus*, was first described by Stelfox (1933) who reported a series of investigations on the species conducted by Mr. C. Winckworth Allen after whom the species is named. This melanic variety of *B. muscorum* is restricted to the Aran Islands in Ireland. Two further melanic varieties of *B. muscorum,* var. *scyllonius* and var. *orcadensis* were subsequently described by Richards (1935) from the Scilly and Orkney Islands, respectively.

There have been previous attempts to clarify the status of these colour varieties of *B. muscorum*. The majority of bumblebee species (such as *B. pascuorum, B. lucorum, B. sylvarum*) can be differentiated at species level based on morphological differences observed in the male genitalia and female sting sheaths (Williams et al., 2008). These have been compared between the colour varieties of *Bombus muscorum*, and, based on these characters, there does not seem to be any variation in these characteristics that suggest they are distinct species (e.g. Løken 1973). Previous genetic work has been performed on *B. muscorum* using microsatellites to establish the relationship between the blonde, intermediate and melanic form (Darvill et al., 2006; Judge, 2007). Both Darvill et al. (2006) and Judge (2007) used microsatellites to study the gene flow between sampled populations of *B. muscorum.* Darvill et al. (2006) examined populations in Britain that included three varieties of *B. muscorum*, var. *pallidus* (blonde), var. *sladeni* (blonde) and var. *smithianus* (melanic). Judge (2007) examined *B. muscorum* populations in Britain and Ireland which included the four varieties, var. *allenellus* (melanic, Aran Islands), var. *pallidus* (Irish mainland), var. *agricolae* (melanic, Scotland), and var. *scyllonius* (melanic, Scilly Islands). Neither study yielded significant genetic differences between the colour morphs that would warrant special species status for the colour morphs (Darvill et al., 2006; Judge, 2007). However Darvill et al. (2006) reported significant inbreeding within the isolated island *B. muscorum* populations in Britain. These studies highlight the need for further studies on *B. muscorum* and Darvill et al. (2006) called for further studies to examine the mtDNA of the color morphs. The need for the conservation of *B. muscorum* var. *allenellus* was noted by Fitzpatrick et al. (2006). For appropriate conservation strategies to be developed for this group of bees it is imperative that the taxonomic and phylogenetic status of the *B. muscorum* varieties are clarified.

This study aims to clarify the status of these colour morphs by sequencing regions of the mtDNA and the nuclear ITS region and identify the haplotypes present for each location sampled. CO1 barcodes are compared for representatives from a number of the varieties of *B. muscorum* in an attempt to determine the amount of nucleotide polymorphism existing within the sampled group. A low number of polymorphisms would be interpreted as indicating that the various varieties included in this study are representatives of a single species. It has been suggested for ecologically and morphologically similar groups that separate species status can be bestowed if there is a difference of at least 26 substitutions per 891 basepairs (0.030-0.043 in Tamura-Nei distance) (Bertsch et al., 2005). Previously generated sequence data for a cryptic species aggregate of *Bombus* revealed intra- and interspecific estimates of variation of 0.36% and 2.8%, respectively, with the latter value being used as a reference in this study as a value for species differentiation (Murray et al., 2008).

The null hypothesis under investigation in this chapter is

- There is no genetic variation between the melanic and blonde varieties of *B. muscorum*

4.3 Materials and Methods

4.3.1 Specimens

Bumblebee populations along the coast and on islands off the British Isles were sampled for specimens of *B. muscorum* (Table 23 and Fig. 26). Irish population samples were obtained from the Aran Islands, Co. Clare, Clare Island, Co. Mayo, Aghany, Co. Mayo, Baltimore Co. Cork, Sherkin Island, Co. Cork during the summer of 2009 (Aislinn Deenihan, collector) and the Irish Botanic Gardens, Dublin city during the spring of 2010 (Dr. Jim Carolan, National University of Ireland, Maynooth, collector). British samples were collected from the Outer and Inner Hebrides, the Shetland Islands, and islands in the Isles of Scilly during the summer of 2004. Samples from Britain were kindly donated by Prof. Dave Goulson and Dr. Ben Darvill of the Bumblebee Conservation Trust/ University of Stirling. Voucher specimens, collected from each of these locations were killed and preserved in 70% ethanol according to the recommendations of the Centre for Barcoding of Life guidelines (CBOL). All specimens were stored at 4°C or frozen at -20°C. Irish specimens of *B. muscorum* from the 1970s were also sequenced (Prof. John Breen, personal collection), but were only analysed superficially and not included in the main sequencing body.

Table 23 - Sampling locations, variety of *B. muscorum* and number of specimens sequenced.

Country	Location	Variety	N
Ireland (Mainland)	Co. Clare	*pallidus*	3
Ireland (Mainland)	Dublin	*pallidus*	6
Ireland (Mainland)	Baltimore	*pallidus*	1
Ireland	Clare Islands	*pallidus*	4
Ireland	Sherkin Island	*pallidus*	3
Ireland	Aghany	*pallidus*	1
Ireland (Aran Islands)	Inis Mor	*allenellus*	4
Ireland (Aran Islands)	Inis Meain	*allenellus*	4
Ireland (Aran Islands)	Inis Oirr	*allenellus*	8
Scotland (inner Hebrides)	Coll	*agricolae*	4
Scotland (inner Hebrides)	Tiree	*agricolae*	2
Scotland (Outer Hebrides)	South Uist	*agricolae*	2
Scotland	Shetlands	*agricolae*	6
England (Scilly Islands)	St Angus	*scyllonius*	5
England (Scilly Islands)	Samson	*scyllonius*	3

Fig. 26 - Sampling locations for *B. muscorum*. Detailed maps of the Hebrides (A) Galway Bay/Aran Islands (B) are also shown.

4.3.2 DNA extraction

Two different types of DNA extraction techniques were used according to how the specimens had been preserved. For specimens which were stored according to the CBOL guidelines a middle leg was removed from each individual and placed in a 1.5ml Eppendorf tube. The forceps used were cleaned in bleach, distilled water and

70% ethanol prior to removing each leg. The QIAGEN DNeasy® Tissue Kit was then used following the (QIAGEN DNeasy® bench) protocol for animal tissues. The 1970s samples had their DNA extracted following the Qiagen Insect PBS extraction protocol (Anonymous, 2006a) with the protocol modification of changing the incubation time from 10 min to 4 h. All extracted DNA samples were stored at -20°C.

4.3.3 DNA regions examined

The CO1 barcode region, cytochrome b, and ITS region were PCR amplified. For the CO1, a partial region of c.650bp was amplified using the primers LCO_Hym and Nancy_Short (Magnacca and Brown, 2010). In the cytochrome B region a partial region of 490bp was amplified using the forward primer Cytb_F1 and the reverse primer Cytb_R (Schwarz et al., 2004) and in the nuclear ITS region, a region of 600 bp was amplified using the forward primer CAS18SF1 and reverse primer CAS5p8sB1d (Ji et al., 2003). The primer names and corresponding primer sequence are listed in Table 24.

Table 24 - Primer names and sequences to amplify DNA in the CO1 barcode region, cytochrome b, and ITS region

PRIMER NAME	PRIMER SEQUENCE
Nancy_Short	5' CCCGGTAAAATTAAAATATAAAC-3’
LCO_Hym	5'–TATCAACCAATCATAAAGATATTGG–3'
CAS18SF1	5'-TACACACCGCCCGTCGCTACTA-3'
CAS5p8sB1d	5'-ATGTGCGTTCRAAATGTCGATGTTCA-3'
Cytb_F1	5'-TATGTACTACCATGAGGACAAATATC-3'
Cytb_ R	5'-ATTACACCTCCTAATTTATTAGGAAT-3’

4.3.4 DNA amplification and sequencing

DNA was amplified under different conditions according to the DNA regions being amplified. For the CO1 region: initial denaturation for 3 min at 94°C; 32 cycles of 60 s denaturation at 95°C, 60 s annealing at 48°C and 60 s elongation at 72°C, and a final extension for 4 min at 72°C. For the cytochrome b region: initial denaturation for 3 min at 94°C, 32 cycles of 60s denaturation at 95°C, 60 s annealing at 48°C and 60 s elongation at 72°C, and a final extension for 4 min at 72°C. For the ITS region: initial denaturation for 3 min at 94°C; 32 cycles of 60 s denaturation at 95°C, 60 s annealing at 60°C and 60 s elongation at 72°C; and a final extension for 4 min at 72°C.

PCR products were run on a 2% agarose gel at 120v for 30 min to establish the success of each of the DNA amplification prior to sequencing. Following confirmation of PCR DNA amplification, 5 µl of the PCR product was treated with 5 µl of an ExoSap mastermix (comprising 2 µl alkaline phosphatase (1U/ µl), 0.3 µl exonuclease (20U/ µl) and 2.7 µl H_2O per reaction) and incubated at 37°C for 30 min followed by 82°C for 20 min to remove unused primers and dNTPs. Sequencing products were purified using 100% ethanol precipitation and 2 µl 3M NaOAC for each reaction (see Appendix 1). DNA was sequenced using an ABI 3730xl capillary sequencer (Applied Biosystems), in both directions, using BigDye Terminator V 3.1 chemistry (Applied Biosystems). Chromatograms were edited in Bioedit v 7.0.9.1 and contiguous sequences were generated from the forward and reverse strands. All polymorphic nucleotides were inspected manually to ensure correct base calling. The first 10 to 20 nucleotides were unreadable and were trimmed from the sequence. In cases where only a forward or reverse sequence was available, a string of N's was inserted into the sequence to account for ambiguous regions caused by excess dye peaks. The edited and trimmed CO1 sequence alignment was imported into Mega version 4.0 (Tamura et al., 2007) for phylogenetic analysis. After sequence trimming, a total of 610 base pairs were available for analysis. Nucleotide and polymorphism positions were determined by comparing to the full CO1 gene sequence from the mitochondrial genome of *B. hypocrita* (GenBank accession no. NC011923; positions 1996 to 3555). Sequence divergences were calculated using the Kimura 2-parameter (K2P) distance model (Kimura 1980) using MEGA 4 after partioning the sequences into the following groups: 1) British and Irish; 2) geographic location and 3) blonde or melanic. The final aligned CO1, ITS and cytochrome B matrices, for specimens included in this study are provided in Appendix 2, 3 and 4 respectively.

4.4 Results:

The CO1 barcode was sequenced for 56 melanic and non-melanic (blonde) specimens. Three partial sequences derived from historic specimens (c.f. Chapter 3). All sequences have been deposited in Genbank (Submission # 1447887). A comparative alignment against the *B. hypocrita* CO1 gene was carried out which indicated that the trimmed sequence alignment begins at position 68 of the CO1 gene. The final edited alignment comprised 610 nucleotides excluding the sequences obtained from the historic specimens. Excluding gaps and missing nucleotides a total of 567 nucleotides were available for analysis. Within the CO1 alignment seven variable sites were observed, two of which were found in a single specimen *B. mus* T612 (Co. Clare, blonde) comprising an A at position 524 (all other specimens have G) and *B. mus* T746 (Dublin, blonde) comprising a G at position 606 (all other specimens have A). Three specimens, *B.mus.* BM008 (Co. Clare, blonde), *B.mus.* T653 (Dublin, blonde) and *B. mus.* BM011 (Sherkin, Blonde) comprised an A at position 99 (all other specimens have G). The two identical specimens from Tiree (*B. mus.* BM045 and BM046) comprised three shared unique polymorphisms, a T instead of an A at position 347, an A instead of a G at position 421 and a T instead of an A at position 342. The single nucleotide polymorphism (SNP) at position 144 was more widely distributed with all specimens having either a C or T at position144 (Fig. 27). Of the 59 specimens included in the final CO1 alignment, 34 and 25 comprised either T or C respectively at this position. This polymorphism revealed that there are two different haploytpes of *B. muscorum* in the British Isles. The different haplotypes distribute themselves (though not exclusively) according to country, with the C type polymorphism being found mostly in Britain and the T type polymorphism being found mostly in the samples from Ireland (Table 25). However, C-type polymorphisms were found in the following sampling locations in Ireland: Co. Mayo, Co. Dublin, Inis Oirr, and Co. Cork. All British specimens included in this study were of the T haplotype except for the two representatives from Tiree.

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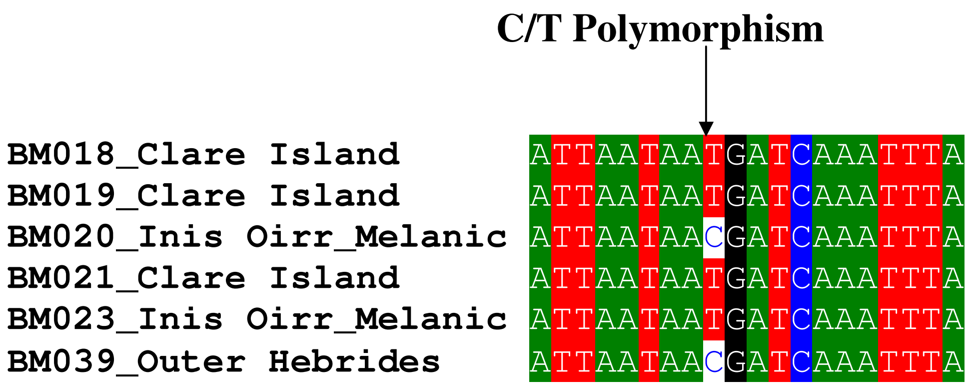

Fig. 27 - Screenshot of the DNA barcode alignment matrix indicating a single nucleotide polymorphism (SNP) at position 144 of either a C/T polymorphism.

Table 25 - Sampling location, variety and number of each haplotype

Country	Location	Variety	C-Haplotype	T-Haplotype
Ireland (Mainland)	Co. Clare	*pallidus*	0	3
Ireland (Mainland)	Dublin	*pallidus*	2	4
Ireland (Mainland)	Baltimore	*pallidus*	1	0
Ireland	Clare Island	*pallidus*	0	4
Ireland	Aghany	*pallidus*	1	0
Ireland	Sherkin Island	*pallidus*	0	3
Ireland (Aran Islands)	Inis Mor	*allenellus*	0	4
Ireland (Aran Islands)	Inis Meain	*allenellus*	0	4
Ireland (Aran Islands)	Inis Oirr	*allenellus*	1	7
Scotland (Inner Hebrides)	Coll	*agricolae*	4	0
Scotland (Inner Hebrides)	Tiree	*agricolae*	0	2
Scotland (Outer Hebrides)	South Uist	*agricolae*	2	0
Scotland	Shetlands	*agricolae*	6	0
England (Scilly Islands)	St Angus	*scyllonius*	5	0
England (Scilly Islands)	Samson	*scyllonius*	3	0

Sequences were partitioned based on geographic location or melanic status to permit inter- and intra-group comparisons. However the inter- and intra-group genetic distance values obtained using the K2P distance model (Kimura 1980) were extremely low indicating that insufficient polymorphism existed to permit an informative population level or phylogenetic analysis. Distance values are calculated as the average number of base differences per site over all sequence pairs with the group. Intra-group mean distance values for British and Irish representatives were 0.0013 and 0.001 respectively. The intergroup distance value was 0.0022. A mean intergroup distance value of 0.0015 was obtained for both the blonde and melanic forms indicating that no significant difference between both groups exists. Variation between the groups was extremely low with an intergroup distance value of 0.0018. Intra-group and intergroup distance values are given as Table 26 and Table 27 respectively with intra-group distance values ranging from 0 to 0.0024. The intergroup distance values indicate that the Tiree and Dublin specimens are the most variable in CO1 sequence for British and Irish *B. muscorum* respectively.

Table 26 - Within group distance (D) values for 13 Irish and British geographic locations from which *B. muscorum* was sampled.

	D
Co. Clare	0.0024
Dublin	0.0022
Inis Oirr	0.0005
Sherkin	0.0012
Clare Is.	0
Inis Mor	0
Inis Meain	0
Coll	0
South Uist	0
Shetlands	0
St Agnus	0
Samson	0
Tiree	0

Table 27 -Estimates of inter-group divergences based on mean distance values calculated from pairwise analysis of representatives from 13 locations of Irish and British *B. muscorum*.

	Co. Clare	Dublin	Inis_Oirr	Sherkin	Clare_Is.	Inis_Mor	Inis_Meain	Coll	South_Uist	Shetlands	St_Agnus	Samson	Tiree
Co. Clare	-	-	-	-	-	-	-	-	-	-	-	-	-
Dublin	0.0022	-	-	-	-	-	-	-	-	-	-	-	-
Inis_Oirr	0.0014	0.0013	-	-	-	-	-	-	-	-	-	-	-
Sherkin	0.0014	0.0016	0.0008	-	-	-	-	-	-	-	-	-	-
Clare_Is.	0.0012	0.0012	0.0002	0.0006	-	-	-	-	-	-	-	-	-
Inis_Mor	0.0012	0.0012	0.0002	0.0006	0	-	-	-	-	-	-	-	-
Inis_Meain	0.0012	0.0012	0.0002	0.0006	0	0	-	-	-	-	-	-	-
Coll	0.003	0.0018	0.0016	0.0024	0.0018	0.0018	0.0018	-	-	-	-	-	-
South_Uist	0.003	0.0018	0.0016	0.0024	0.0018	0.0018	0.0018	0	-	-	-	-	-
Shetlands	0.003	0.0018	0.0016	0.0024	0.0018	0.0018	0.0018	0	0	-	-	-	-
St_Agnus	0.003	0.0018	0.0016	0.0024	0.0018	0.0018	0.0018	0	0	0	-	-	-
Samson	0.003	0.0018	0.0016	0.0024	0.0018	0.0018	0.0018	0	0	0	0	-	-
Tiree	0.0066	0.0066	0.0056	0.006	0.0054	0.0054	0.0054	0.0072	0.0072	0.0072	0.0072	0.0072	-

Sampling undertaken in Ireland for this study confirmed the Aran Islands (all three islands) as the single source of the melanic colour morph of *B. muscorum.* The blonde forms are known from the Saltee Islands, Co. Wexford (Prof John Breen, personal communication), Cape Clear Island, Co. Cork, Sherkin Island, Co. Cork, Clare Island, Co. Mayo (Aislinn Deenihan, personal observation) and Aranmore Island, Co. Donegal (Prof. John Breen, personal communication). Previously generated sequence data for a cryptic species aggregate of *Bombus* (Murray et al., 2007) revealed intra- and interspecific estimates of variation of 0.36% and 2.8%, respectively, the study's results (1.1% intraspecific variation) indicate that British and Irish *B. muscorum* (whether melanic or blonde) are a single species. Additionally a DNA bee barcoding study by Sheffield et al. (2009) reported a DNA barcoding gap of approx. 0.5% between bee species, again supporting the single species status of the blonde and melanic varieties of *B. muscorum.* Conversely, a study by Kuhlmann et al. (2007) reported no barcoding gap in three *Collettes* species, highlighting problems with the barcoding method. Using a DNA barcoding gap of 0.5% for delineating species, results from this study argue for a single species status for the blonde and melanic varieties of *B. muscorum*.

There were no differences between the 1970s' specimens and the more recent samples. It was impossible to estimate the current population health using the data due to the small and uneven sample sizes. Museum specimens from Ireland that were successfully sequenced all exhibited the T haplotype. Six DNA samples that were variable for CO1 sequence were sequenced in the Cytochrome B and ITS region. Analysis of sequences showed no polymorphisms (see appendices 3 and 4 for sequence alignments). Thus sequencing of the other DNA samples was not pursued.

4.5 Discussion

A number of melanic and blonde specimens of *B.* muscorum were collected from various geographic locations across the British Isles to determine whether melanic and blonde varieties are divergent genetically from each other. Although the small sample sizes (n=1 to n=7) inhibited the ability to conduct informative population level genetic analysis, some information regarding variability of *B. muscorum* at different geographic locations across the British Isles could be gleaned by inter- and intra-group estimates of genetic distances. Of the British island representatives, the Tiree specimens are distinct, even though very little geographic distance separates this location from Coll (both Inner Hebrides Islands). However, if these specimens were removed from the analysis, all remaining British specimens were shown to be identical for CO1 barcode sequence. Overall, Irish *B. muscorum* seems to be more divergent. The most informative polymorphism identified was the C/T polymorphism at position 144 and although the C haplotype was the predominant form found in Irish representatives, the T haplotype was also present in a number of Irish collection sites. Although this study highlights some interesting population level characteristics for British and Irish *B. muscorum* further insight into these characteristics can only be achieved by studies involving increased sample sizes and more appropriate molecular markers. However the purpose of this study was to compare melanic and blonde in order to determine whether melanic or blonde specimens are divergent genetically from each other and in this case the CO1 barcode sequence seems to be an appropriate choice to help resolve phylogenetic and taxonomic issues that have arisen with respect to the blonde and melanic varieties of *B. muscorum.*

The lack of genetic variation between melanic and non-melanic samples used in this study argues against theories previously postulated (e.g. Richards, 1935; Rasmont and Adamski, 1995) that melanism in *B. muscorum* was due to species differentiation. Ellis et al. (2005) highlighted the pitfalls of using mtDNA for taxonomic delimitation; however, this study also partially examined the nuclear

DNA region the internal transcribed spacer (ITS). There are likely to be differences in the frequencies of SNPs between nuclear and mtDNA, as there is a lower Ne in mtDNA than nuclear DNA, therefore a lower number of SNPs in mtDNA. In this study, no differences were found in the number of polymorphisms between nuclear and mitochondrial regions. Thus based on the sequence data presented here, and the fact that very low levels of polymorphism were observed, it can be argued that melanic and non-melanic specimens are the same species. The distinct colour morphs of *B. muscorum* had caused authors such as Richards (1935) to regard the melanic, and intermediate colour morphs as separate sympatric species. Results from this study show that this melanic trait is due to convergent evolution and that these colour morphs are most like to have arisen from a product of unknown environmental factors present on islands. This melanism has evolved through congruence five times on Islands around the British Isles (Aran Islands, Channel Islands, Hebride Islands, Scilly Isles and Shetland Islands). Postulations had been put forth that they may be remnants of a once widespread species that survived the last glaciations (Midlandian and Devensian) in Ireland and Britain on islands, since the melanic and intermediate versions of *B. muscorum* are geographically dispersed on Islands which have an arc distribution. However, the results from this study invalidate this theory.

The convergent evolution resulting in the melanism of *B. muscorum* may be due to selection factors that are not present on the mainland of the British Isles. Island habitats have a number of environmental characteristics that make them distinct from their mainland counterparts (MacArthur and Wilson, 1967), and it is similarities between the habitats occupied by *B. muscorum* on different islands around the British Isles that may be responsible for the repeated convergent evolution of the melanic form. Islands' unique geography (small size, barriers to dispersal and an altered climatic variability) can result in the evolution of distinctive traits in island organisms that differentiate from its non-island counterparts (MacArthur and Wilson, 1967). Island melanism has been reported for other bumblebee species such as *B. terrestris* (Chittka et al., 2004), and *B.*

trifasciatus (Williams, 1991). Selection factors such as climate and ecology may be responsible for the occurrence of melanism in *B. muscorum*.

It is possible that melanic phenotypes of *B. muscorum* could have arise due to climatic factors such as temperature and exposure to UV light. Stiles (1979) suggested that thermoregulation may be an important variable in determining the colour of pubescent bumblebees. Exposure to UV light may also play a role in bumblebee melanism as studies have found that that colour patterns of bumblebees depend strongly on geographic origin (Plowright and Owen, 1980; Williams, 2007a). A trend of bumblebee species with progressively darker colour patterns occurring from a mid latitude to higher northern latitudes was reported by Williams (2007a). The islands where the melanic *B. muscorum* occur may receive a larger proportion of UV light due to increased reflection from the sea surrounding the islands and the present of a light reflecting rock on the islands. For example, on the Aran Islands, the bedrock is limestone and it is exposed across a large proportion of the islands resulting in an increase in the reflection of sunlight (Aislinn Deenihan, personal observation). However, such factors are also present on other islands without the melanic form of *B. muscorum* around the British Isles. Intensive study of the geography of islands around the British Isles where the melanic *muscorum* colour morphs occur and where only blonde colour morphs occur would have to take place to investigate this possibility.

Predation and parasitism have long been recognised as factors that drive selection (Darwin, 1859). Different ecological factors such as absence of certain predators may also be present in the islands where the melanic *B. muscorum* occur. Williams (2007) suggested that bumblebees with a yellow-brown colouration that nest on the ground may use crypsis to conceal themselves during the months when grassland is drying and herb stems turn yellow-brown. This time period also co-insides with the time when the nest bumblebee nest populations are at a peak. *B. muscorum* fulfils these requirements. Another possible reason for the island melanism of *B. muscorum* is the absence of certain predators. It is known that islands often contain a different species assemblage than it nearest mainland counterpart (MacArthur and Wilson, 1967). Thus it may be possible to infer that islands where these melanic bumblebees are found may not contain a predator that is present on the mainland. To obtain a definitive explanation for the melanism of *B. muscorum* on these islands is outside the scope of this project, but our results clearly demonstrate the utility of barcoding and the pitfalls associated with its use – as the genetic techniques utilised in barcoding are useful in deriving information on the status of a species but not in explaining the expression of phenotypic characters.

Conservation has been defined as the artificial control of ecological relationships and environment to promote the preservation and maintenance of a particular balance of species (Allaby, 2003). The DNA barcoding technique was designed to aid taxonomic classifications and as such improve the understanding and conservation of biodiversity (e.g. Hebert et al., 2004a; Hajibabaei et al., 2007; Valentini et al., 2009). The very low levels of genetic variation that we observed in the CO1 region indicates that, classification of the melanic colour morphs as different species is not supported. The DNA barcoding results study support the recognition of different varieties representing the different melanic and blonde forms within a single species. The taxonomic resolution of *B. muscorum* and its varieties should assist in the development of appropriate conservation efforts for *B. muscorum,* as no specific form will seem more deserving of conservation over another. If for example, the results of this barcoding study indicated that the melanic and blonde forms were indeed separate species or had significant genetic differences, alternative conservation strategies would be needed e.g. preservation of the Aran Islands bumblebee *Bombus muscorum* var. *allenellus* may have become more noteworthy than preserving *Bombus muscorum* var. *pallidus* in Ireland. Instead, funds that become available for conservation of the species can be directed at the preservation of the entire spectrum of varieties of the species. The conservation of the entire spectrum of varieties of *B. muscorum* would help the species maintain its range, rather than a particular variety of the species in one area. The results of this study show that DNA barcoding is important for conservation efforts through taxonomic clarification of varieties that could otherwise inhibit the appropriate distribution of conservation funds through lack of knowledge of species status.

Knowledge of genetic differentiation within an insect species is important for its conservation as genetic homogenization of a species compromises its adaptive responses (Thompson et al., 2007). Previous studies of endangered bumblebees have found significant levels of genetic differentiation even at small distance ranges (>10km) (Darvill et al., 2006; Ellis et al., 2006). Our study supports such results by the finding of the two different haplotypes which even occur sympatrically in Co. Clare, Inis Oirr, and Dublin. It can thus be suggested that Co. Clare, Inis Oirr, and Dublin have higher effective population sizes (Ne), implying that these *B. muscorum* populations have a greater gene diversity and better population health. The concurrent appearance of these two haplotypes in Ireland indicate that the populations in Ireland have a greater Ne, and thus better population health, than the populations sampled in Britain for *B. muscorum,* as no location in Britain sampled for this study reported the occurrence of both haplotypes from the same location. Even with our modest sampling it seems that the C type haplotype occurs at a higher frequency in Britain. The T haplotype seems more common in Ireland. These haplotypes occur due to a SNP. Single nucleotide polymorphisms can be used in genetic applications relevant to conservation management, and have been used for the honeybee, *Apis mellifera* (Zayed, 2009). Conservation of both haplotypes of *B. muscorum* is important to help the survival of this endangered species survive. Thus it seems that a higher degree of genetic variation exists within Irish populations compared to their British counterparts. In Britain, *B. muscorum* is viewed as a declining bee with decreasing numbers in populations (Darvill et al., 2006; Goulson, 2010), while in Ireland the situation is not considered as bad, though the species is also recognised to be declining (Fitzpatrick et al., 2006b).

A greater understanding of the genetic differentiation within a species can often be found by examining DNA from museum specimens (e.g. Cano 1996; Pääbo et al., 2004; Strange et al., 2009). However, the problems that can arise from using the sequences from museum specimens of rare species are evident in this study. It was impossible to directly compare the sequences from the museum specimens and recently collected specimens of *B. muscorum* due to the small sample size of museum specimens and differences in collection locations. However, superficial comparisons of haplotypes were possible between the sequences from the museum specimens and recently collected *B. muscorum* samples. The information derived from these sequences only allowed analysis into the haplotype present in the samples i.e. whether the C or T haplotype was present. However this information can still be deposited in the BOLD database. Zayed (2009) advocated the use genetics on museum samples for deriving information on bee declines through estimations of parameters such genetic diversity and differentiation. However as this study demonstrated deriving such data is not an easy task technically.

4.6 Conclusion

From this study's results it can be argued that British and Irish *B. muscorum* (whether melanic or blonde) represent a single species. The presence of melanic forms on islands is most likely due to convergence although phenotypic plasticity induced by the island habitat can not be ruled out. The environmental factors associated with island life and selective pressures that contribute to this convergent (and presumably adaptive) trait remain, as yet undetermined.

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Chapter 5 Discussion and Conclusion

5.1 Overview

In contrast to previous studies, which have concentrated on intensive and semi-natural agricultural systems and widespread bumblebees (e.g. Svensson et al., 2000; Svensson, 2002; Lye et al., 2009), this study has produced data on the foraging and nest-site seeking behaviour of both rare and common bumblebee queens in what is potentially their optimum habitats. The confusion surrounding the status of the melanic colour varieties of *B. muscorum* has been evaluated through this project. The overall conclusions from each section are

- Prime landscapes are important for insect conservation because they provide data on the ecological niches of rare species;
- DNA can be extracted from museum specimens but killing and preservation techniques should be noted on labelling;
- Melanic and non-melanic colour varieties of *B. muscorum* are the same species.

These conclusions contribute towards our knowledge of bumblebee ecology and genetics that can lend itself ultimately towards improved conservation of bumblebees.

5.2 Bumblebee Nest Site Seeking, Diversity and Foraging within the Burren Region

Bumblebee diversity in the Burren region in Ireland represents an ideal that might have been present elsewhere in Ireland had it not been for the introduction of intensive farming methods. The association of the nest-site seeking activities of certain bumblebee species with calcareous type grassland and stonewall boundaries highlights the importance of the intertwined nature of the extensive farming landscape and the region's exceptional biodiversity. Calcareous grassland and stonewall boundaries are an integral part of the landscape of the Burren and calcareous grassland is key to supporting the biodiversity with the Burren (e.g Dunford and Feehan, 2001; Dunford 2002; O'Rourke, 2005). Bumblebees as important pollinators, themselves have played a role in maintaining the biodiversity within the Burren. Jeffrey (2003) highlighted the role of vertebrate and invertebrate grazing in the Burren in plant dynamics. The important part played by man in shaping this landscape was highlighted by Dunford (2002), who acknowledged the importance of the extensive farming in the region for maintaining this landscape.

The importance of this region for biodiversity is further highlighted by the presence of the melanic colour variety of *B. muscorum* on the Aran Islands. The delicate dynamic between the human and environmental factors that maintain the Burren landscape needs to be maintained and protected to avoid further damage by anthropogenic influences. These anthropogenic influences are not directly the threats posed by intensive farming. Problems are now occurring in the Burren which are due to inappropriate maintenance of calcareous grassland, such as under-grazing leading to the advance of scrub (ERA-Maptec et al., 2006; Parr et al., 2007; Deenihan et al., 2009), and further research should be conducted on the effect the current scrub encroachment problems are having on the flora and the fauna of the Burren. More research needs to be undertaken to learn about the genetic health of and ecosystems that support these rare bumblebees.

5.3 The Aran Island Bumblebee, Bombus muscorum var. *allenellus*

Stelfox (1933) was the first to officially document the occurrence of the Aran Island Bumblebee, *B. muscorum* var. *allenellus*. Based on the results obtained through DNA barcoding in this study, it can be said that that *B. muscorum* var. *allenellus* is a variety of *Bombus muscorum,* and not a different species of bumblebee. A variety in this study is defined as a group of organisms of the same species with morphological differences from the main population. These groups are capable of interbreeding with the main population but do not do so, due to geographic isolation. The conclusive classification of the melanic colour morphs should aid conservation efforts of all *B. muscorum* varieties as no variety will seem more deserving of conservation over another due to confusion and concern over its species status. Even though the question of whether *B. muscorum* var. *allenellus* is a separate species has been answered, this colour variety is still noteworthy. The persistence of this colour variety on the Aran Islands when its mainland counterpart is experiencing a rapid decline, can be attributed to the high nature value of the extensive farming on the Aran Islands. This provides direct evidence of the correlation between habitat loss and bumblebee decline.

The melanism of *B. muscorum* is most likely due to unknown environmental factors present on the Aran Islands that are allowing convergent evolution to occur. This convergent evolution highlights the exceptional nature of these islands within Ireland. Research to identify the distinct environmental conditions of the Aran Islands was outside the scope of this project. The possibility of the Irish Meteorological Service, MET Eireann, establishing a permanent weather monitoring station on the Islands in the future might allow for certain unique environmental factors to be identified. However, only one weather monitoring station is planned and it will be based on the smallest Island Inis Oirr (Paddy Ni Chathain, Inis Oirr Comhar Cumman Chairperson, personal communication, 2009).

5.4 DNA extractions from museum samples

To ascertain if the same haplotype of *B. muscorum* was present in the 1970s, a number of historical specimens of *B. muscorum* had their DNA extracted. To have a historical specimen typed is important as it allows for type referencing of unidentified historical samples and can contribute to the study and promotion of conservation genetics. Museums and private collections can provide information but museum curators will only allow specimens to be semi-destructively and even destructively sampled if they can be assured of the quality of the information output. Historical specimens can easily become damaged and discoloured in attempts to extract DNA and valuable time and money can be wasted through the application of inappropriate methods as demonstrated by the failure of five methods trialled during this study. More species specific methods need to be identified, and non-destructive methods similar to those described by Gilbert et al.

(2007) need to be developed to ensure more access to samples by museum curators and private collections. A method suitable for bumblebee samples that undergo harsh chemical treatment was developed as part of this study. This method can be recommended for use to those undertaking DNA extractions from samples that may have been subjected to harsh chemical treatments (e.g. cyanide killing).

Museum curators and specimen collectors need also to document more information for each specimen to allow for less damage to specimens through sampling for DNA. Standardised killing, preservation and preservation treatment methods need to be developed for each insect type that allow DNA to be easily extracted from specimens in the future. Extreme difficulties were encountered during the laboratory work for the DNA extractions from the museum specimens due to a lack of knowledge regarding the treatment methods the samples had been subjected to. Several trial samples were destroyed during the efforts to extract DNA from the museum specimens. More detailed labelling of specimens, particularly those not preserved accord to CBOL guidelines, is essential to prevent loss of these valuable sources of genetic information.

5.5 Relevance of the results

This thesis has produced valuable data on the ecology of bumblebees in prime habitats and has determined conclusively the status of the melanic varieties of *B. muscorum.* This information can contribute to bumblebee conservation. The ecological information on rare bumblebees in the Burren region could form part of future agri-environmental schemes for the Burren. The ecological information gathered could help other research projects on the Burren region or research on pollinators in prime regions. More research needs to be conducted in prime landscape locations to determine the ideal conditions for bumblebee conservation so that data can be extrapolated to help their conservation in non-ideal environments. This project supplements previous ecological research on rare bumblebees in prime/agri-environmental locations including studies by Carvell (2002), in southern England, and Redpath et al. (2010) in northwest Scotland, but since it is the first to include an emphasis on nest-site seeking behaviour in the prime landscapes for bumblebees of calcareous grassland, further research is called for on nest-site seeking behaviour in prime landscapes elsewhere.

Data produced during this study can aid in bumblebee conservation through their sole and combined use with other bumblebee genetic and ecological projects. The sole use of this genetic information is that the conclusive classification of the melanic colour morphs of *B. muscorum* should aid conservation efforts of all *B. muscorum* varieties as no variety will seem more deserving of conservation over another due to confusion and concern over its species status. Instead any funds that become available for conservation of the species can be directed at the preservation of the entire spectrum of varieties of the species. However, the information derived may also be combined with other projects to help promote bumblebee conservation. Genetic sequences and barcodes from this project are available on BOLD and Genbank.

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Chapter 7 Appendices

Appendix 1- Protocol for purifying PCR products to be sequenced using 100% ethanol precipitation and 2 µl 3M sodium acetate (Anonymous, 2009).

Cycle Sequence Product Clean-Up (Ethanol Precipitation)

This protocol involved two wash steps (one with 100% ethanol and sodium acetate, and a second with 70% ethanol).

Steps:

1. (a) The master-mix wash buffer was prepared with each reaction requiring:

50 µl 100% ethanol
2 µl 3M NaOAC (sodium acetate)

A master mix was made with these volumes multiplied by number of reactions (+10% to allow for pipetting error). They were homogenated, and aliquots were distributed to each cycle sequencing reaction.

2. They were then left stand at room temperature for 15 min.

3. Following this they were placed on ice for 30 min to 1 h.

4. Following this they were centrifuged for 30 min at 4000rpm

5. Immediately after spinning samples down, the samples were inverted in the centrifuge bucket. The centrifuge was set to 180 rcf and then the samples were returned to their upright position.

6. A 70% ethanol wash buffer was made (30 µl per sample, including 10% extra margin for error). There was a 30 µl aliquot of the wash buffer added to each sample.

7. The samples were placed in the centrifuge bucket and spun for 20 min at 4000rpm. The samples were then gently inverted and placed in the centrifuge bucket.

8. The centrifuge was set to 1000rpm for 1 min and the samples were spun. Immediately after this the tubes were removed from the bucket and placed the right way up. The tubes were then uncovered (without caps) for at least 25 min to ensure that all traces of ethanol have evaporated from the pelleted DNA.

Appendix 2 DNA sequence from the mitochondrial DNA for B. muscorum, using the CO1 mitochondrial DNA sequence for Bombus hypocrita as a reference

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                                     10        20        30        40        50        60        70        80
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita ATAAAAAAATGACTAATATCAACTAATCATAAAAATATTGGAATAATATATTTTATTTTCGCTATATGATCAGGAATAAT
B.mus_T611_Clare_(B)          -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T612_Clare_(B)          -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM008_Clare_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T653_Dublin_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T651_Dublin_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T684_Dublin_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus T748_Dublin_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T747_Dublin_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T746_Dublin_(B)         -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM020_Inis Oirr_(M)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM011_Sherkin_(B)       -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM001_Baltimore (B)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM018_Clare Is._(B)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM017_Clare Is._(B)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus BM019_Clare_Is._(B)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM021_Clare Is._(B)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM010_Sherkin_(B)       -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM002_Sherkin_(B)       -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM061_Inis_Mor_(I)      -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM062_Inis_Mor_(M)      -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM063_Inis_Mor(M)       -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T605_Inis_Mor (M)       -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T605_Inis_Meain (M)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T666_Inis Meain_(M)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T667_Inis Meain_(M)     -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM005_Inis Meain_(M)    -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM003_Aghany (B)        -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM015_Inis_Oirr_(M)     -------------------------------------------------------------------GATCAGGAATAAT
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B.mus_T603_Inis Oirr_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T623_Inis Oirr_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T646_Inis Oirr_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T604_Inis_Oirr_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM006_Inis Oirr_(M)            -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM023_Inis Oirr_(M)            -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T606_Coll_(M)                  -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T616_Coll_(M)                  -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T681_Coll_(M)                  -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T682_Coll_(M)                  -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM039_South_Uist_(M)           -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM044_South Uist_(M)           -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T607_Shetlands_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T614_Shetlands_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T613_Shetlands_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T678_Shetlands_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T679_Shetlands_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T680_Shetlands_(M)             -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T619_St Agnus_(M)              -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T620_St Agnus_(M)              -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T621_St Agnus_(M)              -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T622_St Agnus_(M)              -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T608_St Agnus_(M)              -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T609_Samson_(M)                -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T617_Samson_(M)                -------------------------------------------------------------------GATCAGGAATAAT
B.mus_T610_Samson_(M)                -------------------------------------------------------------------GATCAGGAATAAT
B.mus_BM045_Tiree_(M)                -------------------------------------------------------------------------------T
B.mus_BM046_Tiree_(M)                -------------------------------------------------------------------------------T
B.mus_Cape Clear(26/7/1972(B)        ----------------------------------------------------------------------------TAAT
B.mus_Saltee_Is.(20/8/1972)(B)       ----------------------------------------------------------------------------TAAT
B.mus_Kilglass(8/9/1972)(B)          ----------------------------------------------------------------------------TAAT

                                              90        100       110       120       130       140       150       160
                                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita        TGGTTCATCCATAAGTTTATTAATTCGAATAGAATTAAGTCATCCCGGAATATGAATTAATAATGATCAAATTTATAATT
B.mus_T611_Clare_(B)                 TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T612_Clare_(B)                 TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
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B.mus_BM008_Clare_(B)	TGGATCTTCAATAAGATTATTAATTCGAATAGAATTAAGTNNNNNNGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T653_Dublin_(B)	TGGATCTTCAATAAGATTATTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T651_Dublin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T684_Dublin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus T748_Dublin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T747_Dublin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T746_Dublin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM020_Inis Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_BM011_Sherkin_(B)	TGGATCTTCAATAAGATTATTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM001_Baltimore (B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_BM018_Clare Is._(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM017_Clare Is._(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus BM019_Clare_Is._(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM021_Clare Is._(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM010_Sherkin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM002_Sherkin_(B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM061_Inis_Mor_(I)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM062_Inis_Mor_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM063_Inis_Mor(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T605_Inis_Mor (M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T605_Inis_Meain (M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T666_Inis Meain_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T667_Inis Meain_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM005_Inis Meain_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM003_Aghany (B)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_BM015_Inis_Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T603_Inis Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T623_Inis Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T646_Inis Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T604_Inis_Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM006_Inis Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM023_Inis Oirr_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_T606_Coll_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T616_Coll_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T681_Coll_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T682_Coll_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_BM039_South_Uist_(M)	TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT

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B.mus_BM044_South Uist_(M)        TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T607_Shetlands_(M)          TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T614_Shetlands_(M)          TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T613_Shetlands_(M)          TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T678_Shetlands_(M)          TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T679_Shetlands_(M)          TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T680_Shetlands_(M)          TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T619_St Agnus_(M)           TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T620_St Agnus_(M)           TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T621_St Agnus_(M)           TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T622_St Agnus_(M)           TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T608_St Agnus_(M)           TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T609_Samson_(M)             TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T617_Samson_(M)             TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_T610_Samson_(M)             TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAACGATCAAATTTATAATT
B.mus_BM045_Tiree_(M)             TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_BM046_Tiree_(M)             TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGTCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_Cape Clear(26/7/1972(B)     TGGATCTTCAATAAGATTGTTAATTCGAATAGAANNNNNNCATCCTGGTATATGAATTAATAATGATCAAATTTATAATT
B.mus_Saltee_Is.(20/8/1972)(B)    TGGATCTTCAATAAGATTGTTAATTCGAATAGAATNNNNNNNNNNNNNNNNNNGAATTAATAATGATCAAATTTATAATT
B.mus_Kilglass(8/9/1972)(B)       TGGATCTTCAATAAGATTGTTAATTCGAATAGAATTAAGNNNTCCTGGTATATGAATTAATAATGATCAAATTTATAATT

                                          170       180       190       200       210       220       230       240
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita      CTTTAGTAACTAGACATGCATTTTTAATAATTTTTTTTATAGTAATACCATTTATAATTGGAGGATTTGGAAATTACTTA
B.mus_T611_Clare_(B)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T612_Clare_(B)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM008_Clare_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T653_Dublin_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T651_Dublin_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T684_Dublin_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus T748_Dublin_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T747_Dublin_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T746_Dublin_(B)             CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM020_Inis Oirr_(M)         CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM011_Sherkin_(B)           CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM001_Baltimore (B)         CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM018_Clare Is._(B)         CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
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B.mus_BM017_Clare Is._(B)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus BM019_Clare_Is._(B)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM021_Clare Is._(B)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM010_Sherkin_(B)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM002_Sherkin_(B)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM061_Inis_Mor_(I)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM062_Inis_Mor_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM063_Inis_Mor(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T605_Inis_Mor (M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T605_Inis_Meain (M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T666_Inis Meain_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T667_Inis Meain_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM005_Inis Meain_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM003_Aghany (B)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM015_Inis_Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T603_Inis Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T623_Inis Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T646_Inis Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T604_Inis_Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM006_Inis Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM023_Inis Oirr_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T606_Coll_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T616_Coll_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T681_Coll_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T682_Coll_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM039_South_Uist_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM044_South Uist_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T607_Shetlands_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T614_Shetlands_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T613_Shetlands_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T678_Shetlands_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T679_Shetlands_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T680_Shetlands_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T619_St Agnus_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T620_St Agnus_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T621_St Agnus_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T622_St Agnus_(M)	CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA

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B.mus_T608_St Agnus_(M)            CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T609_Samson_(M)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T617_Samson_(M)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_T610_Samson_(M)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM045_Tiree_(M)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_BM046_Tiree_(M)              CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_Cape Clear(26/7/1972(B)      CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_Saltee_Is.(20/8/1972)(B)     CTTTAGTTACTANNCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA
B.mus_Kilglass(8/9/1972)(B)        CTTTAGTTACTAGTCATGCATTTTTAATAATTTTTTTTATAGTTATACCTTTTTTAATTGGTGGTTTTGGAAATTATTTA

                                             250       260       270       280       290       300       310       320
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita      ATTCCATTAATACTAGGATCACCAGATATAGCTTTTCCCCGAATAAATAATATTAGATTTTGACTTTTACCTCCATCACT
B.mus_T611_Clare_(B)               ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T612_Clare_(B)               ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM008_Clare_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T653_Dublin_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T651_Dublin_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T684_Dublin_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus T748_Dublin_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T747_Dublin_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T746_Dublin_(B)              ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM020_Inis Oirr_(M)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM011_Sherkin_(B)            ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM001_Baltimore (B)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM018_Clare Is._(B)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM017_Clare Is._(B)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus BM019_Clare_Is._(B)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM021_Clare Is._(B)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM010_Sherkin_(B)            ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM002_Sherkin_(B)            ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM061_Inis_Mor_(I)           ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM062_Inis_Mor_(M)           ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM063_Inis_Mor(M)            ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T605_Inis_Mor (M)            ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T605_Inis_Meain (M)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T666_Inis Meain_(M)          ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
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B.mus_T667_Inis Meain_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM005_Inis Meain_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM003_Aghany (B)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM015_Inis_Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T603_Inis Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T623_Inis Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T646_Inis Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T604_Inis_Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM006_Inis Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM023_Inis Oirr_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T606_Coll_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T616_Coll_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T681_Coll_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T682_Coll_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM039_South_Uist_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM044_South Uist_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T607_Shetlands_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T614_Shetlands_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T613_Shetlands_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T678_Shetlands_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T679_Shetlands_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T680_Shetlands_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T619_St Agnus_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T620_St Agnus_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T621_St Agnus_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T622_St Agnus_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T608_St Agnus_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T609_Samson_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T617_Samson_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_T610_Samson_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM045_Tiree_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_BM046_Tiree_(M)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT
B.mus_Cape Clear(26/7/1972(B)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGA--------------------------------------
B.mus_Saltee_Is.(20/8/1972)(B)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGA--------------------------------------
B.mus_Kilglass(8/9/1972)(B)	ATTCCTTTAATATTAGGATCACCAGATATAGCATTTCCTCGAATAAATAATATTAGATTTTGAATTTTACCTCCTTCATT

330 340 350 360 370 380 390 400

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NC_011923.1| Bombus hypocrita    ATTTATATTACTATTAAGAAATACATTTACACCTAATGTAGGAACAGGATGAACTATTTATCCTCCTTTATCTTCCTACC
B.mus_T611_Clare_(B)             AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T612_Clare_(B)             AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM008_Clare_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T653_Dublin_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T651_Dublin_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T684_Dublin_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus T748_Dublin_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T747_Dublin_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T746_Dublin_(B)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM020_Inis Oirr_(M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM011_Sherkin_(B)          AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM001_Baltimore (B)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM018_Clare Is._(B)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM017_Clare Is._(B)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus BM019_Clare_Is._(B)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM021_Clare Is._(B)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM010_Sherkin_(B)          AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM002_Sherkin_(B)          AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM061_Inis_Mor_(I)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM062_Inis_Mor_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM063_Inis_Mor(M)          AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T605_Inis_Mor (M)          AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T605_Inis_Meain (M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T666_Inis Meain_(M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T667_Inis Meain_(M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM005_Inis Meain_(M)       AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM003_Aghany (B)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM015_Inis_Oirr_(M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T603_Inis Oirr_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T623_Inis Oirr_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T646_Inis Oirr_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T604_Inis_Oirr_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM006_Inis Oirr_(M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM023_Inis Oirr_(M)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T606_Coll_(M)              AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
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B.mus_T616_Coll_(M)                AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T681_Coll_(M)                AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T682_Coll_(M)                AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM039_South_Uist_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM044_South Uist_(M)         AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T607_Shetlands_(M)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T614_Shetlands_(M)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T613_Shetlands_(M)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T678_Shetlands_(M)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T679_Shetlands_(M)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T680_Shetlands_(M)           AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T619_St Agnus_(M)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T620_St Agnus_(M)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T621_St Agnus_(M)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T622_St Agnus_(M)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T608_St Agnus_(M)            AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T609_Samson_(M)              AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T617_Samson_(M)              AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_T610_Samson_(M)              AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM045_Tiree_(M)              AATATTATTATTATTAAGAAATTTATTTACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_BM046_Tiree_(M)              AATATTATTATTATTAAGAAATTTATTTACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT
B.mus_Cape Clear(26/7/1972(B)      --------------------------------------------------------------------------------
B.mus_Saltee_Is.(20/8/1972)(B)     --------------------------------------------------------------------------------
B.mus_Kilglass(8/9/1972)(B)        AATATTATTATTATTAAGAAATTTATATACACCTAATGTTGGAACAGGTTGAACAGTTTATCCTCCATTATCTTCATATT

                                             410       420       430       440       450       460       470       480
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita      TATTTCATTCATCCCCATCAATTGATATTGCAATTTTTTCTTTACATATATCAGGAATTTCCTCTATTATTGGATCATTA
B.mus_T611_Clare_(B)               TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T612_Clare_(B)               TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM008_Clare_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T653_Dublin_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T651_Dublin_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T684_Dublin_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus T748_Dublin_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T747_Dublin_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T746_Dublin_(B)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
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B.mus_BM020_Inis Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM011_Sherkin_(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM001_Baltimore (B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM018_Clare Is._(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM017_Clare Is._(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus BM019_Clare_Is._(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM021_Clare Is._(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM010_Sherkin_(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM002_Sherkin_(B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM061_Inis_Mor_(I)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM062_Inis_Mor_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM063_Inis_Mor(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T605_Inis_Mor (M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T605_Inis_Meain (M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T666_Inis Meain_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T667_Inis Meain_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM005_Inis Meain_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM003_Aghany (B)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM015_Inis_Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T603_Inis Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T623_Inis Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T646_Inis Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T604_Inis_Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM006_Inis Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM023_Inis Oirr_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T606_Coll_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T616_Coll_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T681_Coll_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T682_Coll_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM039_South_Uist_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM044_South Uist_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T607_Shetlands_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T614_Shetlands_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T613_Shetlands_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T678_Shetlands_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T679_Shetlands_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T680_Shetlands_(M)	TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA

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B.mus_T619_St Agnus_(M)            TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T620_St Agnus_(M)            TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T621_St Agnus_(M)            TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T622_St Agnus_(M)            TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T608_St Agnus_(M)            TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T609_Samson_(M)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T617_Samson_(M)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_T610_Samson_(M)              TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM045_Tiree_(M)              TATTTCATTCATCACCATCTATTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_BM046_Tiree_(M)              TATTTCATTCATCACCATCTATTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA
B.mus_Cape Clear(26/7/1972)(B)     --------------------------------------------------------------------------------
B.mus_Saltee_Is.(20/8/1972)(B)     --------------------------------------------------------------------------------
B.mus_Kilglass(8/9/1972)(B)        TATTTCATTCATCACCATCTGTTGATATTGCAATTTTTTCTCTTCATATAACAGGAATTTCTTCTATTATTGGATCTTTA

                                            490       500       510       520       530       540       550       560
                                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita      AATTTTATTGTTACAATTCTTATAATAAAAAATTTTTCATTAAATTATGATCAAATTAATTTATTCTCATGATCAGTATG
B.mus_T611_Clare_(B)               AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T612_Clare_(B)               AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAATTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM008_Clare_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T653_Dublin_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T651_Dublin_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T684_Dublin_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus T748_Dublin_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T747_Dublin_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T746_Dublin_(B)              AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM020_Inis Oirr_(M)          AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM011_Sherkin_(B)            AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM001_Baltimore (B)          AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM018_Clare Is._(B)          AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM017_Clare Is._(B)          AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus BM019_Clare_Is._(B)          AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM021_Clare Is._(B)          AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM010_Sherkin_(B)            AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM002_Sherkin_(B)            AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM061_Inis_Mor_(I)           AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM062_Inis_Mor_(M)           AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
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B.mus_BM063_Inis_Mor(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T605_Inis_Mor (M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T605_Inis_Meain (M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T666_Inis Meain_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T667_Inis Meain_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM005_Inis Meain_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM003_Aghany (B)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM015_Inis_Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T603_Inis Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T623_Inis Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T646_Inis Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T604_Inis_Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM006_Inis Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM023_Inis Oirr_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T606_Coll_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T616_Coll_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T681_Coll_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T682_Coll_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM039_South_Uist_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM044_South Uist_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T607_Shetlands_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T614_Shetlands_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T613_Shetlands_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T678_Shetlands_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T679_Shetlands_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T680_Shetlands_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T619_St Agnus_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T620_St Agnus_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T621_St Agnus_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T622_St Agnus_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T608_St Agnus_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T609_Samson_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T617_Samson_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_T610_Samson_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM045_Tiree_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_BM046_Tiree_(M)	AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAAGTTATGATCAAATTAATTTATTTTCATGATCAGTTTG
B.mus_Cape Clear(26/7/1972(B)	--

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B.mus_Saltee_Is.(20/8/1972)(B)    --------------------------------------------------------------------------------
B.mus_Kilglass(8/9/1972)(B)       AATTTTATTGTTACAATTATATTAATAAAAAATTATTCTTTAANNNNTGATCAAATTAATTTATTTTCATGATCAGTTTG

                                          570       580       590       600       610       620       630       640
                                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NC_011923.1| Bombus hypocrita     TATTACTGTAATTCTATTAATTTTATCTTTACCAGTATTAGCTGGAGCAATTACTATACTTCTTTTTGATCGAAATTTTA
B.mus_T611_Clare_(B)              TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T612_Clare_(B)              TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM008_Clare_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T653_Dublin_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T651_Dublin_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T684_Dublin_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus T748_Dublin_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T747_Dublin_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T746_Dublin_(B)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGGGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM020_Inis Oirr_(M)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM011_Sherkin_(B)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM001_Baltimore (B)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM018_Clare Is._(B)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM017_Clare Is._(B)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus BM019_Clare_Is._(B)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM021_Clare Is._(B)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM010_Sherkin_(B)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM002_Sherkin_(B)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM061_Inis_Mor_(I)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM062_Inis_Mor_(M)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM063_Inis_Mor(M)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T605_Inis_Mor (M)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T605_Inis_Meain (M)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T666_Inis Meain_(M)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T667_Inis Meain_(M)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM005_Inis Meain_(M)        TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM003_Aghany (B)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM015_Inis_Oirr_(M)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T603_Inis Oirr_(M)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T623_Inis Oirr_(M)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T646_Inis Oirr_(M)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
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B.mus_T604_Inis_Oirr_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM006_Inis Oirr_(M)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM023_Inis Oirr_(M)           TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T606_Coll_(M)                 TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T616_Coll_(M)                 TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T681_Coll_(M)                 TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T682_Coll_(M)                 TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM039_South_Uist_(M)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM044_South Uist_(M)          TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T607_Shetlands_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T614_Shetlands_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T613_Shetlands_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T678_Shetlands_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T679_Shetlands_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTNNNCTGGAGCAATTACAATATTACTTTTTGANNNNNNNNNNN
B.mus_T680_Shetlands_(M)            TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTNNNCTGGAGCAATTACAATATTACTTTTTGATCGAAANNNNN
B.mus_T619_St Agnus_(M)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T620_St Agnus_(M)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T621_St Agnus_(M)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T622_St Agnus_(M)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T608_St Agnus_(M)             TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T609_Samson_(M)               TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T617_Samson_(M)               TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_T610_Samson_(M)               TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM045_Tiree_(M)               TATTACTGTAATTTTATTAATTTTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_BM046_Tiree_(M)               TATTACTGTAATTTTATTAATTTTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
B.mus_Cape Clear(26/7/1972)(B)      --------------------------------------------------------------------------------
B.mus_Saltee_Is.(20/8/1972)(B)      --------------------------------------------------------------------------------
B.mus_Kilglass(8/9/1972)(B)         TATTACTGTAATTTTATTAATATTATCTTTACCTGTTTTAGCTGGAGCAATTACAATATTACTTTTTGATCGAAATTTTA
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                                          650       660       670
                                    ....|....|....|....|....|....|....|..
NC_011923.1| Bombus hypocrita       ATACTTCATTCTTTGATCCTATAGGAGGAGGAGATCC
B.mus_T611_Clare_(B)                ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T612_Clare_(B)                ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM008_Clare_(B)               ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T653_Dublin_(B)               ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
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B.mus_T651_Dublin_(B)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T684_Dublin_(B)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus T748_Dublin_(B)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T747_Dublin_(B)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T746_Dublin_(B)          ATACATCTTTTTTTGAT--------------------
B.mus_BM020_Inis Oirr_(M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM011_Sherkin_(B)        ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM001_Baltimore (B)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM018_Clare Is._(B)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM017_Clare Is._(B)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus BM019_Clare_Is._(B)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM021_Clare Is._(B)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM010_Sherkin_(B)        ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM002_Sherkin_(B)        ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM061_Inis_Mor_(I)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM062_Inis_Mor_(M)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM063_Inis_Mor(M)        ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T605_Inis_Mor (M)        ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T605_Inis_Meain (M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T666_Inis Meain_(M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T667_Inis Meain_(M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM005_Inis Meain_(M)     ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM003_Aghany (B)         ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM015_Inis_Oirr_(M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T603_Inis Oirr_(M)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T623_Inis Oirr_(M)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T646_Inis Oirr_(M)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T604_Inis_Oirr_(M)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM006_Inis Oirr_(M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM023_Inis Oirr_(M)      ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T606_Coll_(M)            ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T616_Coll_(M)            ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T681_Coll_(M)            ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T682_Coll_(M)            ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM039_South_Uist_(M)     ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM044_South Uist_(M)     ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T607_Shetlands_(M)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
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B.mus_T614_Shetlands_(M)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T613_Shetlands_(M)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T678_Shetlands_(M)          ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T679_Shetlands_(M)          NNNCATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T680_Shetlands_(M)          NNNCATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T619_St Agnus_(M)           ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T620_St Agnus_(M)           ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T621_St Agnus_(M)           ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T622_St Agnus_(M)           ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T608_St Agnus_(M)           ATACATCTTTTTTTGATCCTATAGGAGGTGGTGA---
B.mus_T609_Samson_(M)             ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T617_Samson_(M)             ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_T610_Samson_(M)             ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM045_Tiree_(M)             ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_BM046_Tiree_(M)             ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
B.mus_Cape Clear(26/7/1972(B)     -------------------------------------
B.mus_Saltee_Is.(20/8/1972)(B)    -------------------------------------
B.mus_Kilglass(8/9/1972)(B)       ATACATCTTTTTTTGATCCTATAGGAGGTGGTGATCC
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Note*****

Is. = Island

B.mus = *Bombus muscorum*

B= of a blonde colour variety

M= of a melanic colour variety

Appendix 3 Cytochrome B DNA sequence for B. muscorum

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                            10        20        30        40        50        60        70        80
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001       --------------------------------------------------------------------------------
Bom_mus_BM003       --------------------------------------------------------------------------------
Bom_mus_Bm008       --------------------------------------------------------------------------------
Bom_mus_Bm011       --------------------------------------------------------------------------------
Bom_mus_Bm020       --------------------------------------------------------------------------------
Bom_mus_T606        --------------------------------------------------------------------------------

                            90       100       110       120       130       140       150       160
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001       ----------TTCTTTTTATTTTTTAATTATATATTTACATATTGCACGAAATATATTTTATTATTCTTTTAAATTACAT
Bom_mus_BM003       ----------TTCTTTTTATTTTTTAATTATATATTTACATATTGCACGAAATATATTTTATTATTCTTTTAAATTACAT
Bom_mus_Bm008       ----------TTCTTTTTATTTTTTAATTATATATTTACATATTGCACGAAATATATTTTATTATTCTTTTAAATTACAT
Bom_mus_Bm011       ----------TTCTTTTTATTTTTTAATTATATATTTACATATTGCACGAAATATATTTTATTATTCTTTTAAATTACAT
Bom_mus_Bm020       ----------TTCTTTTTATTTTTTAATTATATATTTACATATTGCACGAAATATATTTTATTATTCTTTTAAATTACAT
Bom_mus_T606        ----------TTCTTTTTATTTTTTAATTATATATTTACATATTGCACGAAATATATTTTATTATTCTTTTAAATTACAT

                           170       180       190       200       210       220       230       240
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001       CAAGTATGAATAATTGGAGTAACAATTTTATTTTTATCAATAGCAACAGCATTTTTAGGATATGTTTTACCATGAGGTCA
Bom_mus_BM003       CAAGTATGAATAATTGGAGTAACAATTTTATTTTTATCAATAGCAACAGCATTTTTAGGATATGTTTTACCATGAGGTCA
Bom_mus_Bm008       CAAGTATGAATAATTGGAGTAACAATTTTATTTTTATCAATAGCAACAGCATTTTTAGGATATGTTTTACCATGAGGTCA
Bom_mus_Bm011       CAAGTATGAATAATTGGAGTAACAATTTTATTTTTATCAATAGCAACAGCATTTTTAGGATATGTTTTACCATGAGGTCA
Bom_mus_Bm020       CAAGTATGAATAATTGGAGTAACAATTTTATTTTTATCAATAGCAACAGCATTTTTAGGATATGTTTTACCATGAGGTCA
Bom_mus_T606        CAAGTATGAATAATTGGAGTAACAATTTTATTTTTATCAATAGCAACAGCATTTTTAGGATATGTTTTACCATGAGGTCA

                           250       260       270       280       290       300       310       320
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001       AATATCATTTTGAGGAGCAATAGTAATTACAAATCTAATTTCAGCATTACCTTATATTGGCCAATTTACAGTTGAATGAA
Bom_mus_BM003       AATATCATTTTGAGGAGCAATAGTAATTACAAATCTAATTTCAGCATTACCTTATATTGGCCAATTTACAGTTGAATGAA
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Bom_mus_Bm008        AATATCATTTTGAGGAGCAATAGTAATTACAAATCTAATTTCAGCATTACCTTATATTGGCCAATTTACAGTTGAATGAA
Bom_mus_Bm011        AATATCATTTTGAGGAGCAATAGTAATTACAAATCTAATTTCAGCATTACCTTATATTGGCCAATTTACAGTTGAATGAA
Bom_mus_Bm020        AATATCATTTTGAGGAGCAATAGTAATTACAAATCTAATTTCAGCATTACCTTATATTGGCCAATTTACAGTTGAATGAA
Bom_mus_T606         AATATCATTTTGAGGAGCAATAGTAATTACAAATCTAATTTCAGCATTACCTTATATTGGCCAATTTACAGTTGAATGAA

                               330       340       350       360       370       380       390       400
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001        TTTGAGGTGGATTTTCAATTAATAATGATACATTAAATCGATTTTATTCATTTCATTTCATTTTACCATTTATTATTCTT
Bom_mus_BM003        TTTGAGGTGGATTTTCAATTAATAATGATACATTAAATCGATTTTATTCATTTCATTTCATTTTACCATTTATTATTCTT
Bom_mus_Bm008        TTTGAGGTGGATTTTCAATTAATAATGATACATTAAATCGATTTTATTCATTTCATTTCATTTTACCATTTATTATTCTT
Bom_mus_Bm011        TTTGAGGTGGATTTTCAATTAATAATGATACATTAAATCGATTTTATTCATTTCATTTCATTTTACCATTTATTATTCTT
Bom_mus_Bm020        TTTGAGGTGGATTTTCAATTAATAATGATACATTAAATCGATTTTATTCATTTCATTTCATTTTACCATTTATTATTCTT
Bom_mus_T606         TTTGAGGTGGATTTTCAATTAATAATGATACATTAAATCGATTTTATTCATTTCATTTCATTTTACCATTTATTATTCTT

                               410       420       430       440       450       460       470       480
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001        ATATTAGTTTTTATTCATTTAATAGTTTTACATATTACAGGTTCTTCAAATCCTATACATTCAAAATTAAATATTTATAA
Bom_mus_BM003        ATATTAGTTTTTATTCATTTAATAGTTTTACATATTACAGGTTCTTCAAATCCTATACATTCAAAATTAAATATTTATAA
Bom_mus_Bm008        ATATTAGTTTTTATTCATTTAATAGTTTTACATATTACAGGTTCTTCAAATCCTATACATTCAAAATTAAATATTTATAA
Bom_mus_Bm011        ATATTAGTTTTTATTCATTTAATAGTTTTACATATTACAGGTTCTTCAAATCCTATACATTCAAAATTAAATATTTATAA
Bom_mus_Bm020        ATATTAGTTTTTATTCATTTAATAGTTTTACATATTACAGGTTCTTCAAATCCTATACATTCAAAATTAAATATTTATAA
Bom_mus_T606         ATATTAGTTTTTATTCATTTAATAGTTTTACATATTACAGGTTCTTCAAATCCTATACATTCAAAATTAAATATTTATAA

                               490       500       510       520       530       540       550       560
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001        AATTAATTTTCATCCTTATTTTACAATTAAAGATTTAATTACAATTATTTTTACATTTTCAATATTTATAATTATTAATC
Bom_mus_BM003        AATTAATTTTCATCCTTATTTTACAATTAAAGATTTAATTACAATTATTTTTACATTTTCAATATTTATAATTATTAATC
Bom_mus_Bm008        AATTAATTTTCATCCTTATTTTACAATTAAAGATTTAATTACAATTATTTTTACATTTTCAATATTTATAATTATTAATC
Bom_mus_Bm011        AATTAATTTTCATCCTTATTTTACAATTAAAGATTTAATTACAATTATTTTTACATTTTCAATATTTATAATTATTAATC
Bom_mus_Bm020        AATTAATTTTCATCCTTATTTTACAATTAAAGATTTAATTACAATTATTTTTACATTTTCAATATTTATAATTATTAATC
Bom_mus_T606         AATTAATTTTCATCCTTATTTTACAATTAAAGATTTAATTACAATTATTTTTACATTTTCAATATTTATAATTATTAATC

                               570       580       590       600       610       620       630       640
                     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001        TTCAATTTCCATATATTTTAGGAGACCCAGATAATTTTAAAATAGCAAATTCAATAATTACTCCTATTCATATTAAACCT
Bom_mus_BM003        TTCAATTTCCATATATTTTAGGAGACCCAGATAATTTTAAAATAGCAAATTCAATAATTACTCCTATTCATATTAAACCT
Bom_mus_Bm008        TTCAATTTCCATATATTTTAGGAGACCCAGATAATTTTAAAATAGCAAATTCAATAATTACTCCTATTCATATTAAACCT
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Bom_mus_Bm011        TTCAATTTCCATATATATTTTAGGAGACCCAGATAATTTTAAAATAGCAAATTCAATAATTACTCCTATTCATATTAAACCT
Bom_mus_Bm020        TTCAATTTCCATATATATTTTAGGAGACCCAGATAATTTTAAAATAGCAAATTCAATAATTACTCCTATTCATATTAAACCT
Bom_mus_T606         TTCAATTTCCATATATATTTTAGGAGACCCAGATAATTTTAAAATAGCAAATTCAATAATTACTCCTATTCATATTAAACCT

                              650       660       670
                     ....|....|....|....|....|....|....
Bom_mus_BM001        GAATGATATTTTTTATTTGCATATTCAATTTTAC
Bom_mus_BM003        GAATGATATTTTTTATTTGCATATTCAATTTTAC
Bom_mus_Bm008        GAATGATATTTTTTATTTGCATATTCAATTTTAC
Bom_mus_Bm011        GAATGATATTTTTTATTTGCATATTCAATTTTAC
Bom_mus_Bm020        GAATGATATTTTTTATTTGCATATTCAATTTTAC
Bom_mus_T606         GAATGATATTTTTTATTTGCATATTCAATTTTAC
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Appendix 4 ITS DNA sequence for B. muscorum

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                                   10        20        30        40        50        60        70        80
                           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc    --------------------------------------------------------------------------------
Bom_mus_BM003_CAS5.8SFc    --------------------------------------------------------------------------------
Bom_mus_Bm008_CAS5.8sFc    --------------------------------------------------------------------------------
Bom_mus_Bm011_CAS5.8sFc    --------------------------------------------------------------------------------
Bom_mus_Bm020_CAS5.8sFc    --------------------------------------------------------------------------------
Bom_mus_T606_CAS5.8sFc     --------------------------------------------------------------------------------

                                   90       100       110       120       130       140       150       160
                           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc    --------------------------------------------------------------------------------
Bom_mus_BM003_CAS5.8SFc    --------------------------------------------------------------------------------
Bom_mus_Bm008_CAS5.8sFc    --------------------------------------------------------------------------------
Bom_mus_Bm011_CAS5.8sFc    --------------------------------------------------------------------------------
Bom_mus_Bm020_CAS5.8sFc    --------------------------------------------------------------------------------
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Bom_mus_T606_CAS5.8sFc   --------------------------------------------------------------------------------

                                    170       180       190       200       210       220       230       240
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc  -----------------------------CAGTCGAGGGCTCGTACGCGACGTACGAGCGATTGTTGGACGTTCGTCGGC
Bom_mus_BM003_CAS5.8SFc  -----------------------------CAGTCGAGGGCTCGTACGCGACGTACGAGCGATTGTTGGACGTTCGTCGGC
Bom_mus_Bm008_CAS5.8sFc  -----------------------------CAGTCGAGGGCTCGTACGCGACGTACGAGCGATTGTTGGACGTTCGTCGGC
Bom_mus_Bm011_CAS5.8sFc  -----------------------------CAGTCGAGGGCTCGTACGCGACGTACGAGCGATTGTTGGACGTTCGTCGGC
Bom_mus_Bm020_CAS5.8sFc  -----------------------------CAGTCGAGGGCTCGTACGCGACGTACGAGCGATTGTTGGACGTTCGTCGGC
Bom_mus_T606_CAS5.8sFc   -----------------------------CAGTCGAGGGCTCGTACGCGACGTACGAGCGATTGTTGGACGTTCGTCGGC

                                    250       260       270       280       290       300       310       320
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc  GTTCGTTGCGGTGTAGTGCCGAAGCACCATTCGTGGCAGAATCGTCGGCATGCTCGATGCAAATATACAAAATCTATCGC
Bom_mus_BM003_CAS5.8SFc  GTTCGTTGCGGTGTAGTGCCGAAGCACCATTCGTGGCAGAATCGTCGGCATGCTCGATGCAAATATACAAAATCTATCGC
Bom_mus_Bm008_CAS5.8sFc  GTTCGTTGCGGTGTAGTGCCGAAGCACCATTCGTGGCAGAATCGTCGGCATGCTCGATGCAAATATACAAAATCTATCGC
Bom_mus_Bm011_CAS5.8sFc  GTTCGTTGCGGTGTAGTGCCGAAGCACCATTCGTGGCAGAATCGTCGGCATGCTCGATGCAAATATACAAAATCTATCGC
Bom_mus_Bm020_CAS5.8sFc  GTTCGTTGCGGTGTAGTGCCGAAGCACCATTCGTGGCAGAATCGTCGGCATGCTCGATGCAAATATACAAAATCTATCGC
Bom_mus_T606_CAS5.8sFc   GTTCGTTGCGGTGTAGTGCCGAAGCACCATTCGTGGCAGAATCGTCGGCATGCTCGATGCAAATATACAAAATCTATCGC

                                    330       340       350       360       370       380       390       400
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc  GTTCACGGGAGGCTCGTGCAACCTACCTCTGTCTCTCTCTGTCCCTTGTTGCGTCGTCAGTGCCCCGCCGTCCGTTCGTC
Bom_mus_BM003_CAS5.8SFc  GTTCACGGGAGGCTCGTGCAACCTACCTCTGTCTCTCTCTGTCCCTTGTTGCGTCGTCAGTGCCCCGCCGTCCGTTCGTC
Bom_mus_Bm008_CAS5.8sFc  GTTCACGGGAGGCTCGTGCAACCTACCTCTGTCTCTCTCTGTCCCTTGTTGCGTCGTCAGTGCCCCGCCGTCCGTTCGTC
Bom_mus_Bm011_CAS5.8sFc  GTTCACGGGAGGCTCGTGCAACCTACCTCTGTCTCTCTCTGTCCCTTGTTGCGTCGTCAGTGCCCCGCCGTCCGTTCGTC
Bom_mus_Bm020_CAS5.8sFc  GTTCACGGGAGGCTCGTGCAACCTACCTCTGTCTCTCTCTGTCCCTTGTTGCGTCGTCAGTGCCCCGCCGTCCGTTCGTC
Bom_mus_T606_CAS5.8sFc   GTTCACGGGAGGCTCGTGCAACCTACCTCTGTCTCTCTCTGTCCCTTGTTGCGTCGTCAGTGCCCCGCCGTCCGTTCGTC

                                    410       420       430       440       450       460       470       480
                         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc  TTAGAACGTTTCAGAGTTGGTGGACGTGCGCAAGGAGAAGGATACGAATAAGAGCGGAGAGAACCCCGTCGTCGTTGTCG
Bom_mus_BM003_CAS5.8SFc  TTAGAACGTTTCAGAGTTGGTGGACGTGCGCAAGGAGAAGGATACGAATAAGAGCGGAGAGAACCCCGTCGTCGTTGTCG
Bom_mus_Bm008_CAS5.8sFc  TTAGAACGTTTCAGAGTTGGTGGACGTGCGCAAGGAGAAGGATACGAATAAGAGCGGAGAGAACCCCGTCGTCGTTGTCG
Bom_mus_Bm011_CAS5.8sFc  TTAGAACGTTTCAGAGTTGGTGGACGTGCGCAAGGAGAAGGATACGAATAAGAGCGGAGAGAACCCCGTCGTCGTTGTCG
Bom_mus_Bm020_CAS5.8sFc  TTAGAACGTTTCAGAGTTGGTGGACGTGCGCAAGGAGAAGGATACGAATAAGAGCGGAGAGAACCCCGTCGTCGTTGTCG
Bom_mus_T606_CAS5.8sFc   TTAGAACGTTTCAGAGTTGGTGGACGTGCGCAAGGAGAAGGATACGAATAAGAGCGGAGAGAACCCCGTCGTCGTTGTCG
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                                    490       500       510       520       530       540       550       560
                             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Bom_mus_BM001_CAS5.8SFc  ACACGCGGTTATCGAATACGCACAAGTTTTCCTCGTGGCCGATATTTTTTTTTTGTGTCAGCCTCTGTCGCGTTTTGTCG
Bom_mus_BM003_CAS5.8SFc  ACACGCGGTTATCGAATACGCACAAGTTTTCCTCGTGGCCGATATTTTTTTTTTGTGTCAGCCTCTGTCGCGTTTTGTCG
Bom_mus_Bm008_CAS5.8sFc  ACACGCGGTTATCGAATACGCACAAGTTTTCCTCGTGGCCGATATTTTTTTTTTGTGTCAGCCTCTGTCGCGTTTTGTCG
Bom_mus_Bm011_CAS5.8sFc  ACACGCGGTTATCGAATACGCACAAGTTTTCCTCGTGGCCGATATTTTTTTTTTGTGTCAGCCTCTGTCGCGTTTTGTCG
Bom_mus_Bm020_CAS5.8sFc  ACACGCGGTTATCGAATACGCACAAGTTTTCCTCGTGGCCGATATTTTTTTTTTGTGTCAGCCTCTGTCGCGTTTTGTCG
Bom_mus_T606_CAS5.8sFc   ACACGCGGTTATCGAATACGCACAAGTTTTCCTCGTGGCCGATATTTTTTTTTTGTGTCAGCCTCTGTCGCGTTTTGTCG

                                    570       580       590       600       610
                             ....|....|....|....|....|....|....|....|....|....|...
Bom_mus_BM001_CAS5.8SFc  GTGTTCGCACGAAGGTCCGCTCCCCCGACGTCGTCTTAAATGAATTTTTTTTT
Bom_mus_BM003_CAS5.8SFc  GTGTTCGCACGAAGGTCCGCTCCCCCGACGTCGTCTTAAATGAATTTTTTTTT
Bom_mus_Bm008_CAS5.8sFc  GTGTTCGCACGAAGGTCCGCTCCCCCGACGTCGTCTTAAATGAATTTTTTTTT
Bom_mus_Bm011_CAS5.8sFc  GTGTTCGCACGAAGGTCCGCTCCCCCGACGTCGTCTTAAATGAATTTTTTTTT
Bom_mus_Bm020_CAS5.8sFc  GTGTTCGCACGAAGGTCCGCTCCCCCGACGTCGTCTTAAATGAATTTTTTTTT
Bom_mus_T606_CAS5.8sFc   GTGTTCGCACGAAGGTCCGCTCCCCCGACGTCGTCTTAAATGAATTTTTTTTT
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Note****

B.mus = *Bombus muscorum*